Investigations into Metal-Binding Groups to Probe the DNA Repair Nuclease SNM1A

A thesis submitted to the School of Chemistry, Trinity College Dublin, The University of Dublin, for the degree of Doctor of Philosophy

Eva-Maria Dürr, B.A. (Mod)

Under the supervision of Prof. Joanna F. McGouran

Trinity College Dublin, The University of Dublin
2021
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, other than where acknowledged.

I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Eva-Maria Dürr
Abstract

SNM1A is a nuclease that is implicated in DNA interstrand crosslink repair and as such is of importance to regular cellular function. However, little is known about its roles, regulation, interaction partners or posttranslational modifications. Research into the function of SNM1A is complicated by its low expression levels, and because overexpression in mammalian cells leads to cytotoxicity. In addition, there is redundancy among repair pathways.

Activity-based protein profiling is a promising approach to further the knowledge about this enzyme. As the active site of SNM1A contains a metal centre, the design of activity-based probes (ABPs) requires the incorporation of metal-binding groups into an oligonucleotide scaffold. To identify suitable metal-binding groups for such probes, insights from metal-binding groups in medicinal chemistry and phosphate bioisosteres were combined. Modified nucleosides, dinucleosides and oligonucleotides were designed and synthesised. These compounds were evaluated for binding to SNM1A to identify the most potent metal-binding groups.

Within this work, results of the synthesis and screening of a series of nucleosides with 5'-modifications are described. Hydroxamic acid modifications emerged as the most potent group, and the positioning of the group relative to the nucleoside core affected the binding strength.

The impact of an oligonucleotide scaffold on the binding affinity was also investigated. Using some of the previously evaluated modifications, the results highlighted the significant interaction between the oligonucleotide scaffold and the enzyme. Squaramide-functionalised oligonucleotides were found to weakly bind to SNM1A, despite the corresponding nucleosides having no activity. A novel synthetic strategy to install these modifications was developed as part of this work.

Finally, the synthesis and testing of a family of malonate-derived nucleosides are described. An alternative strategy to install hydroxamic acid groups was explored, as well as different positioning relative to the nucleoside core. The strongest inhibitor was a 5'-modified nucleoside containing a hydroxamic acid group. Additionally, dinucleosides were synthesised and evaluated with promising results.
Overall, the work presented in this thesis provides insights into suitable metal-binding groups for ABPs targeting the repair nuclease SNM1A. Based on these results, probes consisting of an oligonucleotide scaffold and a hydroxamic acid or malonate hydroxamic acid modification are likely to achieve strong binding. Such probes are expected to aid the study of this enzyme.
Acknowledgements

I would like to thank my supervisor Prof. Joanna McGouran for giving me the opportunity to start this project and trusting me to be one of the first PhD students in her research group. Her guidance and support have been invaluable during my time in the lab. I would also like to thank Trinity College Dublin for funding this research.

I am grateful to the technical staff in the School of Chemistry, particularly Dr. John O’Brien and Dr. Manuel Ruether for their tireless support with NMR spectroscopy. I would also like to thank Dr. Gary Hessman and Dr. Martin Feeney for their help with mass spectrometry. I would like to express my gratitude to Prof. Tom Brown and his group at the University of Oxford for welcoming me into their lab for a research visit and the Royal Society of Chemistry for funding it.

I would like to extend a massive thank you to all McGouran group members, past and present (although there aren’t that many past members yet). Special thanks to William, who taught me a lot in the lab and who braved the phosphoramidite synthesis with me and to Werner, who didn’t let the squaramides get him down. Mark, you are a great scientist and friend; thank you for all the chats and bouncing ideas off each other. Ellen and Susie, the deadly duo, you two are a joy to be around and have made the lab such a nice work environment. Sean, thanks for the productive discussions, and Connor and Valerio, although our time in the group didn’t overlap much, I’m sure you are great additions to the lab.

My lovely friends Jess, Deirdre and Katie, our chats and cups of tea or coffee were what I needed to destress, and I have really missed them since March. Also thank you to Elaine for introducing me to climbing, which, thanks to the folks at Gravity and particularly my fellow German Friedrich, kept me sane during these four years.

Finally, I would like to thank my family who always believe in me and do whatever they can to support me from a distance. Neil, you have been the most wonderful person to go through this process with and I can’t thank you enough for that.
Contents

Declaration ................................................................................................................................. i
Abstract ..................................................................................................................................... ii
Acknowledgements ................................................................................................................... v
Contents ..................................................................................................................................... vi
Abbreviations .......................................................................................................................... x
Explanatory note ....................................................................................................................... xvi

1 Introduction .......................................................................................................................... 1
  1.1 DNA interstrand crosslink repair ...................................................................................... 1
    1.1.1 DNA interstrand crosslink repair pathways .............................................................. 2
    1.1.2 The role of SNM1A in crosslink repair ..................................................................... 3
  1.2 SNM1A – Structure and function .................................................................................... 6
    1.2.1 Biochemical characterisation ................................................................................... 7
    1.2.2 Structure and substrate binding .............................................................................. 8
    1.2.3 Tools to study SNM1A and reported inhibitors ....................................................... 11
    1.2.4 Need for probes targeting SNM1A ......................................................................... 12
  1.3 Inhibitors and probes for metalloenzymes ..................................................................... 14
    1.3.1 Inhibitors of metalloenzymes ................................................................................ 14
      1.3.1.1 Inhibitors of phosphodiesterases ................................................................... 18
      1.3.1.2 Inhibitors of matrix metallopeptidases ......................................................... 20
    1.3.2 Activity-based probes for metalloenzymes ............................................................ 24
      1.3.2.1 Activity-based probes for metallopeptidases ............................................... 25
      1.3.2.2 Activity-based probes for histone deacetylases ........................................... 27
      1.3.2.3 Probes for DNA repair enzymes and nucleases ......................................... 29
  1.4 Phosphate replacements in medicinal chemistry and nucleic acids ............................ 31
    1.4.1 Phosphate bioisosteres .......................................................................................... 32
    1.4.2 Alternative backbones for nucleic acids .............................................................. 36
1.5 Objectives: Identification of metal-binding group for probes targeting SNM1A

2 Hydroxamic acid and squaramide-based nucleoside inhibitors

2.1 Design and Synthesis

2.1.1 Design of modified nucleosides

2.1.1.1 Hydroxamic acid derivatives

2.1.1.2 Squaramide derivatives

2.1.1.3 Target compounds

2.1.2 Synthesis of modified nucleosides

2.2 Biological evaluation

2.2.1 Optimisation of gel-based assay

2.2.2 Gel-based assays

2.2.3 IC50 determination

2.2.4 Membrane permeability assay

2.3 Hydroxamic acid-containing nucleosides inhibit SNM1A

3 Modification of oligonucleotides for binding to SNM1A

3.1 Design and synthesis of modified oligonucleotides

3.1.1 Design of modified oligonucleotides

3.1.1.1 Hydroxamic acid derivatives

3.1.1.2 Squaramide derivatives

3.1.1.3 Target compounds

3.1.2 Synthesis of phosphoramidites and incorporation into oligonucleotides

3.1.2.1 Phosphoramidite synthesis

3.1.2.2 Solid-phase synthesis

3.1.3 Post-solid phase synthesis of squaramides

3.1.3.1 Optimisation of reaction conditions

3.1.3.2 Synthesis on oligonucleotides
3.1.4 Design and synthesis of further squaramide-modified oligonucleotides ................................................................. 75

3.1.4.1 Design of target structures ................................................................. 75

3.1.4.2 Optimisation of reaction conditions ...................................................... 75

3.1.4.3 Synthesis on oligonucleotides ............................................................ 76

3.2 Biological evaluation .................................................................................. 77

3.2.1 Stability to SNM1A ................................................................................. 77

3.2.2 Binding to SNM1A ................................................................................ 79

3.2.2.1 Gel-based inhibition assay ................................................................. 80

3.2.2.2 Electrophoretic mobility shift assay .................................................. 85

3.3 Squaramide-modified oligonucleotides bind to SNM1A ......................... 86

4 Malonate-based mono- and dinucleoside inhibitors .................................. 89

4.1 Design and synthesis of malonate-based nucleosides ............................ 90

4.1.1 Design of malonate-based nucleosides ................................................ 91

4.1.2 Synthesis of modified mononucleosides .............................................. 93

4.1.2.1 Synthesis of 5’-modified nucleosides ................................................ 95

4.1.2.2 Synthesis of 3’-modified nucleosides .............................................. 107

4.1.3 Synthesis of modified dinucleosides .................................................... 113

4.2 Biological evaluation as inhibitors of SNM1A ....................................... 120

4.3 Malonate-modified nucleosides and dinucleosides bind to SNM1A ....... 129

5 Conclusions and future directions ............................................................. 132

6 Experimental procedures ........................................................................... 136

6.1 Synthesis of modified nucleosides and dinucleosides ........................... 136

6.1.1 Hydroxamic acid and squaramide-based nucleoside inhibitors .......... 137

6.1.1.1 Hydroxamic acid derivatives ............................................................. 137

6.1.1.2 Squaramide derivatives .................................................................... 143

6.1.2 Synthesis related to modified oligonucleotides ................................ 149

6.1.2.1 Synthesis of phosphoramidites ........................................................ 149
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1.2.2</td>
<td>Test reactions for synthesis of squaryl diamide oligonucleotides</td>
<td>153</td>
</tr>
<tr>
<td>6.1.3</td>
<td>Synthesis of malonate-based mono- and dinucleosides</td>
<td>155</td>
</tr>
<tr>
<td>6.1.3.1</td>
<td>Synthesis of 5’-modified nucleosides</td>
<td>155</td>
</tr>
<tr>
<td>6.1.3.2</td>
<td>Synthesis of 3’-modified nucleosides</td>
<td>188</td>
</tr>
<tr>
<td>6.1.3.3</td>
<td>Synthesis of modified dinucleosides</td>
<td>204</td>
</tr>
<tr>
<td>6.2</td>
<td>Synthesis of modified oligonucleotides</td>
<td>221</td>
</tr>
<tr>
<td>6.3</td>
<td>Biological experimental procedures</td>
<td>224</td>
</tr>
<tr>
<td>6.3.1</td>
<td>Gel-based assays</td>
<td>224</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Membrane permeability assay</td>
<td>225</td>
</tr>
<tr>
<td>7</td>
<td>References</td>
<td>226</td>
</tr>
</tbody>
</table>
Abbreviations

°C  degree Celsius
53BP1  TP53-binding protein 1
A  adenine
Å  Ångström
ABP  activity-based probe
Ac  acetyl
Ala  alanine
AMP  adenosine monophosphate
AP  apurinic/apyrimidinic
APCI  atmospheric-pressure chemical ionisation
app.  apparent
aq.  aqueous
ar  aromatic
Arg  arginine
Asn  asparagine
Asp  aspartate
ATP  adenosine triphosphate
AZT  3’-azido-3’-deoxythymidine
Bn  benzyl
BOM  benzyloxymethyl
bs  broad singlet
BSA  bovine serum albumin
C  cytosine
conc.  concentrated
CPSF  cleavage and polyadenylation specificity factor
CSB  Cockayne syndrome B protein
CuAAC  copper(I)-catalysed azide-alkyne cycloaddition
Cy3  cyanine3
d  doublet
DCLRE1A  DNA cross-link repair 1A protein

x
dd doublet of doublets
Ddc1 DNA damage checkpoint protein 1
ddd doublet of doublets of doublets
dec. decomposed
DEPT distortionless enhancement by polarization transfer
DIAD diisopropyl azodicarboxylate
DIPEA N,N-diisopropylethylamine
DMAP 4-dimethylaminopyridine
DME 1,2-dimethoxyethane
DMF N,N-dimethylformamide
DMSO dimethyl sulfoxide
DMTr 4,4'-dimethoxytrityl
DNA deoxyribonucleic acid
DNA-PKcs DNA-dependent protein kinase, catalytic subunit
dt doublet of triplets
DTT 1,4-dithiothreitol
EDC N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA ethylenediaminetetraacetic acid
EMSA electrophoretic mobility shift assay
eq. equivalents
ESI electrospray ionisation
Et ethyl
et al. et alií, and others
EXSY exchange spectroscopy
FEN1 flap endonuclease 1
Fmoc fluorenylmethyloxycarbonyl
FRET Förster resonance energy transfer
G guanine
glmS ribozyme glucosamine-6-phosphate riboswitch ribozyme
Gly glycine
Glu glutamic acid
h  hour
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
his  histidine
HIV  human immunodeficiency virus
HMBC  heteronuclear multiple bond correlation
HOAt  1-hydroxy-7-azabenzotriazole
HPLC  high performance liquid chromatography
HRMS  high resolution mass spectrometry
HSQC  heteronuclear single quantum correlation
i.e.  *id est*, that is
IC₅₀  half maximal inhibitory concentration
ICL  interstrand crosslink
Ile  isoleucine
i-Pr  isopropyl
IR  infrared
lac  lactose
Leu  leucine
LG  leaving group
lit.  literature
Lys  lysine
M  metal
m  multiplet
m/z  mass-to-charge ratio
M6P  mannose-6-phosphate
MBL  metallo-β-lactamase
MDO1  macrodomain-containing protein 1
Me  methyl
Met  methionine
min  minute
MMP  matrix metallopeptidase
mp  melting point
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Ms</td>
<td>methanesulfonyl</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>ON</td>
<td>oligonucleotide</td>
</tr>
<tr>
<td>OTf</td>
<td>trifluoromethanesulfonate</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMPA</td>
<td>parallel artificial membrane permeation assay</td>
</tr>
<tr>
<td>PARP1</td>
<td>poly [ADP-ribose] polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>Pe</td>
<td>effective permeability</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PhthN</td>
<td>phthalimide</td>
</tr>
<tr>
<td>pKa</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>qC</td>
<td>quaternary carbon</td>
</tr>
<tr>
<td>quant.</td>
<td>quantitative</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>Rf</td>
<td>retention factor</td>
</tr>
<tr>
<td>Rho</td>
<td>rhodamine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>sat.</td>
<td>saturated</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SNM1A</td>
<td>sensitive to nitrogen mustard 1A</td>
</tr>
<tr>
<td>SNM1B</td>
<td>sensitive to nitrogen mustard 1B</td>
</tr>
<tr>
<td>SNM1C</td>
<td>sensitive to nitrogen mustard 1C</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>T4 PNK</td>
<td>T4 polynucleotide kinase</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-n-butylammonium fluoride trihydrate</td>
</tr>
<tr>
<td>TBDMS</td>
<td>tert-butyldimethylsilyl</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/borate/EDTA</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>td</td>
<td>triplet of doublets</td>
</tr>
<tr>
<td>TEG</td>
<td>triethyleneglycol</td>
</tr>
<tr>
<td>Tf₂O</td>
<td>trifluoromethanesulfonic anhydride</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>ti</td>
<td>incubation time</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TP53</td>
<td>Cellular tumor antigen p53</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>vis</td>
<td>visible</td>
</tr>
<tr>
<td>vs</td>
<td>versus, against</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>β-CASP</td>
<td>metallo-β-lactamase-associated CPSF Artemis</td>
</tr>
<tr>
<td>SNM1/PSO2</td>
<td></td>
</tr>
</tbody>
</table>
Explanatory note

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

Numbering of atoms for modified nucleosides and dinucleosides
Chapter 1 – Introduction

1 Introduction

1.1 DNA interstrand crosslink repair

Deoxyribonucleic acid (DNA) in living cells is continually subjected to endogenous and exogenous agents that can damage it and impair important cellular processes. A particularly dangerous type of DNA damage is the covalent linking of the two opposing strands of a DNA duplex, as this prevents strand separation for replication or transcription. These interstrand crosslinks (ICLs) can be caused by endogenous metabolites such as aldehydes\(^1\) or external agents such as chemotherapeutics.\(^2,3\) Due to the cytotoxicity of ICLs and their structural diversity, cells have evolved an intricate network of repair pathways to combat them.\(^4\)

Defective ICL repair is associated with a range of diseases.\(^5\) Fanconi anaemia is a hereditary disorder that is characterised by a sensitivity to crosslinking agents.\(^6\) This condition is caused by mutations in a group of over twenty genes that encode the Fanconi anaemia proteins, which are important components of ICL repair.\(^6\) Among the symptoms of Fanconi anaemia are an increased incidence of different types of cancers,\(^7\) as well as bone marrow failure.\(^6\) Xeroderma pigmentosum is another disorder related to defective DNA damage repair.\(^8\) This condition leads to a heightened risk of cancer, particularly in tissues that are exposed to ultraviolet (UV) radiation.\(^8\) Cockayne syndrome is associated with impaired transcription-coupled nucleotide excision repair,\(^8\) a pathway that is involved in removing ICLs.\(^9\) Unlike other diseases resulting from defective DNA damage repair proteins, patients with Cockayne syndrome are not predisposed to cancers.\(^8\)

Intact ICL repair is not only crucial to maintaining normal cellular function, but it is also an important factor in determining sensitivity to crosslinking agents in cancer chemotherapy.\(^10\) Lower levels of ICL repair are associated with a stronger response to crosslinking agents,\(^11\) and such therapeutics are of limited benefit in cancers expressing high levels of the repair enzyme ERCC1.\(^12\) This observation highlights the potential for inhibiting ERCC1 and related repair enzymes to overcome resistance to chemotherapeutic crosslinking agents.\(^13,14\)

Since blocking these very important repair processes can lead to dramatic consequences, as evident in Fanconi anaemia, Xeroderma pigmentosum and
Cockayne syndrome, the exact mechanisms and pathways need to be well understood prior to any therapeutic intervention. The following sections provide a brief introduction to the current understanding of ICL repair pathways.

1.1.1 DNA interstrand crosslink repair pathways

In vertebrates, the detection of ICLs can be divided into three subgroups: transcription-dependent, replication-dependent or transcription- and replication-independent. The choice of repair pathway depends on which phase of the cell cycle the ICL is detected in and the type of lesion. In G0/G1 phase, in the absence of replication, ICLs are repaired via a transcription-coupled nucleotide excision repair pathway, combined with translesion synthesis. In the S-phase, repair occurs by replication-dependent pathways, which are thought to be activated by stalling of replication forks at ICLs. However, replication can also continue past lesions and repair can occur following replication. In the absence of replication and transcription, distortion of the duplex structure resulting from ICLs can be recognised by repair factors to initiate different repair pathways. The repair of ICLs can be achieved by cleavage of the crosslink, through excision of the nucleobase, or via removal of the nucleotide by hydrolysing the phosphodiester backbone (Figure 1.1).

![Figure 1.1: ICL repair can occur via cleavage of the crosslink, the glycosidic linkage or the phosphate backbone.](image)

Removal of ICLs via cleavage of the crosslink (Figure 1.1) was recently discovered as a repair mechanism that is specific to acetaldehyde crosslinks. Enzymes involved in translesion synthesis were found to participate in this pathway, but...
further research is required to identify the enzyme that cleaves the crosslink. This repair mechanism restores one of the two strands, leaving a monoadduct on the other strand, which is subsequently removed.

In a different pathway, ICL repair is achieved by cleaving the glycosidic linkage between the nucleobase and the deoxyribose sugar at the site of the crosslink, creating an abasic site on one strand and a monoadduct on the other strand \((\text{Figure 1.1})\). This pathway involves the DNA glycosylase NEIL3\(^{22,23}\) which also participates in the base excision repair pathway\(^{24}\). The role of NEIL3 in ICL repair was only recently discovered\(^{22,23}\) and this pathway appears to overlap with those that employ nucleases\(^{22}\).

Finally, in some repair pathways nucleases are used to excise the ICL from DNA by hydrolysing the phosphate backbone \((\text{Figure 1.1})\). Several nucleases have been implicated in ICL repair\(^{25}\). Accumulating evidence points towards XPF-ERCC1 as a key endonuclease in multiple repair pathways\(^{26,27}\). This endonuclease has been postulated to make the first incision near an ICL at a stalled replication fork\(^{25}\). Following this incision, either other endonucleases are postulated to cleave the backbone on the other side of the ICL, or an exonuclease such as SNM1A can digest past the ICL from the incision\(^{25}\). The roles of many nucleases in ICL repair are not fully understood yet\(^{25}\).

Despite significant advances in the field of ICL repair in recent years, the roles of many repair proteins and the sequence of events are still under investigation. Overlap and redundancy among the different repair pathways pose a substantial challenge in deciphering the roles of the individual proteins\(^{27}\).

### 1.1.2 The role of SNM1A in crosslink repair

The nuclease SNM1A (also known as DCLRE1A) is involved in the repair of ICLs, as is evident by the increased sensitivity of cells depleted in SNM1A to various crosslinking agents\(^{28-31}\). SNM1A has been suggested to act in several different pathways, all of which are still under investigation\(^{32}\). These pathways include SNM1A acting both as an exonuclease\(^{33}\) and an endonuclease\(^{34}\) \((\text{Figure 1.2})\). Additionally, interactions with transcription-dependent repair pathways\(^{9}\) and a role in cell cycle checkpoints\(^{35,36}\) which delay progression through the cell cycle if DNA is extensively damaged, have been reported.
Figure 1.2: Proposed replication-dependent pathways that SNM1A participates in. a) Following replication fork convergence or replication fork traverse, the replication fork reverses to reform a stretch of double-stranded DNA. XPF-ERCC1 makes an incision following RPA binding, and SNM1A digests past the lesion from the incision. Further repair restores the replication products. b) SNM1A is recruited to a stalled replication fork by Ub-PCNA, where it makes an incision and digests past the ICL. Further repair restores the replication products.
In vitro characterisation described the 5’-3’ exonuclease activity of SNM1A on single- and double-stranded DNA and its ability to digest past lesions. One proposed role of SNM1A is the digestion of DNA strands past an ICL following an incision by an endonuclease as part of the replication-dependent Fanconi anaemia pathway (Figure 1.2a). Work by the McHugh group and the Walter group points towards a pathway in which an incision by XPF-ERCC1 precedes SNM1A activity. Significant insights into this pathway have been obtained from a Xenopus model system developed by the Walter group. Their initial proposed model requires the convergence of two replication forks at the ICL (Figure 1.2a). More recent insights show that following this convergence, one replication fork reverses to reform a stretch of double-stranded DNA near the ICL. This structure was found to be a substrate for XPF-ERCC1 in the presence of replication protein A (RPA), and following incision by XPF-ERCC1, SNM1A was able to digest past the ICL in such a structure. Similarly, a single stalled replication fork results in the same intermediate and can be processed in an analogous manner. It is worth noting that while stalling of replication forks is observed in this model, reports by the Seidmann group describe traversing of ICLs by the replication machinery (Figure 1.2a). However, this traversing also results in an identical structure to two replication forks stalling at the ICL and is postulated to be processed by XPF-ERCC1 and SNM1A in the same manner as before (Figure 1.2a). The processed nucleic acid is then a substrate for translesion synthesis and further repair.

A recent publication by the Junop group reports structure-specific endonuclease activity of SNM1A. The authors reported that on synthetic oligonucleotide substrates with a blocked 5’-end, SNM1A acts as an endonuclease in single-stranded regions. They postulate that SNM1A acts both as an endonuclease and an exonuclease during ICL repair, making an incision to generate a substrate for exonuclease activity and then further processing the DNA strand (Figure 1.2b). A potential mechanism for the recruitment of SNM1A independent of the Fanconi anaemia pathway was reported by Yang et al., who detected binding of SNM1A to monoubiquitinated proliferating cell nuclear antigen (Ub-PCNA). Monoubiquitination of PCNA is a consequence of RAD18 activation in a replication-dependent mechanism to recruit polymerases for translesion synthesis. SNM1A could potentially participate in this repair pathway by incising the DNA strand and digesting past the ICL to provide a substrate for translesion synthesis.
In transcription-dependent ICL repair, SNM1A has been found to be recruited to sites of damage by Cockayne syndrome B protein (CSB).\textsuperscript{9} Transcription-dependent ICL repair uses nucleotide excision repair combined with translesion synthesis to repair crosslinks.\textsuperscript{16} Coimmunoprecipitation identified a direct interaction between CSB and SNM1A, and CSB enhanced the affinity of SNM1A for single-stranded DNA, highlighting a potential mechanism of directing nuclease activity towards damaged structures. While no detailed pathway has been proposed that involves SNM1A and CSB, this finding shows that the role of SNM1A is not limited to replication-dependent repair pathways.

A different role for SNM1A was proposed by the Legerski group,\textsuperscript{35,36,44} who observed that SNM1A binds to TP53-binding protein 1 (53BP1).\textsuperscript{44} SNM1A and 53BP1 were also found to interact with the anaphase-promoting complex, and SNM1A deficient cells failed to arrest at a mitotic checkpoint.\textsuperscript{36} In a more recent publication, it was reported that recruitment of SNM1A is dependent on the kinase ATM and that SNM1A is a phosphorylation substrate of ATM.\textsuperscript{35} Cells depleted in SNM1A did not arrest in the G1 phase following treatment with ionising radiation, and phosphorylation of cellular tumour antigen p53 (TP53) by ATM was reduced upon depletion of SNM1A. These results indicate that SNM1A plays a role in a mitotic checkpoint and the G1 checkpoint.

While some interaction partners of SNM1A have been identified, research into the exact components of the different pathways is difficult due to some redundancy between the proteins. It is plausible that SNM1A has different roles in different pathways, depending on interactions partners or posttranslational modifications. The low expression levels of SNM1A pose an additional obstacle to cellular studies.\textsuperscript{30} However, as is evident by the consequences of depleting it, its role in maintaining genome integrity is substantial.

\textbf{1.2 \hspace{1em} SNM1A – Structure and function}

Insights into the function of SNM1A can be gained from analysing its structure and the structures of its homologues, SNM1B and SNM1C.\textsuperscript{45} All three enzymes are orthologues of the yeast nuclease Pso2, which is involved in the repair of ICLs. Pso2 was initially characterised as a 5’-exonuclease\textsuperscript{46} but structure-specific endonuclease activity of Pso2 has since been reported.\textsuperscript{47} The SNM1 enzymes in
vertebrates are also nucleases, but they differ in their substrates and roles within the cell. SM1A has 5'-exonuclease activity \textit{in vitro}, and as for Pso2, structure-specific endonuclease activity has also been reported. SM1B is a 5'-exonuclease involved in telomere maintenance and is also implicated in ICL repair and checkpoint activation. SM1C acts both as an exonuclease and endonuclease, with its endonuclease activity depending on DNA-dependent protein kinase, catalytic subunit (DNA-PKcs). While all three vertebrate enzymes are homologues, they appear to carry out vastly different functions.

These different roles highlight the intricacy of ICL repair pathways in vertebrates compared to yeast and show that a variety of functions can be carried out by the same enzyme, depending on its interaction partners and/or modifications. Deciphering the functions of these repair proteins is therefore a challenging endeavour. The following sections summarise the current knowledge on the structure and function of SNM1A.

1.2.1 Biochemical characterisation

Several studies of the enzymatic activity of SNM1A \textit{in vitro} have been published. These reports have elucidated different aspects of SNM1A activity. Some of these studies used full-length SNM1A, while others employed the truncated catalytic domain. Similar activity was observed for both the truncated and full-length enzymes.

SNM1A is active as a monomer \textit{in vitro} and digests both single-stranded and double-stranded DNA, although it has a higher affinity for single-stranded DNA. It also processes ribonucleic acid (RNA), while short oligonucleotides (<10 nucleotides) are not digested efficiently. Longer substrates such as plasmids (~3000 base pairs) are hydrolysed with increased efficiency due to processive activity, where the substrate is not released fully between two hydrolysis events. A 5'-phosphate group was found to be essential for exonuclease activity. Importantly for its proposed biological function, SNM1A can hydrolyse oligonucleotides containing ICLs past the lesion. This activity was observed for the synthetic crosslinker SJG-136 and a triazole linkage. While depletion of SNM1A in the presence of crosslinking agents mitomycin C and cisplatin led to poorer survival in cellular assays, these ICLs cannot be introduced in a
site-specific manner, and activity of SNM1A towards substrates containing such lesions was therefore not confirmed in *in vitro* assays.

Variation of the buffers used highlighted the requirement for divalent cations such as Mg\(^{2+}\) or Mn\(^{2+}\) ions, while excess Zn\(^{2+}\) or Fe\(^{3+}\) ions inhibit SNM1A.\(^{37,39}\) The nuclease is particularly sensitive to the metal chelator \(\alpha\)-phenanthroline, and it was also inhibited by EDTA at higher concentrations.\(^{39}\) These findings point towards a metal-dependent hydrolysis mechanism.

More recently, endonuclease activity of SNM1A was reported.\(^{34}\) SNM1A showed hairpin opening activity when expressed in yeast. *In vitro* experiments showed that this endonuclease activity is specific to single-stranded regions and requires blocking of the 5′-end. This is in contrast to other studies, which did not observe any endonuclease activity.\(^{28,37}\) The difference between the reports is likely due to different concentrations used. In the experiments that detected endonuclease activity,\(^{34}\) stoichiometric amounts of enzyme compared to the DNA substrate were used, highlighting that endonuclease activity of SNM1A *in vitro* is substantially poorer than its exonuclease activity. However, this activity could potentially be enhanced by posttranslational modifications or interactions with other cellular components *in vivo* and might be one of several ways in which SNM1A contributes to ICL repair.

### 1.2.2 Structure and substrate binding

Crystal structures of the catalytic subunit of SNM1A and its homologue SNM1B have identified structural features related to the role of SNM1A in ICL repair.\(^{50}\) SNM1A and its homologues are members of the metallo-β-lactamase-associated CPSF Artemis SNM1/PSO2 (β-CASP) family.\(^{52}\) This family is a group of nucleic acid processing enzymes found in eukaryotes that are part of the metallo β-lactamase (MBL) fold superfamily. The MBL fold superfamily encompasses a large number of enzymes across different organisms that act on a wide range of substrates.\(^{53}\) Proteins of the MBL fold superfamily typically contain five motifs (motif 1-5) that participate in binding to the metal centre, but in the β-CASP family, motif 5 is replaced by the β-CASP domain.\(^{52}\) This domain contains several conserved motifs that differ between DNA- and RNA-processing enzymes and is inserted within the MBL fold domain.\(^{52}\)
The crystal structure of the catalytic domain of SNM1A (amino acids 676-1040) reported in 2015 provided further insights into the activity of SNM1A (Figure 1.3a).\(^{50}\) The ubiquitin-binding zinc-finger domain and PCNA interacting protein box described by Yang et al.\(^{42}\) are not part of this truncated structure. On its surface, SNM1A contains predominantly positively charged residues in the area surrounding the active site (Figure 1.3b), which is thought to permit processive digestion of large negatively charged DNA substrates. The active site of SNM1A is placed in a wide binding groove, which allows for processing bulky lesions such as ICLs. The introduction of point mutations in this groove identified several residues in this region that are required for efficient binding of the DNA substrate.\(^{50}\)

![Figure 1.3](image)  
**Figure 1.3:** a) Crystal structure of the catalytic domain of SNM1A (PDB: 4B87), b) electrostatic potential map of SNM1A, yellow arrow points at active site (image reproduced with kind permission from Oxford University Press).\(^{50}\)

The active site of SNM1A is thought to contain a di-metal centre, although only one zinc ion was observed in the crystal structure. The presence of two metal ions is supported by the pervasiveness of di-nuclear metal centres across the whole MBL fold superfamily.\(^{53}\) In addition, the nuclease SNM1B was crystallised with two zinc ions in its active site (Figure 1.4a),\(^{50}\) which is highly similar in structure to that of SNM1A. In both enzymes, one metal ion is in an octahedral environment, with four coordinating amino acid residues, three histidine residues and one aspartate residue.\(^{50}\) The remaining two coordination sites are occupied by a tartrate molecule from the crystallisation buffer in the structure of SNM1B. The other metal ion in the
active site of SNM1B is also in an octahedral coordination sphere and is held by three amino acid residues, one histidine and two aspartate residues, with one aspartate bridging the metal ions. Tartrate and a water molecule occupy the remaining coordination sites (Figure 1.4a). SNM1A contains the same residues in the active site but differs in the orientation of the non-bridging aspartate residue (highlighted in red, Figure 1.4b). This difference in orientation was postulated to cause weaker binding of the metal ion and may explain the lack of a second metal ion in the crystal. The aspartate residue (highlighted in red, Figure 1.4b) was shown to be required for enzymatic activity, as its mutation leads to complete loss of activity. This indicates that a second metal ion is involved in catalysis, analogous to SNM1B, although the identity of that metal ion is not confirmed. However, a group of more recent crystal structures of SNM1A also only observed one metal ion.\textsuperscript{54} In all 26 of these structures, a malonate anion was found to coordinate to the two free coordination sites of the single metal ion, occupying the sites that are postulated to be used by the phosphodiester group and an activated water molecule.

Based on these crystal structures, the following mechanism of phosphodiester hydrolysis can be proposed (Figure 1.5).\textsuperscript{55} The phosphodiester group coordinates to the two metal ions through two of its oxygen atoms, activating the group for nucleophilic attack and stabilising the 3’-oxygen as a leaving group. A bridging coordination site is occupied by a water molecule or hydroxide ion, which then attacks the phosphodiester, leading to hydrolysis. This mechanism is based on the hydrolysis mechanism of other MBL fold containing enzymes\textsuperscript{55} and the importance of the aspartate residue highlighted in Figure 1.4b to enzymatic activity.
Figure 1.5: Proposed mechanism of phosphodiester hydrolysis by SNM1A.

From this binding mode, hydrogen bonding interactions between the 5’-phosphate group and SNM1B were modelled and showed that the 5’-phosphate group forms hydrogen bonds with a serine residue and the amide backbone. Similar interactions are expected in SNM1A.

Overall, the structure of SNM1A exhibits several features that explain its observed activity. The positively charged surface aids with processivity of large substrates and the wide binding groove allows for the accommodation of bulky lesions. A binding pocket for the 5’-phosphate group explains the selectivity for substrates containing this group. Although the postulated di-metal centre could not be observed in the crystal structure, the analysis of its homologue SNM1B supports the presence of a second metal ion. While much insight has been gained from the crystal structure of SNM1A, its exact role in ICL repair and potential other cellular functions are still elusive.

1.2.3 Tools to study SNM1A and reported inhibitors

To aid the identification of inhibitors of SNM1A, a Förster resonance energy transfer (FRET) assay was developed by the McHugh group. This assay exploits the tolerance for bulky lesions and monitors the digestion of an oligonucleotide substrate containing a fluorophore and a quencher. Digestion by SNM1A removes the fluorescently-tagged nucleotide from the quencher and an increase in fluorescence is observed. Using this assay, four cephalosporins were identified as inhibitors of SNM1A. The strongest inhibitor, ceftriaxone (Figure 1.6a), was found to inhibit SNM1A with an IC$_{50}$ value of 4 μM and co-crystal structures with the enzyme were obtained. Two different binding modes of ceftriaxone were observed; in one of the binding modes the dicarbonyl moiety of ceftriaxone is coordinating to a metal ion in the active site (PDB: 5NZW, Figure 1.6b), while the other binding mode does not involve the metal centre (PDB: 5NZX).
This assay is a valuable tool for the high-throughput screening of inhibitors; however, as it does not capture the enzyme covalently, it does not provide any information about modulation of its activity via posttranslational modifications or interactions with other proteins.

1.2.4 Need for probes targeting SNM1A

SNM1A has been proposed to play several different roles in various stages of the cell cycle, and loss of its activity is associated with a variety of diseases. The *SNM1A* gene was identified as part of a functional module of genes that are significantly associated with survival of ovarian cancer patients, with higher expression linked to poorer prognosis. A study on familial breast cancer identified a non-sense mutation of the *SNM1A* gene as a candidate for predisposition to breast cancer. A single-nucleotide mutation in the *SNM1A* gene was found to be weakly associated with small cell lung carcinoma. In age-related cataract, the expression of *SNM1A* in lens epithelial cells was reduced compared to healthy controls. Knockout of the *SNM1A* gene in mice led to more bacterial infections and tumorigenesis. It is therefore apparent that both increased and decreased activity of SNM1A are related to disease.

SNM1A, like other DNA damage repair proteins, is also a potential target for overcoming resistance to crosslinking agents in cancers. SNM1A was found to be a determinant of sensitivity to chlorambucil and cisplatin in breast cancer; thus inhibiting SNM1A is a promising strategy to sensitisate resistant tumours towards...
crosslinking agents. However, much is yet to be discovered about its functions, posttranslational modifications and interaction partners. Significant impediments to research into SNM1A are its low expression levels and the toxicity resulting from overexpression.30 There is therefore an unmet need for tools to elucidate the functions of this nuclease, its modifications and its interaction partners.

Activity-based probes (ABPs) have emerged as powerful tools for quantifying enzymatic activity, as they measure the abundance of active enzyme by binding to the target in an activity-dependent manner.64 ABPs contain a recognition element that allows for recognition as a substrate, and a reactive group, often an electrophile, that forms a covalent bond to the enzyme. A fluorophore or affinity label then permits visualisation, or isolation and analysis (Figure 1.7a). In probes targeting metalloenzymes, the reactive group is replaced by a metal-binding group and often supplemented with a photo-crosslinking group to achieve covalent labelling.65,66 Applied to the nuclease SNM1A, such probes would consist of an oligonucleotide scaffold as a recognition element, a metal-binding group in place of a phosphodiester group, a photo-crosslinking group, and a fluorophore or affinity label (Figure 1.7b).

Figure 1.7: a) Structure of ABPs targeting enzymes that form a covalent enzyme-substrate intermediate, b) structure of probes targeting SNM1A.

While probes for other metalloenzymes have been reported,65-69 there are no reported probes for metallonucleases such as SNM1A to date. Key to their successful design is the incorporation of a metal-binding group into a nucleoside core in a manner that achieves strong binding while retaining recognition by the enzyme. Insights into such groups can be drawn from metal-coordinating inhibitors for metalloenzymes as well as probes for these enzymes. Additionally, phosphate bioisosteres used in medicinal chemistry are a valuable guide in identifying suitable metal-binding groups.
1.3 Inhibitors and probes for metalloenzymes

Metal ions are required for catalysis by approximately 40% of enzymes, as reported by Andreini et al. in 2008. Their analysis showed that in all enzyme classes, over 30% of enzymes contained a catalytic metal ion, excluding ions that provide structural stability. Some metals predominantly occur in one enzyme class, such as iron, copper and molybdenum which are most frequently found in oxidoreductases. Two thirds of the enzymes containing a catalytic zinc ion in this study are hydrolases, but zinc occurs in all enzyme classes. Magnesium and manganese on the other hand are found across all classes. The preference for certain metals in specific enzyme classes points at the different roles that metal ions play in catalysis. Non-redox active metal ions such as magnesium, calcium and zinc ions catalyse reactions by activating substrates and stabilising reactive species, transition states or intermediates. The ubiquity of metal ions in biological systems and their many roles in catalysis illustrate their importance in cellular function. Consequently, many metalloenzymes are implicated in disease and are therefore potential drug targets. The following section provides an overview of inhibitors and molecular tools that target metalloenzymes through binding to their active site metal ions.

1.3.1 Inhibitors of metalloenzymes

Inhibitors of metalloenzymes often contain a metal-binding group to anchor the inhibitor within the active site and a range of pharmacophores that bind in different pockets of the active site. Metal-binding groups can generally be described as mono- or bidentate ligands with one or two charged or polarised nitrogen, oxygen, sulfur or phosphorus atoms. Metalloenzyme inhibitors in clinical use often contain various metal binding groups, as summarised in recent reviews on metalloenzyme inhibitors for therapeutic applications. Metalloenzyme inhibitors that are currently in clinical use include those shown in Table 1.1, which were chosen to illustrate the different metal binding groups used to target different enzymes. Table 1.1 only includes compounds with crystallographic evidence for metal coordination and is limited to one example per metal-binding group, even if several analogues are in clinical use.
Table 1.1: Drugs in clinical use that inhibit metalloenzymes through a metal-binding group as confirmed by X-ray crystallography. a: active metabolite of fosinopril prodrug, b: structure of closely related analogue AN2898.

The structures and metal binding modes of the inhibitors listed in Table 1.1 are shown in Figure 1.8 for single metal ions and in Figure 1.9 for inhibitors targeting enzymes with di-metal centres.
Chapter 1 – Introduction

Figure 1.8: Binding of metalloenzyme inhibitors to the single metal ion of their target based on co-crystal structures. Metal binding groups are highlighted in red, curved lines represent the coordination sphere of the metal ion. a: active metabolite of fosinopril obtained by hydrolysis of the phosphinate ester.

Voriconazole (3, Table 1.1, entry 1) and ritonavir (4, Table 1.1, entry 2) are drugs that target enzymes containing heme iron in their active site and therefore bind to the metal ion in a monodentate manner. Acetazolamide (5, Table 1.1, entry 3) is one of many sulfonamide-based carbonic anhydrase inhibitors that bind to the active site zinc ion through the nitrogen of the sulfonamide group. This prevents a water molecule from binding to the metal centre and subsequently attacking a carbon dioxide molecule to form bicarbonate. Carbonic anhydrase inhibitors are frequently used in the treatment of glaucoma and epilepsy, among others. Vorinostat (6, Table 1.1, entry 4) is a hydroxamic acid-containing histone deacetylase inhibitor that is used as an anti-cancer drug. The angiotensin converting enzyme inhibitors captopril (7, Table 1.1, entry 5), fosinoprilat (8, Table 1.1, entry 6) and lisinopril (9, Table 1.1, entry 7) use different zinc-binding groups to inhibit the same enzyme. While captopril contains a thiol, fosinoprilat
uses a phosphinate\textsuperscript{79} and lisinopril employs a carboxylic acid group\textsuperscript{80} to coordinate to the active site metal ion. All three are used to treat cardiovascular diseases. The phosphinate group of fosinoprilat, the active metabolite, is masked as an ester in the prodrug fosinopril and released by hydrolysis after oral administration.\textsuperscript{87}

Three inhibitors binding to di-metal centres are shown in Figure 1.9.

![Figure 1.9: Binding of metalloenzyme inhibitors to the di-metal centre of their target based on co-crystal structures. Metal binding groups are highlighted in red, curved lines represent the coordination sphere of the metal ion. a: active form of crisaborole obtained by attack of hydroxide ion in active site.](image)

Crisaborole (10, Table 1.1, entry 8) is a boron-based inhibitor of phosphodiesterase 4 used to treat eczema.\textsuperscript{88} Phosphodiesterase 4 cleaves cyclic AMP at a di-metal active site using a bridging hydroxide ion. The benzoxaborole is attacked by this hydroxide ion, resulting in a tetrahedral boron centre with strong binding to the two metal ions.\textsuperscript{81} Dolutegravir (11, Table 1.1, entry 9) and elvitegravir (12, Table 1.1, entry 10) are HIV-1 integrase inhibitors.\textsuperscript{89} HIV-1 integrase contains two bivalent metal ions in its active site, thought to be Mg\textsuperscript{2+}, that form key interactions with the inhibitors. Both compounds contain a tri-oxygen scaffold that allows for chelation of two bivalent metal ions. Dolutegravir employs a β-hydroxy-γ-keto amide whereas elvitegravir uses a β-keto acid to chelate the two metal ions.

As illustrated by the range of metal-binding groups in Table 1.1 and Figures 1.8 and 1.9, the specific binding requirements of each enzyme need to be considered in the development of inhibitors. For instance, inhibitors targeting heme iron are unlikely to benefit from a bidentate ligand due to the lack of available coordination sites. Hydroxamic acids are a strong metal-binding group in histone deacetylase inhibitors due to their chelation to the zinc ion through two oxygen atoms.
Surprisingly, in carbonic anhydrase inhibitors this group binds through the
deprotonated nitrogen atom, leading to much weaker binding.\textsuperscript{90}

Two enzyme classes with inhibitors in clinical use are of particular interest for the
design of probes targeting SNM1A. Phosphodiesterases such as HIV-1 integrase
contain a di-metal centre similar to SNM1A. Even though the exact structure of the
active site differs among phosphodiesterases,\textsuperscript{91} using groups to chelate two metal
ions simultaneously is an approach that is potentially applicable to SNM1A. Matrix
metallopeptidases are a class of zinc metalloenzymes that have been the subject of
significant research efforts with limited success.\textsuperscript{92} While many inhibitors were
identified \textit{in vitro}, the structural similarity between the different enzymes led to
problems with selectivity and toxicity and only one inhibitor has been approved for
clinical use.\textsuperscript{93} Nonetheless, the large volume of research on these enzymes
provides insights that can aid the development of probes for other metalloenzymes.

\subsection{1.3.1.1 Inhibitors of phosphodiesterases}

Phosphodiesterases have many different roles in physiology.\textsuperscript{91} They are implicated
in various conditions such as eczema,\textsuperscript{88} respiratory diseases,\textsuperscript{94} cardiovascular
disease\textsuperscript{95} and erectile dysfunction.\textsuperscript{96} Of the phosphodiesterase inhibitors approved
for clinical use, only five are metal-binding (see Section 1.3.1). These inhibitors
target phosphodiesterase 4 and HIV-1 integrase.

Phosphodiesterase 4 hydrolyses cyclic AMP and is implicated in inflammatory
diseases in particular. Only one Of the phosphodiesterase 4 inhibitors in clinical use
is metal-binding.\textsuperscript{72} Crisaborole (\textbf{10}) mimics the tetrahedral phosphodiester group of
the substrate through a benzoxaborole scaffold that reacts with the coordinated
water molecule.\textsuperscript{81} Its development was a departure from the established
pharmacophores of other phosphodiesterase inhibitors, which exploit the
hydrophobic interactions of the binding pocket but do not bind the metal ions.\textsuperscript{97,98}
Some phosphodiesterase inhibitors interact with the active site metal ions through
a coordinated water molecule, and only a small number bind to the metal ion(s)
directly (\textbf{Figure 1.10}).\textsuperscript{98}
Figure 1.10: Phosphodiesterase inhibitors that bind directly to the metal ions. Metal-binding group are highlighted in red.

Zardaverine (13) has been cocrystallised with phosphodiesterase 4D in different conformations, with two of them involving direct coordination of the carbonyl group to the zinc ion. The chelating binding mode of benzoxaboroles crisaborole (10) and AN2898\(^\text{81}\) (15, 16) that exploits the coordinated water molecule to generate the active tetrahedral form (Figures 1.9 and 1.10) is unique among clinically approved phosphodiesterase inhibitors.

HIV-1 integrase is a nuclease that integrates the viral genome into the host genome. This involves two processes: cleavage of two nucleosides from the 3'-end of the viral reverse-transcribed DNA and incorporation of this viral DNA into the host DNA. Both processes, hydrolysis of the viral DNA and activation of the resulting 3'-hydroxyl group, and activation of the phosphodiester group of the host DNA for attack, involve metal catalysis.\(^99\)

Currently four inhibitors of HIV-1 integrase are approved for clinical use.\(^89\) All approved integrase inhibitors prevent the strand transfer into the host DNA by coordination of the metal centre and displacement of the viral DNA, ensuring that the viral DNA is not positioned correctly for attack.\(^99\) Early inhibitors can be divided into natural products and synthetic compounds, with many of the natural products containing a diketo- or β-hydroxy-keto motif.\(^100,101\) Similarly, in the synthetic compounds a diketo acid motif was identified to inhibit the strand transfer reaction\(^102\) through interaction with the active site metal ions.\(^103\) Figure 1.11 shows the structures of several integrase inhibitors that were evaluated in clinical trials.
The most potent inhibitor of the initial series, L-731,988 (16), inhibits HIV-1 replication with an IC\textsubscript{50} of 1 \(\mu\)M\textsuperscript{102}. This marked the beginning of optimisation efforts, leading to naphthyridine-carboxamide derivates such as L-870,810\textsuperscript{104} (17), GSK-364735\textsuperscript{105} (18) and GS-9160\textsuperscript{106} (19) (Figure 1.11) which all reached clinical trials. The research into pharmacologically potent diketo acid derivatives culminated in the approval of raltegravir (20). Since then, dolutegravir (11, Figure 1.9), elvitegravir (12, Figure 1.9), and bictegravir (21, Figure 1.11) have also successfully completed clinical trials.\textsuperscript{89} Although dolutegravir (11) and raltegravir (20) contain the same metal-binding group, the two inhibitors bind in different orientations. These examples highlight the use of an extended metal-chelating scaffold to achieve potent inhibition of a metalloenzyme.

Progress in the inhibition of phosphodiesterases has been slowed by the ubiquity and similarity of many of these enzymes. The success in targeting viral nucleases with no human orthologue illustrates that these enzymes are druggable, but challenges with selectivity remain. However, the range of pharmacophores binding to metal centres is expanding, as exemplified by the use of the benzoazaborole moiety.

1.3.1.2 Inhibitors of matrix metallopeptidases

Matrix metallopeptidases (MMPs) are one class of metallopeptidases that has been difficult to target, but is of interest to cancer therapy.\textsuperscript{71} There are over twenty MMPs
known to date, with important roles in a large variety of cellular processes, such as ageing, cancers and inflammatory conditions. A significant challenge in targeting MMPs for therapeutic use is the high degree of homology among the different enzymes. Therefore, a large amount of research has been dedicated to the design of selective and potent MMP inhibitors, with many relying on metal chelation. Insights gained from decades of research in this field are valuable in the identification of suitable metal-binding groups for probes targeting nucleases such as SNM1A.

To date, only one MMP inhibitor, doxycycline, has been approved for clinical use, but its mode of inhibition is unlikely to involve binding to the active site zinc ion. Initially, the focus in inhibitor design was on achieving strong zinc-binding. This is evident in a review on MMP inhibitors by Whittaker et al. published in 1999. The first inhibitors were substrate-based peptides, using the peptide sequence of the cleavage site and a zinc-binding group. In these early inhibitors, hydroxamic acids emerged as a particularly potent zinc-binder, and many inhibitors containing this group or its derivatives were synthesised and evaluated (Figure 1.12). The positioning of the hydroxamic group was shown to influence the binding mode, with succinyl hydroxamates such as marimastat displaying a substrate-like binding mode and malonate hydroxamates such as adopting a non-substrate-like binding mode. Structures containing the succinyl hydroxamate motif were generally stronger inhibitors and have been optimised extensively. Two such inhibitors, batimastat and marimastat, were tested in clinical trials but were unsuccessful due to toxicity and lack of therapeutic benefit.

![Figure 1.12: MMP inhibitors with different hydroxamic acid or hydroxamic acid-derived zinc-binding groups. Zinc-binding groups are highlighted in red.](image-url)
More recently, research efforts have been directed away from hydroxamic acids, with examples highlighted in a review by Jacobsen et al.\textsuperscript{112} A popular strategy to replace hydroxamic acid groups but maintain metal binding was to replace the group by other nitrogen- and oxygen-containing derivatives. For example, carboxylic acid-containing MMP inhibitors such as 24 have shown good activity and selectivity.\textsuperscript{113,114} The hydroxamic acid derived hydrazide group and sulfonyl hydrazide have also been explored with some success. Sulfonyl hydrazide 25 for example was reported as a selective MMP-9 inhibitor following optimisation.\textsuperscript{115} N-Hydroxyurea groups surprisingly showed significantly lower potency than their hydroxamic acid analogues when evaluated against MMP-8; this was explained by the monodentate binding mode observed in a co-crystal structure with MMP-8.\textsuperscript{116} A series of squaric acid derivatives were also tested against MMPs as they were expected to form a six-membered ring by chelating the metal ion similarly to hydroxamic acids. The thiocarbonyl N-hydroxysquaramide 26 emerged as the most potent of the series.\textsuperscript{117} This finding illustrates the potential of using sulfur-based zinc-binding groups in addition to oxygen- and nitrogen-containing groups.

The incorporation of sulfur and phosphorus is another commonly used strategy in the design of MMP inhibitors (\textit{Figure 1.13}). The inhibitor rebimastat (27) contains a thiol as its zinc-binding group. It inhibits MMP-2 and MMP-9 and was tested in several clinical trials, up to phase III.\textsuperscript{118} Mercaptosulfides such as compound 28 were found to be active against a range of MMPs. The cyclic pyrrolidine structure provides increased water solubility as well as protection against degradation.\textsuperscript{119}

\textbf{Figure 1.13:} MMP inhibitors with zinc-binding groups based on sulfur and phosphorus. Zinc-binding groups are highlighted in red.
Phosphorus-based inhibitors include phosphonates, phosphinates and carbamoyl phosphonates and have been the subject of a review by Veerendhar et al.\textsuperscript{120} Bisphosphonates, more stable analogues of pyrophosphoric acid, chelate metal ions and have therefore been examined as MMP inhibitors. The bisphosphonate clodronate (29) inhibits MMP-1 with an IC\textsubscript{50} value in the micromolar range.\textsuperscript{121} Other phosphonates such as compound 30 make use of the interactions that were optimised for hydroxamic acid-based inhibitors to achieve higher potency and selectivity.\textsuperscript{122} Phosphinates can also coordinate to the zinc ion\textsuperscript{123} and, depending on the substituents, some selective inhibitors such as 31 have been reported.\textsuperscript{124} Carbamoyl phosphonates mimic the five-membered ring chelation of hydroxamic acids through the installation of a carbonyl group in $\alpha$-position to the phosphonate group, as in compound 32. This small molecule inhibits MMP-1 and MMP-2 with sub-micromolar IC\textsubscript{50} values, despite the lack of large groups to fill the binding pockets.\textsuperscript{125} This finding illustrates how relying primarily on metal coordination can achieve strong binding.

Bidentate binding has also been attained using substituted heterocycles (\textbf{Figure 1.14}). Pyrimidine-2,4,6-trione-containing compounds such as 33 have been found to bind to the active site metal ion of MMPs.\textsuperscript{126} Some more recently explored zinc-binding groups aim to maintain the beneficial five-membered ring chelation of hydroxamic acids, improve the metabolic stability and rigidify the group through cyclisation. For example, the inhibitor 34 contains the hydroxamic acid-like hydroxypiperidine-2,6-dione group for metal coordination and showed nanomolar affinity for MMP-12 and good selectivity over other MMPs.\textsuperscript{127} The addition of thiones was shown to be beneficial by Puerta \textit{et al.} in the screening of a range of heterocyclic zinc binding groups.\textsuperscript{128} Compound 35 lacks any peptidomimetic moieties that could interact with the binding pockets of the enzyme, but had an IC\textsubscript{50} value of 35 $\mu$M in an assay against MMP-3. Inspired by zinc ion sensors, some novel inhibitors such as in inhibitor 36 use a combination of sulfonamides and heterocycles to achieve bidentate chelation.\textsuperscript{129} This compound has an IC\textsubscript{50} value of 6.9 $\mu$M against MMP-2 and is selective over three other MMPs that were evaluated.
Throughout the years of research into MMP inhibitors, the focus has shifted away from combining the most powerful zinc-binding groups with suitable substituents to fill binding pockets towards weaker but more selective binding. The nature of the zinc-binding group itself also affects their potency and selectivity, as was explored by evaluating the same inhibitor with three different zinc-binding groups. Recent reviews of MMP inhibitors highlight the shift in inhibition strategies from small molecule active site inhibitors to allosteric inhibitors, monoclonal antibodies and protein-engineered tissue inhibitors of MMPs. While strong metal chelation may not lead to beneficial pharmacological properties, it is highly desirable in the design of probes targeting metalloenzymes. In the traditional metal-binding inhibitors, the hydroxamic acid group appears to be the most widely applicable group that achieves strong binding. Even though this makes it unfavourable for achieving selectivity, it is a good property for ABPs that provide selectivity through the recognition element.

1.3.2 Activity-based probes for metalloenzymes

Despite the abundance of inhibitors for metalloenzymes, probing them is inherently challenging due to the nature of the interaction between the enzyme and the substrate. In enzymes that form a covalent enzyme-substrate intermediate via attack of a nucleophilic residue, an electrophilic probe can be used to form a covalent enzyme-probe complex (Figure 1.15a). However, when nucleophilic attack is carried out by water instead of an amino acid residue, probe binding relies on weaker, reversible interactions such as metal coordination. A photo-crosslinking group that binds non-selectively to nearby amino acid residues is frequently employed to covalently link the probe to the enzyme following the formation of the reversible enzyme-probe complex (Figure 1.15b). This method has expanded the scope of activity-based protein profiling to metalloenzymes.

Figure 1.14: MMP inhibitors with heterocyclic zinc-binding groups. Zinc-binding groups are highlighted in red.
This methodology was first reported for profiling metallopeptidases\(^{65,69}\) and has since been applied to some other enzyme classes such as histone deacetylases.\(^ {67}\)

The structure of ABPs targeting these enzymes, the insights they have provided and the need for such probes for nucleases are discussed in the following sections.

### 1.3.2.1 Activity-based probes for metallopeptidases

The first ABPs for a metalloenzyme were independently reported by the Cravatt group\(^ {65}\) and the Yao group\(^ {69}\) in 2004. Both probes target metallopeptidases and consist of a hydroxamate-based inhibitor, a photo-crosslinking group and a fluorophore (Figure 1.16). The probe reported by the Cravatt lab\(^ {65}\) (Figure 1.16a) used the structure of known peptidic MMP inhibitors, incorporated a benzophenone group in place of a large hydrophobic group, and tethered a rhodamine dye for visualisation. A probe containing both a dye and a biotin tag was also synthesised. The potency of the probe 37 without crosslinking was found to be similar to that of parent inhibitors marimastat (22) and GM6001, and under crosslinking conditions, labelling of active MMP-2 but not of inactive zymogen or inhibitor-bound enzyme was observed. The probe was also validated in cell lysate spiked with MMP-2,
detecting the enzyme at concentrations where MMP-2 makes up 0.02% of the proteome. This is comparable to the sensitivity of other ABPs. The broad-spectrum activity of the probe was illustrated in cancer cells, where an upregulated metallopeptidase was identified as an unexpected target of GM6001, as it shares no sequence homology with the intended target family. The Yao group used a peptide scaffold containing a hydroxamic acid group and a cyanine dye or a biotin handle to probe metallopeptidases (Figure 1.16b). The peptide scaffold allows for the facile variation of the sequence which can be used to tune the selectivity of this probe. Similar to the probes reported by the Cravatt group, the inhibitory potency of the probe was confirmed and its specificity for metallopeptidases over other enzymes was confirmed. Again, the requirement for active enzyme was validated, and the sensitivity in cell lysate was found to be down to 0.03% of the proteome. This work compared two different photo-crosslinking groups, diazirine and benzophenone, and found that diazirine-containing probes showed higher sensitivity. Variations of the diazirine probe with different sequences were tested against a panel of yeast proteases.

**Figure 1.16:** Structures of selected APBs for metallopeptidases reported by a) Saghatelian et al., b) Chan et al., and c) Sieber et al. Rho = rhodamine dye, Cy3 = cyanine3. Metal-binding groups are highlighted in red, variable residues are highlighted in blue.
Following on from the first reported probe, the Cravatt group published a library of probes to target the whole superfamily of metallopeptidases.\textsuperscript{68} This approach used a hydroxamic acid group bound to a peptide scaffold and an alkyne handle in place of a fluorophore or biotin tag. Three different libraries were designed (\textit{Figure 1.16c}), with each library \textit{39, 40} and \textit{41} having a common scaffold and members of the library differing in one amino acid residue. Incubation of the different probes with cell lysate revealed distinct labelling patterns. A comparison with the rhodamine dye-containing probes highlighted the improved labelling of the alkyne probes, stressing the influence of reporter tags on binding.

Further probes reported since then have built on this scaffold\textsuperscript{133-135} and some were validated in an \textit{in vitro} model of posttraumatic osteoarthritis.\textsuperscript{136} Using radiolabels in place of fluorophores improved sensitivity,\textsuperscript{137,138} and such probes were found to be sensitive enough for measurement in a murine disease model.\textsuperscript{139} Immobilisation of hydroxamate-modified peptides was successfully employed to enrich metallopeptidases\textsuperscript{140} and a probe library prepared by click chemistry was used in a microarray to analyse the specificity profile of different metallopeptidases,\textsuperscript{141} highlighting the wealth of potential applications for such probes. Despite the further development and optimisation of these probes, the hydroxamic acid group has remained the metal-binding group of choice, with the exception of reports by the Dive group that use a phosphinate group.\textsuperscript{137-139}

\subsection*{1.3.2.2 Activity-based probes for histone deacetylases}

Histone deacetylases are another class of metalloenzymes that ABPs have been developed for. Their structure is generally based on known inhibitors and, like probes for metallopeptidases, a photo-crosslinking group is used to covalently capture the target (\textit{Figure 1.17}). The first ABP for histone deacetylases was reported by Salisbury and Cravatt\textsuperscript{67} and was based on the known hydroxamic acid inhibitor vorinostat (see Section 1.3.1). This probe \textit{42} (\textit{Figure 1.17a}) utilises the metal-binding hydroxamic acid group, while a benzophenone group was introduced to covalently link the probe to the enzyme upon exposure to light. An alkyne handle was installed to allow for the introduction of fluorophores or affinity labels. Labelling experiments carried out with probe \textit{42} identified not only histone deacetylases but also associated proteins.\textsuperscript{67} Since histone deacetylase activity is highly dependent on interaction with other proteins, this makes this method a valuable tool for
Chapter 1 – Introduction

illuminating the roles of the different interaction partners. Building on this work, a comparison of the initial probe 42 with other closely related probes such as probe 43 (Figure 1.17a) in live cells found the initial probe 42 to be superior.66 Remarkably, probe 42 showed higher selectivity and lower background labelling in live cells compared to other probes, despite their superior labelling in cell fractions. Additionally, inhibitory potency of the probes did not correlate with the labelling strength, showing that other factors such as orientation of the photo-crosslinking group heavily influence labelling efficiency once binding is established.

![Figure 1.17](image)

Figure 1.17: Structures of selected ABPs for histone deacetylases reported by a) Salisbury et al.,66,67 b) Xu et al.,142 c) Albrow et al.,143 d) Diyabalange et al.144 Metal-binding groups are highlighted in red.

Probes 44 (Figure 1.17b) employs a different metal-binding group and was used to confirm histone deacetylase 3 as the target of the parent inhibitor.142 Work by Albrow et al.,143 (Figure 1.17c) focused on the expansion of the probe repertoire to include class IIa histone deacetylases. For this, the non-selective hydroxamic acid-based inhibitor pandacostat was used as the starting point for the design of probe 45, while probe 46 (Figure 1.17c) is derived from class IIa-selective inhibitors, which bind in
their hydrated form. A very different approach to achieving covalent linking as a result of histone deacetylase activity was reported by Diyabalanage et al.\textsuperscript{144} who employ an acetyl-protected enamine as the reactive moiety in their probe \textsuperscript{47} (\textit{Figure 1.17d}). The acetyl group is removed by active histone deacetylases, giving an enamine which can be hydrolysed to the aldehyde \textsuperscript{48}. This reactive aldehyde then reacts non-specifically with amines such as lysine residues on proteins. This strategy does not necessarily provide covalent linking to the target enzyme, but rather traps the probe in the cell in case of histone deacetylase activity, providing a way to trap compounds preferentially in cells with high deacetylase activity.

Inhibitors for histone deacetylases have also been employed for enriching their binding partners without the use of photo-crosslinking groups. The immobilisation of two inhibitors on resin was used as a platform to identify unanticipated targets.\textsuperscript{145} Similarly, libraries of peptide probes containing hydroxamic acids in known acetylation sites were used to probe sequence specificity.\textsuperscript{146,147} Overall, the use of chemical probes for histone deacetylases has shown promise for use in tissues\textsuperscript{148} and disease models\textsuperscript{142} and has helped to elucidate different factors that influence activity in different cell types.\textsuperscript{149} Nonetheless, challenges remain in tuning selectivity.

\subsection*{1.3.2.3 Probes for DNA repair enzymes and nucleases}

Substantial information about the roles of DNA repair proteins has been obtained from studies using nucleic acids containing photo-crosslinking groups (\textit{Figure 1.18}). The first example of using nucleic acids modified with a photo-crosslinker to interrogate their interactions with proteins was reported in 1974 by Lin and Riggs.\textsuperscript{150} The installation of 5-bromodeoxyuridine into the \textit{lac} operator DNA sequence was found to lead to covalent binding to \textit{lac} repressor protein under ultraviolet light and confirmed the specificity of this interaction. Since then, this photoaffinity labelling approach has been expanded to mimic intermediates in repair pathways to capture the proteins that bind to these structures. The roles of several proteins of the base excision repair pathway, namely flap endonuclease 1 (FEN1),\textsuperscript{151-154} RPA,\textsuperscript{151,153,155,156} poly(ADP-ribose) polymerase-1 (PARP1),\textsuperscript{154,157} DNA polymerase \( \beta \),\textsuperscript{154,158-160} apurinic/apyrimidinic (AP) endonuclease,\textsuperscript{154,161-163} DNA polymerase \( \lambda \),\textsuperscript{160} XRCC1,\textsuperscript{154,163} DNA polymerase \( \varepsilon \)\textsuperscript{164} and DNA damage checkpoint protein 1\textsuperscript{164} have been elucidated this way. Similarly, insights into the roles of proteins involved in the nucleotide excision repair pathway such as RPA,\textsuperscript{165-170} XPC-HR23B...
complex,\textsuperscript{166,167,169,171,172} XPA,\textsuperscript{166,167,169,170} UvrA,\textsuperscript{173} UvrB,\textsuperscript{173} PARP1\textsuperscript{174} and centrin 2\textsuperscript{175} have been gained through use of photoaffinity labelling.\textsuperscript{176,177} Of these repair proteins, the human enzymes FEN1, AP endonuclease, DNA polymerase ε, and bacterial proteins UvrA and UVrB have nuclease activity.\textsuperscript{178} The Carrell group have synthesised and evaluated different affinity probes for studying DNA-protein interactions and have successfully trapped proteins involved in the repair of the oxidative DNA lesion 8-oxo-2'-deoxyguanosine.\textsuperscript{179,180} Valuable insights into preferences for certain structures\textsuperscript{181,182} and the interactions between different repair proteins have been obtained using photoaffinity probes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_18}
\caption{Analysis of proteins that interact with nucleic acids using photoaffinity probes.}
\end{figure}

Fluorescence-based assays, among other methods, have been developed to measure nuclease activity and have been applied to DNA repair enzymes.\textsuperscript{183} These assays rely on a quenching mechanism that is altered as a result of nuclease activity, such as cleavage of a duplex required for intercalation of a dye (\textit{Figure 1.19a}) or separation of a fluorophore-quencher pair (\textit{Figure 1.19b}). Work by Wang \textit{et al.}\textsuperscript{184} uses a double-stranded DNA substrate that shows selectivity for micrococcal nuclease over other nucleases and a nucleic acid dye (\textit{Figure 1.19a}). Intercalating of this dye into the intact substrate enhances its fluorescence, but in the presence of micrococcal nuclease, the degradation of the substrate prevents this enhancement. In the context of DNA ICL repair, Thomas \textit{et al.}\textsuperscript{185} developed a FRET assay for XPF-ERCC1 (\textit{Figure 1.19b}). The substrate for this endonuclease consists of a DNA fork structure that contains a fluorophore and quencher on opposite strands, which are separated upon cleavage by XPF-ERCC1. Many similar assays have been developed to target different nucleases and elucidate preferences for different nucleic acid structures.\textsuperscript{183,186}
Figure 1.19: Analysis of DNA repair enzymes and nucleases using fluorescence-based assays with a) an intercalating dye, b) a fluorophore-quencher pair.

Fluorescence assays are useful tools to measure nuclease activity, but because they do not capture the enzymes, they allow for little analysis of the enzyme and potential posttranslational modifications. By combining photoaffinity-labelling with activity assays, like in ABPs, a more complete picture can be obtained. This is expected to be particularly valuable in the context of nucleases that are tightly regulated and poorly characterised.

1.4 Phosphate replacements in medicinal chemistry and nucleic acids

As SNM1A hydrolyses phosphodiesters, any potential metal-binding group for an ABP targeting SNM1A needs to resemble a phosphodiester group to be able to bind to the active site. Phosphate groups are common recognition sites for enzymes, but the enzymatic lability of phosphate groups and the poor membrane permeability conferred by them pose obstacles for their incorporation in therapeutics and probes. Phosphate bioisosteres are used in medicinal chemistry to overcome these problems. They are also used in small interfering ribonucleic acid (siRNA), as the activity of siRNA depends on 5'-terminal phosphorylation by kinases. The need for phosphorylation limits the chemical modification of their backbone since extensive modification can compromise recognition by kinases and thus prevent phosphorylation. Phosphodiester groups in the backbone of nucleic acids are also a point of vulnerability in the application of nucleic acid therapeutics, as they are susceptible to non-specific hydrolysis by nucleases. For this reason, many different alternative nucleic acid backbones have been developed to increase stability while retaining recognition. The following sections discuss phosphate bioisosteres and alternative nucleic acid backbones with a view to identifying suitable metal-binding phosphate replacements for ABPs targeting SNM1A.
1.4.1 Phosphate bioisosteres

Phosphate bioisosteres are used in place of phosphate groups in small molecule inhibitors and siRNA to replace the labile group with a more metabolically stable and often less polar group, while maintaining recognition by the target biomolecule. Many phosphate bioisosteres, such as phosphonates, phosphorothioates,192 and boranophosphates,193,194 are phosphorus-based. In phosphonates (Figure 1.20a), a bridging oxygen atom is replaced by a methylene group. While this renders the group stable to hydrolysis, it also alters the pKₐ and often lowers the biological activity.195 To offset this increase in pKₐ, halogenation of the methylene group is frequently employed.196 This also creates an electrophilic centre for attack by a nucleophilic residue, turning reversible inhibitors into covalent inhibitors. In phosphorothioates, (Figure 1.20b) a non-bridging oxygen is substituted by a sulfur atom. Phosphorothioates have found applications both as a backbone replacement for oligonucleotides as well as a bioisosteres in nucleotides and other small molecules. An example is the use of the phosphorothioate group as part of adenine nucleotide analogues as neuroprotective agents.197 Boranophosphates (Figure 1.20c) contain a negatively charged borane group in place of a non-bridging oxygen, preserving the charge of the group. Their incorporation into nucleoside triphosphates increased specificity for the HIV reverse transcriptase over other polymerases.198

![Figure 1.20](image)

Figure 1.20: Structures of commonly used phosphate bioisosteres. a) phosphonate, b) phosphorothioates, c) boranophosphates, d) malonate, e) squaramide with charged resonance form.

Carbon-based bioisosteres include malonates and squaramides. Malonate-based isosteres (Figure 1.20d) preserve the negative charge of the phosphate group, while squaramides (Figure 1.20e) are neutral replacements. Despite being overall uncharged, squaramides are highly polarised, as illustrated by the aromaticity of the resonance form with two anionic oxygen atoms (Figure 1.20e). Carbon-based phosphate bioisosteres such as malonates and squaramides are of interest as potential metal-binding groups for two reasons. Their structure allows for connection
to the nucleoside core in different positions, and hydroxamic acid-like groups can be installed. Therefore, these two groups of bioisosteres are the focus of the following section.

Malonates have found application as phosphate isosteres in a wide variety of structures (Figure 1.21), such as phosphosugars, nucleotides, phosphorylated amino acids and potential herbicides. Malonate-containing mannose-6-phosphate (M6P) mimic dimers have been evaluated as replicates of biantennary oligosaccharides binding to M6P/Insulin-Like Growth Factor II Receptor. The malonate-based inhibitor (Figure 1.21) of an aldose-ketose isomerase was reported by Foret et al., which contains the malonate group in the 6-position of β-mannose. In work by Fei et al., glucosamine-6-phosphate analogues, including malonyl ether (Figure 1.21) were synthesised and evaluated for their ability to catalyse the self-cleavage of the glmS ribozyme. In this context, the phosphate bioisostere is interacting with nucleic acids rather than amino acids, and metal coordination is thought to be important for positioning. The malonyl ether (Figure 1.21) was found to activate self-cleavage and docking studies showed that the malonyl group chelates two metal ions. The incorporation of a malonate group as part of a pyrophosphate replacement of a nucleoside triphosphate was found to retain recognition by HIV-1 reverse transcriptase, and nucleotide analogue (Figure 1.21) was incorporated into DNA strands by the enzyme. A malonate group incorporated at the 5'-position of siRNA showed similar silencing activity to the natural phosphate analogue, but was more metabolically stable.

Figure 1.21: Examples of biologically active molecules containing a malonate group (highlighted in red) as phosphate bioisosteres.
The use of malonate groups as phosphate bioisosteres in phosphotyrosine analogues has resulted in several inhibitors, for example for growth factor receptor-bound protein 2 (52, Figure 1.21),205 protein tyrosine phosphatase 1B (53, Figure 1.21),206 or purple acid phosphatases.207 The Sikorski group has published multiple papers on 5-enolpyruvoyl-shikimate-3-phosphate synthase inhibitors as potential herbicides, some of which feature malonate groups as phosphate bioisosteres (54, Figure 1.21).208

While squaramides have no overall charge, their carbonyl oxygen atoms carry a partial negative charge.211 Their application as phosphate bioisosteres has been reported for nucleotide analogues,211-217 phosphotyrosine mimics218 and phospholipid analogues.219,220 The first reported successful use of squaramides as phosphate mimics was by Sato et al.211 who used the group as an alternative oligonucleotide backbone. As part of this work, the dinucleoside 55 (Figure 1.22) was synthesised. Analysis of its structure confirmed its similarity to the native phosphodiester bond. The squaramide group appears to coordinate magnesium ions and ab initio calculations found the carbonyl groups to be highly polarised. Following on from this work, the 2,5'-squaramide linked dinucleoside 56 (Figure 1.22) was synthesised.212 Both these dinucleosides were incorporated into oligonucleotides and formed base pairs with a complementary strand. A further publication from the Sekine group213 reported the synthesis of a series of nucleotide and cyclic nucleotide analogues. Cyclic squaramide nucleotide mimics such as compound 57 (Figure 1.22) were found to adopt a similar structure to the natural cyclic nucleotides. Sugar-nucleotide analogues containing a squaramide moiety such as 58 (Figure 1.22), where the sugar moiety is replaced by a nitrobenzyl group, were reported to inhibit a mannosyltransferase found in Trypanosoma brucei.214 In work by Soukarieh et al.,216 a crystal structure of the eukaryotic translation initiation factor 4E with compound 59 bound in the active site was obtained.
Like malonates, squaramides have also been employed in phosphotyrosine mimics. Squaramide 60 (Figure 1.22) was designed in an effort to identify less charged phosphotyrosine mimics for incorporation into protein tyrosine phosphatase inhibitors and exhibited modest inhibition. In the context of larger inhibitors, this group has potential to improve the stability and permeability of phosphotyrosine based inhibitors. Squaramides have also been used as isosteres for phospholipids, and amphiphiles like 61 (Figure 1.22) showed promise for potential applications in liposome-based therapies. The phosphoglycolipid analogue 62 (Figure 1.22) was designed as an activator of G protein coupled receptor 55 and showed activity similar to its natural analogue.

The use of carbon-based phosphate bioisosteres in the literature illustrates the potential of these groups. Nonetheless, the positioning of the group appears to be essential to achieving strong interactions, as is evident by the differences in potency between malonate and malonate ether groups depending on the target enzyme. Squaramides are a particularly promising functional group, as they have been employed as phosphate mimics in nucleotides in multiple contexts and preserve the shape and properties of the parent compound at least partially in many different structures.
1.4.2 Alternative backbones for nucleic acids

In oligonucleotide chemistry, phosphate mimics have successfully been used as alternative backbones, replacing phosphodiester groups (Figure 1.23). Phosphate replacements are introduced for a number of reasons, such as to improve resistance to degradation by nucleases,\(^{221}\) enhance the binding affinity to complementary strands or to increase membrane permeability while retaining water solubility.\(^{187}\) Such properties are of particular importance in the development of oligonucleotide therapeutics.\(^{222}\) Many alternative backbones are phosphorus-based and overlap significantly with phosphate bioisosteres in small molecules. However, frequently these groups are chiral at the phosphorus atom, as one hydroxy group is substituted by a different group. This is the case for all three phosphorus-based backbones (Figure 1.23 a-c) that are discussed in this section.

Figure 1.23: Alternative backbones used in oligonucleotides. a) Phosphorothioate, b) methylphosphonate, c) boranophosphate, d) peptide, e) squaramide, f) triazole.

Phosphorothioates (Figure 1.23a) were the first modification to be widely used in oligonucleotide therapeutics\(^ {222}\) and several oligonucleotide drugs in clinical use are based on phosphorothioate scaffolds.\(^ {223}\) Phosphorothioates can be introduced in solid-phase oligonucleotide synthesis, and the linkage is more stable to nucleases than the native phosphodiester group.\(^ {224}\) Phosphorothioates can also be incorporated into nucleic acid strands by polymerases from their nucleoside triphosphate analogues.\(^ {225}\) However, the chirality of the phosphorothioate group leads to heterogeneous samples if multiple phosphodiester groups are replaced.\(^ {192}\) Oligonucleotides containing methylphosphonates (Figure 1.23b) are stable to nucleases due to the substitution of one negatively charged oxygen atom with a neutral methyl group.\(^ {226}\) The resulting increase in lipophilicity however does not increase cellular uptake. Instead, oligonucleotides containing methylphosphonates are taken up into endosomes and remain there.\(^ {227}\) Again, chirality leads to diastereomers that were found to have different activity.\(^ {228}\) Boranophosphates (Figure 1.23c) contain a borane group in place of a non-bridging oxygen atom, again resulting in a chiral linkage. They can also be incorporated into nucleic acid
strands by polymerases from their nucleoside triphosphate analogues.\textsuperscript{229} While the borane group preserves the charge of the phosphate group, the absence of lone pairs on the borane group means that boranophosphates interact differently with metals and proteins compared to phosphates, impacting for example their hydrolysis.\textsuperscript{230}

Amides have emerged as a suitable backbone, for siRNA in particular, where they enhance stability as well as activity, despite their different geometry and lack of negative charge.\textsuperscript{231,232} In peptide nucleic acids, the phosphate and the deoxyribose are both substituted by an amide backbone that acts as a scaffold for the nucleobases.\textsuperscript{233} Alternatively, an amide linkage can be used to replace only the phosphodiester without replacing the deoxyribose sugar (\textit{Figure 1.23d}).\textsuperscript{231,232} Despite the strikingly different structure of the backbone, the nucleobases can form base pairs with a complementary strand.\textsuperscript{232,233} Squaramides (\textit{Figure 1.23e}) have been employed both as 2'-5' linkages and 3'-5' linkages to replace a single phosphate linkage in oligonucleotides, with the 2'-5' linkage distorting the duplex structure less than the 3'-5' linkage.\textsuperscript{211,212} The strong polarisation and interaction with metal ions (\textit{Section 1.4.1}) makes this group a good phosphodiester replacement in oligonucleotides.

The copper(I)-catalysed azide-alkyne cycloaddition (CuAAC),\textsuperscript{234,235} which is popular for biological applications,\textsuperscript{236,237} has also found application in oligonucleotide synthesis. The biocompatibility of the reaction and control over the timing of the reaction allows for the chemical ligation of synthetic DNA fragments into longer DNA strands in a templated manner. The triazole linkage shown in \textit{Figure 1.23f} led to no observable differences in gene expression in \textit{E. coli} when incorporated into a reporter gene.\textsuperscript{238} However, a duplex with a native oligonucleotide was found to be destabilised\textsuperscript{239} and additional modifications such as locked nucleic acids are required to compensate for the negative effect on duplex stability.\textsuperscript{240}

Many functional groups can function as both phosphate bioisosteres and alternative nucleic acid backbones. The reported properties of these moieties can be used to inform the design of metal-binding groups that replace a phosphodiester bond in an oligonucleotide probe. Medicinal chemistry investigations highlight the general suitability of groups as phosphate bioisosteres. However, they also illustrate the importance of conformational considerations and the positioning of the groups.
Investigations of backbone replacements in nucleic acids have focussed on how the linkages affect the overall structure of an oligonucleotide and whether enzymes recognise the linkage as a natural phosphodiester group. Taken together, squaramides emerge as a particularly strong candidate. While malonates have not been evaluated as nucleic acid backbones, the successful use of amide backbones and the application of malonates in inhibitors suggest that they are a promising option for probes targeting SNM1A.

1.5 Objectives: Identification of metal-binding groups for probes targeting SNM1A

Repair nucleases such as SNM1A are important to cellular integrity. However, tools for the study of SNM1A and similar nucleases are currently lacking. One promising strategy to interrogate the roles and interaction partners of this enzyme is the development of probes that covalently capture the active enzyme upon binding in the active site. The structure of such probes requires the incorporation of metal-binding groups near the 5'-end of oligonucleotides. In order to achieve binding to the metal centre of SNM1A, this group needs to be recognised as a phosphodiester group. The identification of such metal-binding groups is expected to be the crucial part in the design of ABPs for SNM1A, as photo-crosslinking groups and fluorophores are both established modifications of oligonucleotides and are commercially available.

The objective of this work is to evaluate a range of chelating moieties for their potential as metal-binding groups for probes targeting SNM1A. This was achieved through synthesis of various modified nucleosides and oligonucleotides and their evaluation as inhibitors of SNM1A as a measure of their affinity for the active site.

Nucleosides containing different metal-binding groups at the 5'-position were synthesised and evaluated as small molecule inhibitors (Figure 1.24a). This work allowed for the comparison of different groups and is presented in Chapter 2.

Several 5'-modified nucleosides were also evaluated as part of an oligonucleotide to examine whether the additional interaction of the oligonucleotide backbone with the enzyme enhances their affinity for SNM1A (Figure 1.24b). These studies are detailed in Chapter 3.
Finally, a family of malonate-based nucleosides and dinucleosides was synthesised and evaluated as inhibitors. 5’-Modified and 3’-modified nucleosides as well as dinucleosides containing the metal-binding group in place of the phosphodiester linkage were tested to assess the ideal positioning of the metal-binding group (Figure 1.24c). Chapter 4 describes the synthesis and biological evaluation of these compounds.

Taken together, these studies provide insights into which metal-binding groups are suitable for probes for SNM1A, where they should be installed and how the oligonucleotide scaffold influences binding.

Figure 1.24: Investigations into metal-binding groups to inform the design of ABPs for SNM1A. a) 5’-Modified nucleosides discussed in Chapter 2, b) 5’-modified oligonucleotides discussed in Chapter 3, c) malonate-modified nucleosides and dinucleosides discussed in Chapter 4.
2 Hydroxamic acid and squaramide-based nucleoside inhibitors

Modified nucleosides containing metal-binding groups were designed as model systems for ABPs for the nuclease SNM1A. The design of such probes for this nuclease is based on an oligonucleotidic scaffold with a metal-binding group near the 5'-end. Modified nucleosides model the portion of the probe that interacts with the active site, and they are expected to bind in a similar fashion to the 5'-end of the natural substrate of the enzyme (Figure 2.1a) and to inhibit enzymatic activity as a result. Several nucleosides containing hydroxamic acid groups, derivatives of hydroxamic acids and squaramides (Figure 2.2b) were synthesised and evaluated as inhibitors of SNM1A in a gel-based assay. Two compounds 63 and 67, both with hydroxamic acid modifications, were found to inhibit the enzyme with varying potency.

**Figure 2.2:** a) Binding of nucleoside inhibitor to SNM1A compared to substrate binding. b) General structure of potential nucleoside inhibitors.

The two most promising compounds 63 and 67 were evaluated further in a real-time fluorescence assay to determine IC$_{50}$ values and the membrane permeability of the strongest inhibitor was also evaluated. Results presented in this chapter were published in *Organic & Biomolecular Chemistry* in 2019.
2.1 Design and Synthesis

In the design of modified nucleoside inhibitors, three components were considered: the nature of the metal-binding group which influences binding to the metal centre; the nucleobase that may influence enzyme recognition and the linker between the two which determines the positioning of the different groups. For this series of nucleosides, a range of metal-binding groups were explored, while the nucleobase thymine was kept constant and one metal-binding group was introduced with two different linker lengths.

2.1.1 Design of modified nucleosides

The modified nucleosides were designed around a thymidine core. A deoxyribonucleoside was chosen due to the preference of SNM1A for DNA over RNA. As no sequence specificity has been reported for SNM1A, the nucleoside was chosen based on synthetic considerations. Thymidine requires less protection of the nucleobase than other nucleosides due to the lack of an exocyclic amine and was therefore selected for synthetic ease. A range of metal-binding groups were installed in the 5'-position with the aim to occupy a similar space to a phosphodiester bond. The hydroxamic acid analogues 63, 66 and 67 were designed with different linkers, varying by a methylene group each, between the metal-binding group and the deoxyribose core in order to evaluate the relationship between the linker and binding to SNM1A. The selection of metal-binding groups is discussed below.

2.1.1.1 Hydroxamic acid derivatives

Hydroxamic acids feature in many inhibitors and ABPs for metalloenzymes. Early inhibitors of matrix metallopeptidases in particular were primarily based on this metal-binding group. While its strong chelating ability led to broad spectrum activity and made many inhibitors unsuitable for use as therapeutics, this potency is desired for probes. Hydroxamic acids are commonly used as metal-binding group in ABPs for metallopeptidases and histone deacetylases (see Section 1.3.2). Nucleosides containing hydroxamic acid groups and derivatives were designed together with Dr. William Doherty in the McGouran group.

Incorporation of this group into nucleosides can be achieved via oxidation of the 5'-hydroxyl group, such as in compound 63 (Figure 2.2). To explore whether similar
neutral analogues can also bind to the active site of SNM1A, acetylated or methylated hydroxamic acids 64 and 65 were also synthesised. Successful binding of weaker metal-binding groups offers advantages in selectivity, as other components of the recognition element likely make a greater contribution to binding.

Figure 2.2: Hydroxamic acid derivatives resulting from oxidation of the 5'-position.

The comparison of the more labile acetyl group of 64 with the stable methyl group of 65 allows for insights into potential unmasking of the acetyl group under the assay conditions. Substitution of the hydroxyl group of hydroxamic acids by an amine yields the hydrazide group, which has been used in inhibitors for metallopeptidases, although with limited success.\(^\text{115}\) It can be installed in a similar way to the hydroxamic acid via oxidation of the 5'-carbon to give nucleoside 68. While replacing hydroxamic acid groups with the hydrazide group has led to decreased potency in reported inhibitors,\(^\text{243,244}\) as for the acetylated and methylated analogues, lower metal-binding potency has the potential for increased selectivity.

A third group of hydroxamic acid analogues were designed with a view to linking two nucleosides. Oxyamides as in compounds 69 and 70 (Figure 2.3) are analogues of the methylated hydroxamic acid 65, but the N-oxamide group is inverted.

Figure 2.3: Oxyamide nucleosides 69 and 70.

While this group does not have the potential to coordinate through a negatively charged oxygen atom like hydroxamic acids, the amide group alone can be sufficient to achieve recognition as a phosphate. Mononucleotides containing amides as phosphate bioisosteres successfully bind to the O-acetyl-ADP-ribose deacetylase MDO1.\(^\text{217}\) An advantage of this group is that it can withstand solid-phase
oligonucleotide synthesis\textsuperscript{245} which is important for incorporation into the oligonucleotide scaffold of an ABP.

Another factor to consider in the design of modified nucleosides is the distance between the coordinating atoms and the deoxyribose core. The native phosphodiester bond of nucleic acids has three bonds between the coordinating oxygen atoms and the 5'-carbon. Taken together, the bonds between the 5'-carbon atom and the atoms that coordinate the metal ion are 4.64 Å and 4.52 Å long for the two different oxygen atoms (\textit{Figure 2.4}).\textsuperscript{246} Different linker lengths between the metal-binding group and the deoxyribose core were compared for the hydroxamic acid modification, and bond lengths were calculated using the values provided in the CRC Handbook of Chemistry and Physics.\textsuperscript{247} The hydroxamic acid group can be installed via oxidation of the 5'-position, so the 5'-carbon of thymidine is converted into the carbonyl carbon of the hydroxamic acid group, as in analogue 63. This results in the hydroxyl group 2.73 Å away from the 5'-carbon, closer than in the phosphodiester. The carbonyl oxygen is 1.23 Å away, significantly closer to the deoxyribose core than the non-bridging oxygen of a phosphodiester. The corresponding distances for analogues 66 and 67 are 4.24 Å and 2.74 Å for compound 66 and 5.76 Å and 4.26 Å for compound 67.

\textit{Figure 2.4}: Phosphodiester backbone of nucleic acids and hydroxamic acid containing nucleosides with different linkers between the hydroxamic acid group and the deoxyribose core. Bond lengths given in Å, atoms coordinating to metal ions highlighted in red.

While these values are only very rough estimates as they do not account for bond angles, it is apparent that in all hydroxamic acids, the hydroxyl group is 1.50 Å further away from the 5'-carbon atom than the carbonyl oxygen atom. In phosphodiester bonds, the two atoms that coordinate the metal ions are approximately equidistant from the deoxyribose core. With such different spatial arrangements, it is difficult to predict which nucleoside resembles the native phosphate most closely.
The eight nucleosides 63-70 compose a panel of different hydroxamic acid derivatives that will provide insights into the need for charged groups, orientation of the group and linkage between metal-chelator and deoxyribose core. Hydrazide 68 and oxyamides 69 and 70 were designed with a view to using the metal-binding group as an alternative phosphodiester linkage and allow for the connection of two nucleosides.

2.1.1.2 Squaramide derivatives

Squaramides have found applications as phosphate replacements in a range of contexts.211,218-220 Of particular interest is their use as alternative backbones for nucleic acids.211 Squaramides are excellent hydrogen bond donors and acceptors, as hydrogen bonding increases the aromaticity of the system.248 The ability of squaramides to act as hydrogen bond acceptors suggests an ability to chelate metal ions through the oxygen atoms. When incorporated into oligonucleotides, the squaramide linkage was shown to be responsive to magnesium ions with apparent coordination through the oxygen atoms (Figure 2.5).211 The pKa value of squaramides depends heavily on their substituents, with electron-withdrawing aromatic groups resulting in a pKa value of 8.37 and alkyl-substituted squaramides having pKa values of approximately 15.0-16.5.249 Deprotonation of the squaramide group results in increased electron density on the oxygen atoms, thereby increasing the chelating ability of the group.

![Figure 2.5: Squaramide coordinating to metal ions.](image)

The relatively facile synthesis is another attractive property of squaramides. They can be synthesised from squarate esters by sequential substitution with amines.250 Squaryl monoamides and squaryl diamides can therefore both be incorporated into nucleosides. To investigate whether their different electronic properties are reflected in their biological activity, squaryl monoamide 71 as well as squaryl diamide target 72 were designed (Figure 2.6).
Installing the group through an amino group in the 5′-position of the nucleoside is expected to result in the squaramide group occupying a similar space to the native phosphodiester group. This approach was used in previous reports of squaramide-containing nucleosides and nucleotides.\textsuperscript{211-216} The incorporation of squaramide-linked dinucleosides into oligonucleotides\textsuperscript{211,212} also shows that the group has potential for connecting two nucleosides as part of an ABP.

### 2.1.1.3 Target compounds

The nucleoside targets described above make up a panel of ten compounds (Figure 2.7). A variety of different functionalities is included, based on the hydroxamic acid and squaramide motifs. Variation of the linker between the hydroxamic acid targets 63, 66 and 67 is expected to provide insights into the ideal positioning of metal-binding groups for coordination to the metal centre of SNM1A.

Figure 2.7: Target nucleosides to be tested as nuclease inhibitors.

The synthesis of all these targets is based on the functionalisation of the 5′-position of thymidine, with hydroxamic acids 66 and 67 requiring homologation.

### 2.1.2 Synthesis of modified nucleosides

The modified nucleosides containing hydroxamic acid groups and their derivatives (63-68) were synthesised by Dr. William Doherty in the McGouran group. One hydroxamic acid analogue 66 could not be accessed synthetically. The ester
intermediate required for the synthesis of hydroxamic acid 66 underwent epimerisation via ring opening and was only obtained in poor yield, and no viable alternative synthetic strategy could be identified.\textsuperscript{241}

The oxyamide linkage of compounds 69 and 70 has previously been reported as a backbone replacement between two nucleosides.\textsuperscript{251} Although no biological testing of these reported compounds was carried out, the synthetic strategy was adapted to the 5'-modified nucleosides 69 and 70. The synthesis of the 5'-substituted nucleosides 69 and 70 was achieved in 30% and 14% yield over five steps, respectively (\textit{Scheme 2.1}).

\textbf{Scheme 2.1: Synthesis of nucleosides 69 and 70 containing an N-oxyamide group.}

Starting from thymidine (1), the protected aminoxy functionality was introduced using a Mitsunobu reaction with \textit{N}-hydroxypthalimide in 75\% yield. Following tert-butyldimethylsilyl (TBDMS) protection of the 3'-hydroxyl group of 73 in 86\% yield, the aminoxy group of 74 was deprotected using hydrazine in 90\% yield. Amide coupling between the protected 5'-O-aminothymidine 75 and either acetic acid or formic acid was carried out successfully to give compounds 76 and 77 in 71\% and 27\% yield, respectively. The 3'-hydroxyl group was selectively deprotected with tetra-\textit{n}-butylammonium fluoride (TBAF) to give alcohols 69 and 70 in 72\% and 92\% yield, respectively. \textit{N}-Oxyamide 70 exhibits rotamers at room temperature.

For the synthesis of squaramide-modified nucleosides such as 72, the reactivity of squarate esters towards primary or secondary amines was exploited (\textit{Scheme 2.2}).\textsuperscript{250} The squaryl monoamide product is less reactive than the dialkyl squarate, allowing for selectivity. Due to the strong preference of squarate esters
for attack by nitrogen over oxygen,\textsuperscript{252} no protecting group manipulations were required. The squaryl monoamide containing nucleoside $\text{71}$ was synthesised in $36\%$ yield over four steps using modified literature procedures\textsuperscript{213,253,254} and squaryl diamide $\text{72}$ was synthesised in one step from $\text{71}$, giving an overall yield of $27\%$ over five steps for compound $\text{72}$.

\begin{equation}
\text{Scheme 2.2: Synthesis of nucleosides 71 and 72 containing a squaramide group.}
\end{equation}

In order to introduce a squaramide moiety in the 5'-position of thymidine (1), the 5'-hydroxyl group was converted to an amine prior to reaction with diethyl squarate. The intermediate iodothymidine $\text{78}$ was prepared from thymidine (1) in $81\%$ yield in an Appel reaction\textsuperscript{253} and was subsequently reacted with sodium azide\textsuperscript{254} to give compound $\text{79}$ in $76\%$ yield. Azide $\text{79}$ underwent Staudinger reduction to give amine $\text{80}$ in $97\%$ yield. The water solubility of the product was exploited for facile separation from excess reagent and by-products including triphenylphosphine oxide, which can often be problematic to remove.\textsuperscript{255,256} Amine $\text{80}$ was then reacted with diethyl squarate\textsuperscript{213} to give squaryl monoamide $\text{71}$ in $61\%$ yield. In order to obtain the squaryl diamide $\text{72}$, squaryl monoamide $\text{71}$ was reacted with excess diethylamine to give nucleoside $\text{72}$ in $74\%$ yield. Squaramide $\text{72}$ exhibits rotamers at room temperature, a feature commonly observed in squaramides due to the restricted rotation around the C-N bond.\textsuperscript{257} This short and high-yielding synthetic sequence gives access to the two modified nucleosides $\text{71}$ and $\text{72}$ for evaluation as nuclease inhibitors.

48
2.2 Biological evaluation

The activity of SNM1A, and therefore its inhibition, can be evaluated in a gel-based assay. Incubation of the enzyme with an oligonucleotide substrate leads to digestion of the substrate from the 5'-end one nucleotide at a time. Since SNM1A produces 5'-phosphorylated nucleic acids as a result of its activity, the products of hydrolysis are in turn substrates for SNM1A and can be digested further (Figure 2.8). Therefore, activity of SNM1A leads to a range of nucleic acids that have been shortened from the 5'-end to different degrees. By using an oligonucleotide substrate with a fluorescent tag at the 3'-end, the products of the reaction can be visualised after separation by gel electrophoresis. The size of the fluorescently tagged fragments provides a picture of exonuclease activity. For SNM1A, digestion of a 21mer substrate yields oligonucleotides approximately ten nucleotides in length. At this point, the oligonucleotide strand appears to be too short for efficient binding to the positively charged surface residues of SNM1A and is therefore not hydrolysed further.

Figure 2.8. Schematic representation of the exonuclease assay. The product of hydrolysis by SNM1A is in turn a substrate for the enzyme and can be digested further, giving a range of shortened oligonucleotide products as well as single nucleotides.

While endonuclease activity has been reported for SNM1A, exonuclease activity dominates in the presence of a 5'-phosphate group. Additionally, the concentrations of SNM1A required to achieve endonuclease activity were very high at 0.18 µM, and the enzyme was used in excess compared to the substrate. Therefore, the use of a 5'-phosphorylated substrate and the use of lower enzyme-to-substrate ratios ensures that only exonuclease activity is observed.
2.2.1 Optimisation of gel-based assay

A fluorescently tagged 21-nucleotide substrate for the enzymatic assays was designed. The sequence was chosen to have an even distribution of all nucleobases and such that no secondary structure can form. The oligonucleotide 81b was synthesised using solid-phase synthesis and subsequently phosphorylated enzymatically at the 5’-end using T4 Polynucleotide Kinase (T4 PNK) to give oligonucleotide 82 (Scheme 2.3a). The phosphorylation reaction proceeds to high conversion after 30 min at 37 °C, as confirmed by an assay with varying concentrations of SNM1A (Scheme 2.3b).

Scheme 2.3: a) Enzymatic phosphorylation of oligonucleotide 81b by T4 PNK to give the substrate 82. b) Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with varying amounts of SNM1A (1-0.01 pmol, 100-1 nM) for 60 min at 37 °C, analysed by denaturing PAGE. nt = nucleotides.

Depending on its concentration, SNM1A digests oligonucleotide 82 to different degrees (Scheme 2.3b, lanes 2-6), with the optimum at 25 pmol per 10 µL reaction volume (Scheme 2.3b, lane 5). The ratio of undigested (21 nucleotides) to digested oligonucleotide in the optimum conditions indicates a high efficiency of phosphorylation. Confirmation of the presence of the labile phosphate group by mass spectrometry (MS) was unsuccessful.

Optimisation of the gel-based assay to evaluate SNM1A activity was carried out. Parameters that were varied are the enzyme storage buffer, pH of the reaction buffer, addition of dimethyl sulfoxide (DMSO), enzyme concentration and incubation time (Figures 2.9 and 2.10). The amount of oligonucleotide substrate 82 was kept constant at 0.8 pmol, as this concentration is suitable for fluorescence detection. Starting conditions for the assay were based on the conditions reported by Sengerová et al., used for the initial characterisation of SNM1A.
Chapter 2 – Hydroxamic acid and squaramide-based nucleoside inhibitors

Figure 2.9: Optimisation of conditions for SNM1A assay. Digestion of phosphorylated oligonucleotide \textit{82} (0.8 pmol, 80 nM) after incubation with varying amounts of SNM1A for the specified time at 37 °C, analysed by denaturing PAGE. 

\textbf{a)} Incubation in reaction buffer at pH 7.5 with SNM1A (2-0.25 pmol, 200-25 nM) stored in dialysis buffer, for 60 min (lanes 1-5) or 0 min (lanes 6-10). 

\textbf{b)} Incubation in reaction buffer at pH 7.5 with SNM1A (2-0.02 pmol, 200-2 nM) stored in dialysis buffer (lanes 2-6) or reaction buffer (lanes 7-11) for 60 min. 

\textbf{c)} Incubation in reaction buffer at pH 7.5 (lanes 2-6) or pH 7.9 (lanes 7-11) with SNM1A (2-0.02 pmol, 200-2 nM) stored in reaction buffer for 60 min. 

\textbf{d)} Incubation in reaction buffer at pH 7.5 containing 0% DMSO (lanes 2-6) or 4% DMSO (lanes 7-11) with SNM1A (1-0.001 pmol, 100-0.1 nM) stored in reaction buffer for 60 min. \textit{nt} = nucleotides.

Initially, significantly higher amounts of enzyme were required for activity than those reported in the literature (Figure 2.9a, lanes 1-5).\textsuperscript{39,56} Changing the storage buffer of SNM1A from dialysis buffer (1 M HEPES-KOH, pH 7.5, 0.5 M NaCl, 5% glycerol, 1 mM TCEP) (Figure 2.9b, lanes 2-6) to reaction buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM MgCl\textsubscript{2}, 0.05% Triton-X, 0.1 mg/mL BSA, 5% glycerol, 0.5 mM DTT) (Figure 2.9b, lanes 7-11) improved activity significantly. Next, different pH values reported in the literature were compared.\textsuperscript{39,50,56} No discernible difference between pH 7.5 (Figure 2.9c, lanes 2-6) and pH 7.9 (Figure 2.9c, lanes 7-11) was observed. The addition of 4% DMSO (Figure 2.9d, lanes 7-11) also did not improve or hinder hydrolysis compared to reaction buffer without DMSO (Figure 2.9d, lanes 2-6). This finding is important to the screening of inhibitors, which may require the addition of DMSO to aid solubility.
With these optimised conditions, varying concentrations ranging from 1000 fmol to 0.1 fmol of SNM1A per 10 µL reaction were tested (Figure 2.10a, lanes 3-11). In this experiment, the non-phosphorylated oligonucleotide 81b was added as a negative control (Figure 2.10a, lane 1) in addition to a lane containing phosphorylated oligonucleotide 82 and no enzyme (Figure 2.10a, lane 2). The best result was obtained in the range of 10-40 fmol (Figure 2.10a, lanes 6-7); thus 25 fmol of SNM1A was used in all future experiments.

![Figure 2.10: Further optimisation of conditions for SNM1A assay. Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with varying amounts of SNM1A in reaction buffer at pH 7.5 for the specified time at 37 °C, analysed by denaturing PAGE. a) Incubation with varying amounts of SNM1A (1000-0.1 fmol, 100-0.01 nM) for 60 min. b) Incubation with SNM1A (25 fmol, 2.5 nM) for varying times (1-60 min), control experiment without SNMA1 (lane 1). nt = nucleotides.](image)

Lastly, the hydrolysis reaction was carried out using optimised conditions and the incubation was stopped at various time points (Figure 2.10b, lanes 2-10). Again, a control experiment without nuclease was carried out (Figure 2.10b, lane 1). Since hydrolysis appears to cease after 45 min with the conditions used (Figure 2.10b, lane 10), all future experiments were incubated for 60 min to ensure that the results capture the full extent of enzymatic activity. This is significantly longer than the reaction times of around 10 min reported for small oligonucleotides and similar conditions in the literature.39

This optimised assay was used to evaluate the modified nucleosides as inhibitors, with an incubation time of 60 min and 25 fmol of SNM1A. Since the assay gives rise to multiple products, both the disappearance of full-length substrate 82 and the size of the resulting oligonucleotides give insight into the potency of the inhibitors.
2.2.2 Gel-based assays

The following nucleosides were tested as inhibitors of SNM1A (Figure 2.11), using the optimised assay conditions with the addition of 4% DMSO to aid solubility of the nucleosides. Thymidine (1) was included as a control to ensure that any observed effect could be attributed to the metal-binding group. Thymidine (1), oxyamides 69 and 70, hydroxamic acids 63 and 67, hydroxamic acid derivatives 64, 65 and 68 and the two squaramides 71 and 72 were all tested at 1 mM concentrations.

Figure 2.11: Nucleosides evaluated as nuclease inhibitors.

Nucleosides were preincubated with SNM1A for 5 min prior to the addition of oligonucleotide substrate 82 to allow the inhibitors to bind to the enzyme. Following 60 min incubation at 37 °C, the reaction was stopped and the products analysed using gel electrophoresis (Figure 2.12a). The results of this assay (Figure 2.12b) show that the presence of thymidine (1) has no visible effect on the reaction (Figure 2.12b, lane 3), and oligonucleotides approximately ten nucleotides in length are observed as the fluorescently-tagged products of the reaction, consistent with the literature. Of the modified nucleosides, the two hydroxamic acids 63 and 67 inhibited SNM1A (Figure 2.12b, lanes 6 and 10) and formyloxyamide 70 showed marginal activity (Figure 2.12b, lane 5). None of the other nucleosides inhibited enzymatic activity.
Figure 2.12: Evaluation of modified nucleosides as inhibitors of SNM1A using fluorescent substrate 82. a) Schematic representation of inhibition by modified nucleosides. b) Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and the nucleoside inhibitors 63-65 and 67-72 or control thymidine (1) (1 mM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the nucleosides for 5 min at 37 °C prior to the addition of substrate 82. nt = nucleotides.

While no full-length substrate remained with either hydroxamic acid inhibitor, the oligonucleotides remaining after the reaction in the presence of 63 are larger than for compound 67. Hydroxamic acid 67 appears to be less effective than compound 63 at competing with full-length substrate, but it inhibits the digestion of already shortened strands. Since SNM1A relies on electrostatic interactions of the phosphate backbone with its positively charged surface residues, shorter DNA strands bind less strongly to the surface than longer strands. The weaker inhibitor hydroxamic acid 67 can therefore only outcompete shorter substrates. This result is somewhat surprising, given the close proximity of the hydroxamic acid group in nucleoside 63 that is different from the native phosphodiester bond. The longer linker in nucleoside 67 was expected to more closely resemble the phosphodiester linkage and provide more flexibility for the correct orientation within the active site.

To further investigate the most potent inhibitor hydroxamic acid 63, the assay was repeated with lower concentrations of inhibitor, ranging from 1 mM to 3 µM (Figure 2.13). Thymidine (1) was tested at the highest concentration that was used for hydroxamic acid 63 and had no effect on the reaction (Figure 2.13, lane 3).
Chapter 2 – Hydroxamic acid and squaramide-based nucleoside inhibitors

**Figure 2.13.** Concentration dependence of inhibition of nucleoside 63. Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside 63 (1000-3 µM) or thymidine (1) (1 mM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the nucleosides for 5 min at 37 °C prior to the addition of substrate 82. nt = nucleotides.

From this assay it is evident that while the inhibitor 63 is effective at higher concentrations down to 100 µM (**Figure 2.13**, lanes 4-6), it only has a marginal effect at 33 µM (**Figure 2.13**, lane 7) and no effect at concentrations below this (**Figure 2.13**, lanes 8-9). Although this range is not sufficient for therapeutic use, it shows that the hydroxamic acid group is a suitable metal-binding group and the 5'-modified nucleoside 63 is a good candidate for incorporation into ABPs.

### 2.2.3 IC$_{50}$ determination

A real time fluorescence plate reader assay to determine the IC$_{50}$ value of hydroxamic acids 63 and 67 was carried out by Hannah Baddock in the McHugh group at the University of Oxford, using a previously reported procedure.$^{56}$ An oligonucleotide substrate containing a fluorophore and quencher was incubated with SNM1A in the presence of the nucleoside inhibitors. Digestion of the substrate by SNM1A removes the nucleotide containing the quencher, and a fluorescence increase is observed. This assay showed that the IC$_{50}$ of hydroxamic acid 63 is 139 µM. Thymidine (1) was included as a control and showed no inhibition, consistent with gel-based assays. The other hydroxamic acid 67 also showed no inhibition in this assay, despite having some activity in the gel-based assay. This observation can be attributed to the two different assays measuring different outputs. The gel-based assay shows all shortened oligonucleotide products, while the fluorescence assay only measures the removal of the first nucleotide. This difference in readout leads to no apparent inhibition for compound 67 in the
fluorescence assay, where only competition with the full-length substrate is measured. The seeming lack of activity is in line with the gel-based assay that suggests compound 67 is less effective at competing with longer DNA substrates, as discussed previously.

From these results it is apparent that the positioning of the hydroxamic acid group is important for its activity and the mere presence of a zinc-binding group is not sufficient to inhibit SNM1A at low concentrations. The evaluation of the one-carbon homologue 66 would have provided valuable insight into the ideal position of the hydroxamic acid group, but its synthesis by Dr. William Doherty in the McGouran group was unsuccessful.241

2.2.4 Membrane permeability assay

In addition to its potential use in ABPs, hydroxamic acid 63 serves as a lead compound for small molecule inhibitors for SNM1A. One of the important pharmacological properties of small molecule therapeutics is membrane permeability.258 To evaluate the membrane permeability of the strongest inhibitor, compound 63, a parallel artificial membrane permeability assay (PAMPA) was performed.259 This assay was developed for drug discovery to predict membrane permeability of lead compounds in a simple inexpensive assay.260 An artificial membrane is formed by dissolving lecithin in an alkane such as dodecane, and the solution is applied to a filter in a 96-well plate. The compound is dissolved in buffered solution and added on one side of the membrane and incubated for several hours to allow the compound to pass through the membrane into another buffered solution (Figure 2.14). Following incubation, the concentrations of the compound in the different compartments as well as the theoretical equilibrium concentration are measured. Variation in the membrane components and pH of the compartments allows the modelling of a range of physiologically relevant membranes.261

![Figure 2.14: Schematic representation of PAMPA.](image_url)
The membrane permeability of lead compound 63 was evaluated using PAMPA with egg lecithin and dodecane. The concentration in the two compartments was determined by UV/Vis spectroscopy. To determine the limit of detection of the plate reader, the absorption spectrum of thymidine was recorded at different concentrations (Figure 2.15a,b). The limit of detection of 10 µM allows for measurement of concentrations in the micromolar range which is suitable for PAMPA, which typically uses concentrations of 100-500 µM. The limit of detection of compound 63 was then evaluated, using 100 µL or 250 µL of buffered solution per well, corresponding to the volumes of donor and acceptor solutions in PAMPA. The spectra of 100 µL hydroxamic acid solution are shown in Figure 2.15c. Both volumes allow for the determination of concentrations as low as 10 µM (Figure 2.15d).

Figure 2.15: UV/Vis spectroscopy of thymidine (1) (a,b) and hydroxamic acid 63 (c,d). a) Absorption spectrum of thymidine (1) using 100 µL per well. Box highlights data used to determine limit of detection. b) Absorbance vs concentration at 270 nm and 276 nm (with background subtracted). c) Absorption spectrum of 63 using 100 µL per well. Box highlights data used to determine limit of detection. d) Absorbance vs concentration at 260 nm for 100 µL and 250 µL per well (with background subtracted).
To confirm that a permeable membrane was formed in the experiment, two compounds with known permeability, carbamazepine and furosemide, were used as controls. The assay was carried out using a 1% lecithin in dodecane membrane. Following the incubation, samples of the donor and acceptor solution, as well as a solution at the theoretical equilibrium concentration if no membrane was present, were analysed by UV/Vis spectroscopy. The absorption spectra show that carbamazepine is partially permeable (Figure 2.16a) while furosemide does not cross the membrane, as is evident by the lack of absorbance in the acceptor well (Figure 2.16b). This is in agreement with the literature and confirms the formation of an intact membrane.²⁵⁹

![Absorption spectrum of carbamazepine](image1)

**Figure 2.16:** Control experiments for PAMPA using carbamazepine and furosemide. a) Absorption spectra of donor and acceptor wells and equilibrium concentration for carbamazepine after incubation at r.t. for 16 h. b) Absorption spectra of donor and acceptor wells and equilibrium concentration for furosemide after incubation at r.t. for 16 h. Volumes of samples: Donor 100 µL, Acceptor 250 µL, Equilibrium 250 µL.

The spectra obtained from the permeability assay of hydroxamic acid 63 are shown in Figure 2.17. The spectrum obtained from the equilibrium solution is the spectrum that is expected for hydroxamic acid 63 if it was fully permeable. The stark difference between the spectra obtained for the acceptor and equilibrium solutions and the lack of absorption at 260 nm for the acceptor solution shows that the compound did not pass through the membrane.
Figure 2.17: Absorption spectra of donor and acceptor wells and equilibrium concentration for nucleoside 63 after incubation at r.t. for 16 h. Volumes of samples: Donor 100 µL, Acceptor 250 µL, Equilibrium 250 µL.

The concentration of 63 in the acceptor well was calculated to be 1 µM (Table 2.1). This is in contrast with the concentration of 182 µM that is expected if the compound was fully permeable, i.e. the equilibrium concentration. The concentration of the donor well decreased from 500 µM to 488 µM, indicating that some compound either adsorbed to the wells or permeated into the membrane and remained there.

<table>
<thead>
<tr>
<th>Concentration of 63 (µM)</th>
<th>Donor</th>
<th>Acceptor</th>
<th>Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>488</td>
<td>1</td>
<td>182</td>
</tr>
</tbody>
</table>

Table 2.1: Concentrations of nucleoside 63 in donor, acceptor and equilibrium solutions of PAMPA after 16 h incubation at r.t.

The effective permeability $P_e$ of compound 63 was calculated using the following equation:

$$P_e = -\ln(1 - r) \left( \frac{V_D V_A}{(V_D + V_A) A t_i} \right)$$

where

$$r = \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}$$

$V_D = $ volume of donor well, 0.15 cm$^3$

$V_A = $ volume of acceptor well, 0.30 cm$^3$

$A = $ area of the filter, 0.3 cm$^2$

$t_i = $ incubation time, 57 600 s

$$P_e \text{ (Hydroxamic acid 63)} = 3.01727 \times 10^{-8} \text{ cm s}^{-1}$$

$$\log P_e \text{ (Hydroxamic acid 63)} = -7.5$$

Literature log $P_e$ values for the permeable drug carbamazepine and the less permeable compound furosemide are -5.2 and -6.8, respectively. The value
of -7.5 for hydroxamic acid 63 shows the nucleoside is even less permeable than furosemide in this assay. The inability of nucleoside 63 to cross the artificial membrane is presumably due to the hydrophilic nature of the hydroxamic acid moiety. A pro-drug approach where the active moiety is masked by a labile protecting group, as envisaged for compound 64, could be used to overcome this limitation. As evident by the lack of activity of compound 64 (Figure 2.12b, lane 7), the nature of the protecting group is crucial to the successful release of the active compound. The results of the permeability assay indicate that a significant increase in lipophilicity is required for the successful diffusion of derivatives of compound 63 across a membrane. Another obstacle for the use of hydroxamic acids in therapeutics is the metabolism of the hydroxamic acid group in plasma, which could also be prevented by the use of a labile protecting group. For use as a metal-binding group for ABPs however, the permeability of the modified nucleoside is of limited importance. In the context of an oligonucleotide scaffold, the influence of the hydroxamic acid group on cell permeability is expected to be negligible.

2.3 Hydroxamic acid-containing nucleosides inhibit SNM1A

A series of eight modified nucleosides 63-65 and 67-72 was synthesised and their activity as inhibitors of SNM1A was evaluated in a gel-based assay. The results have provided several insights that can guide the design of ABPs for this enzyme. The two most promising compounds 63 and 67 both contain the potent metal-chelating hydroxamic acid group. They were evaluated further in a real-time fluorescence assay and the IC\textsubscript{50} of the strongest inhibitor 63 was found to be 139 µM. The membrane permeability of compound 63 was also evaluated and it was apparent that the nucleoside is too hydrophilic to permeate an artificial membrane. The results highlight that while the nucleoside containing an unmasked hydroxamic acid group is too polar for medicinal chemistry applications, it has potential as a metal binding group for probes targeting SNM1A.

The difference between the two hydroxamic acid analogues illustrates the importance of the position of the metal-binding group. This indicates that the binding is not solely relying on metal coordination, but that other interactions are affecting the stability of the complex. While the strongest inhibitor, compound 63, is not potent enough for therapeutic applications, and is not membrane permeable, it offers scope for optimisation. For example, other larger nucleobases might form more
interactions with the enzyme pocket than thymine and increase potency. Furthermore, masking of the hydroxamic acid moiety using a prodrug approach could overcome the poor permeability. Importantly, for the design of ABPs these studies have validated hydroxamic acids as a strong contender for metal-binding groups. While none of the other modifications showed binding to SNM1A in the assays, incorporation into an oligonucleotide scaffold as part of ABPs could enhance their binding.
3 Modification of oligonucleotides for binding to SNM1A

Oligonucleotides containing modifications at the 5’-end were designed to inhibit SNM1A in a similar manner to the modified nucleosides described in Chapter 2. Despite the low potency of the modified nucleosides, oligonucleotide structures containing these moieties are expected to interact differently with the enzyme. The metal-binding group incorporated at the 5’-end can bind to the active site metal ion(s), while the oligonucleotide strand enhances binding via electrostatic interactions with the positively charged surface of the enzyme (**Scheme 3.1a**). Therefore, an oligonucleotide strand bearing a 5’-modification is expected to bind in a similar manner to the natural substrate and hence more strongly than the corresponding mononucleoside. The oligonucleotides were synthesised using solid-phase synthesis, with the modified nucleoside added as a phosphoramidite. However, this approach was not successful for all modified nucleosides, with some failing to produce the phosphoramidites in the phosphitylation reaction, others not coupling during solid-phase synthesis and some decomposing during deprotection of the oligonucleotide. One oxyamide modification was successfully introduced using this strategy (**Scheme 3.1b**). An alternative approach was therefore investigated to introduce the squaramide modification after solid-phase synthesis, affording differently substituted squaramides (**Scheme 3.1b,c**).

**Scheme 3.1:** a) Binding of modified oligonucleotide to SNM1A compared to substrate binding. b) General structure of modified oligonucleotides which were synthesised and tested. c) Introduction of squaramide group after solid-phase synthesis.
Biological evaluation of these oligonucleotides with SNM1A was carried out to determine their stability to the exonuclease and their ability to bind to the active site of SNM1A and compete with the substrate. Results presented in this chapter were published in *ChemistrySelect* in 2018.  

### 3.1 Design and synthesis of modified oligonucleotides

The modified oligonucleotides were designed to be 21 nucleotides in length, bearing metal-binding groups at the 5'-end. The size of the oligonucleotide was based on reported substrates for SNM1A to ensure that the strand is long enough to interact with the enzyme. Two lysine residues on the surface of the enzyme were found to be crucial to substrate binding, and very short oligonucleotides cannot bind to both the distal binding site and the active site at the same time. The oligonucleotide sequence was the same as that of the substrate used to evaluate nucleoside inhibitors (Chapter 2). It has an even distribution of all four nucleobases and cannot form any secondary structures like hairpins or duplexes. The modifications that were evaluated as part of nucleoside inhibitors (Chapter 2) were also selected for the target oligonucleotides. Solid-phase oligonucleotide synthesis was used to incorporate suitably protected phosphoramidite derivatives. For squaramide derivatives, a post-solid phase approach was developed as an alternative.

#### 3.1.1 Design of modified oligonucleotides

The range of modifications was based on the nucleosides that were tested as inhibitors. Despite only two of the series showing activity against the enzyme, the additional electrostatic interactions of the negatively charged oligonucleotide backbone with the positively charged surface residues of the enzyme were expected to improve binding. As the modified oligonucleotides could act as substrates for SNM1A if their modification was recognised as a 5'-phosphate group, their stability to the nuclease was also examined. To this end, additional versions of each modified oligonucleotide with a fluorescent tag at the 3'-end were synthesised alongside the unlabelled ones. Unlabelled oligonucleotides can be tested as inhibitors in assays using a fluorescent substrate, in a similar way to the modified nucleosides that were evaluated in this way in Chapter 2. Biotin-tagged oligonucleotides were also synthesised to allow for enrichment of bound enzyme, in case of a strong interaction.
3.1.1.1 Hydroxamic acid derivatives

The hydroxamic acid group was the only modification that resulted in biological activity against SNM1A when introduced at the 5’-position of thymidine (Chapter 2).\textsuperscript{241} Therefore, this nucleoside was a promising target for incorporation into oligonucleotides. While the hydroxamic acid group is the most potent chelating group of the series, the additional interaction from the phosphate backbone was expected to enhance the potency of weaker, neutral metal-binding groups. Thus, despite the lack of activity of all other modified nucleosides, the same hydroxamic acid derivatives as for the modified nucleosides were chosen for the target oligonucleotides (Figure 3.1).

\textbf{Figure 3.1: Hydroxamic acid and derivatives chosen as modifications for oligonucleotides.}

A challenge in the incorporation of some of these groups is the need for protecting groups during solid-phase synthesis. Both the hydroxamic acid group and the hydrazide group require masking for solid-phase synthesis.

3.1.1.2 Squaramide derivatives

As for the hydroxamic acid derivatives, the same modifications as in Chapter 2 were chosen for incorporation into oligonucleotides. While the squaramide-modified nucleosides exhibited no activity against SNM1A, the use of squaramides in oligonucleotides\textsuperscript{211,212} and nucleotide mimics\textsuperscript{213-216} in the literature is encouraging. Squaramide-containing oligonucleotides were found to form duplexes with complementary strands and while the duplex structure was bent, they can still be considered viable backbone replacements for nucleic acids.\textsuperscript{211} Their ability to bind magnesium ions in this context\textsuperscript{211} is also promising for their use as metal-chelating groups in ABPs.

The squaryl monoamide modification that was evaluated in mononucleosides is unstable to the conditions used to remove nucleobase protecting groups after solid-
phase oligonucleotide synthesis, as this uses concentrated ammonium hydroxide. Therefore, this modification is not a viable target for incorporation into oligonucleotides using solid-phase synthesis. By treating a squaryl monoamide-containing oligonucleotide with an amine like diethylamine, the group can be transformed into a more stable squaryl diamide. Therefore only one squaramide target (Figure 3.2) was included in the initial target compounds.

![Figure 3.2: Squaramide-modified oligonucleotide target.](image)

3.1.1.3 Target compounds

The target oligonucleotides were 21 nucleotides in length, with the metal-binding modification at the 5'-end. For each 5'-modification, an oligonucleotide with a fluorophore on the 3'-end, an unlabelled oligonucleotide and an oligonucleotide with a biotin-label at the 3'-end were synthesised (Figure 3.3). These 3'-modifications allow for use in stability testing, inhibitor assays and enrichment of bound enzyme in the case of strong binding, respectively. As no sequence specificity was has been observed for SNM1A, the sequence of the oligonucleotide was designed such that no hairpin formation or duplex formation containing more than a single base pair is possible. The metal-binding groups at the 5'-end include all previously tested nucleosides with the exception of the squaryl monoamide which is unstable to the deprotection conditions used after solid-phase synthesis (Figure 3.3).
3.1.2 Synthesis of phosphoramidites and incorporation into oligonucleotides

Solid-phase oligonucleotide synthesis was used to construct the target oligonucleotides (Figure 3.4). In solid-phase oligonucleotide synthesis, oligonucleotides are synthesised in 3' to 5'-direction on a solid support. Initially, the 4,4'-dimethoxytrityl (DMTr) protecting group of the first nucleoside which is bound to the solid support is removed with trichloroacetic acid. The next nucleoside is then added as a phosphoramidite and coupling is performed using tetrazole as a catalyst. Any unreacted 5'-hydroxyl groups are capped with acetic anhydride. This avoids the formation of oligonucleotides containing a point deletion and thus simplifies purification. After the capping step, the phosphite-triester is oxidised to the stable phosphotriester using iodine in H2O and pyridine in THF and the next cycle of deprotection, coupling, capping and oxidation is carried out. Coupling efficiency is monitored by measuring the absorbance of the DMTr-carbocation that is formed during deprotection. After the final cycle, the oligonucleotide is cleaved from the resin and the nucleobases and phosphate backbone are deprotected.
Figure 3.4: Schematic overview of solid-phase oligonucleotide synthesis.

As oligonucleotides are synthesised from the 3'-end, the modified nucleoside is added last. Consequently, the modification is not exposed to multiple washes of deprotection and coupling reagents and less likely to decompose during solid-phase synthesis. Since the modified nucleosides do not contain a DMTr-protecting group, no deprotection in acidic conditions is required. However, this also precludes the measurement of the coupling yield by measuring the absorption of the dimethoxytrityl cation after deprotection. Following solid-phase synthesis, the oligonucleotides are removed from the solid support and the nucleobases are deprotected in basic conditions.

For any modifications to be incorporated successfully in solid-phase synthesis, they need to withstand the coupling reagents, the capping and oxidation steps and the basic conditions used to deprotect the nucleobases. Any additional protecting groups are ideally removed alongside the nucleobase protecting groups and their removal should not require conditions that degrade oligonucleotides.
3.1.2.1 Phosphoramidite synthesis

Phosphoramidites were prepared from nucleosides with a free 3'-hydroxyl group and a phosphitylating agent, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. The oxyamides 69 and 70 did not require protecting groups. The acetamide derivative 69 underwent phosphitylation in a mixture of dichloromethane and N,N-dimethylformamide (DMF) in 98% yield (Scheme 3.2), requiring four equivalents of phosphitylating agent to drive the reaction to completion. The formamide derivative 70 proved to be unstable to N,N-diisopropylethylamine (DIPEA), which is required for the phosphitylation reaction and thus was not incorporated into an oligonucleotide (Scheme 3.2).

\[ \text{Scheme 3.2: Synthesis of phosphoramidites from N-oxyamide-modified nucleosides 69 and 70.} \]

The presence of the phosphoramidite group in 83 was confirmed by $^{31}$P NMR spectroscopy. Two signals at the characteristic value of 147-148 ppm were observed, corresponding to the two diastereoisomers. Compound 83 was stored under argon and used in solid-phase synthesis without further characterisation.

The hydroxamic acids 63 and 67 and hydrazide 68 were the only targets that were expected to require protecting groups. Acetylation of the hydroxamic acid group present in nucleosides 63 and 67 was anticipated to be stable to solid-phase conditions and be removed during the nucleobase deprotection step. Nucleoside 64 was therefore a precursor for oligonucleotides containing 63 and 64. However, this nucleoside proved unstable to the phosphitylation conditions (Scheme 3.3) and acetylation was therefore not attempted for hydroxamic acid 67. Phosphitylation of the methylated analogue 65 resulted in a complex mixture of products and the phosphoramidite 86 was not isolated (Scheme 3.3).
Scheme 3.3: Attempted synthesis of phosphoramidites from protected hydroxamic acid-containing nucleosides 64 and 65.

The hydrazide nucleoside 68 was protected using the base-labile fluorenylmethyloxycarbonyl (Fmoc) protecting group and phosphitylated by Dr. William Doherty in the McGouran group.

Squaryl monoamide 71 was successfully phosphitylated in tetrahydrofuran (THF) in 70% yield (Scheme 3.4) despite its poor solubility in this solvent. THF was chosen since compound 71 was insoluble in dichloromethane. Phosphitylation of the squaryldiamide 72 was also attempted, but no reaction was observed, possibly due to the poor solubility of compound 72 in THF (Scheme 3.4).

Scheme 3.4: Synthesis of phosphoramidites from squaramide-modified nucleosides 71 and 72.

The presence of the phosphoramidite group in 87 was confirmed by $^{31}$P NMR spectroscopy. Four signals in the region of 147-148 ppm were observed due to the presence of diastereoisomers as well as rotamers that were also observed for compound 71. Compound 87 was stored under argon and used in solid-phase synthesis without further characterisation. The squaryl monoamide group of 87 is not stable to the deprotection of the oligonucleotide in concentrated ammonium hydroxide after solid-phase oligonucleotide synthesis. The group therefore needs to be converted into the more stable squaryl diamide by reaction with diethylamine after solid-phase synthesis and prior to deprotection (Scheme 3.5).
Scheme 3.5: Envisaged modification of the squaryl monoamide after solid-phase synthesis.

Three phosphoramidites 83, 87 and 89 shown in Figure 3.5 of the initial eight were prepared successfully and were used in solid-phase synthesis.

Figure 3.5: Phosphoramidites used in solid-phase synthesis.

3.1.2.2 Solid-phase synthesis

The modified nucleosides were incorporated at the 5'-end of oligonucleotides using solid-phase oligonucleotide synthesis (Figure 3.6). For each type of 5'-modification, two different modifications, fluorophore Cy3 and affinity label Biotin-triethyleneglycol (TEG), were incorporated at the 3'-end, and an oligonucleotide without 3'-modification was also synthesised. The fluorescent label allows for detection in stability assays while the biotin-tagged oligonucleotides can be used for enrichment in case of strong binding. The three phosphoramidites were expected to give the nine oligonucleotides 90a-c, 91a-c and 92a-c shown in Figure 3.6 alongside the controls 81a-c, which contain thymidine in place of the modified nucleoside.

<table>
<thead>
<tr>
<th>modified nucleoside with zinc-binding group</th>
<th>oligonucleotide strand</th>
<th>fluorophore or affinity tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>X = modified nucleoside, Thymidine</td>
<td>Y = OH</td>
<td></td>
</tr>
<tr>
<td>90a: Y = OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90b: Y = Cy3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90c: Y = Biotin-TEG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.6:** Anticipated structures of modified oligonucleotides resulting from solid-phase synthesis.

As the 5'-modified nucleosides do not contain a DMTr-protecting group, yields for the final coupling could not be determined prior to deprotection and analysis by MS. The acetamide phosphoramidite 83 did not couple in a high yield, as MS analysis after deprotection showed a mixture of 21mer and 20mer (X = OH). Purification by reversed-phase HPLC was unsuccessful in separating the two oligonucleotides. Both the non-modified oligonucleotide 90a and fluorescently-tagged modified oligonucleotide 90b exist as a 2:3 mixture of 21mer and 20mer. The 3'-Biotin-TEG-modified oligonucleotide 90c was isolated as a 3:2 mixture of 21mer and 20mer after HPLC purification. However, since SNM1A shows no activity towards oligonucleotides without a 5'-phosphate, the 20mer with a 5'-hydroxyl group does not cause false positive results in the biological assay and the oligonucleotides 90a-c were therefore used as a mixture with the 20mer.

The squaramide phosphoramidite 87 did not couple successfully during solid-phase synthesis and the protected hydrazide phosphoramidite 89 decomposed during deprotection of the nucleobases. Oligonucleotides 90a-c and controls 81a-c were successfully synthesised using solid-phase synthesis. The squaramide-containing oligonucleotides 92a-c were synthesised using a different approach.

### 3.1.3 Post-solid phase synthesis of squaramides

The squaramide phosphoramidite 87 was not added successfully to the oligonucleotide in solid-phase synthesis, so a different synthetic strategy was used. As the reaction of dialkyl squarate with amines proceeds selectively and rapidly, oligonucleotides containing a primary amine in place of the 5'-hydroxyl group can
be reacted with dialkyl squarate after solid phase synthesis. Reaction of the resulting squaryl monoamide with an amine of choice gives the desired squaramide modifications (Scheme 3.6). The strong preference of squarates and squaryl monoamides for nitrogen nucleophiles over oxygen nucleophiles allows for this reaction to be carried out in aqueous solutions.\(^{252}\)

![Scheme 3.6: Strategy for the introduction of squaramide-modifications on oligonucleotides after solid-phase synthesis.](image)

This strategy was used to obtain the diamide oligonucleotide that could not be synthesised using the phosphoramidite strategy. As well as giving access to that target, this approach also allows for evaluation of squaryl monoamides as 5’-modifications and comparison of primary and secondary amine substituents.

### 3.1.3.1 Optimisation of reaction conditions

While the addition of the squarate ester had previously been optimised by collaborators in Prof. Tom Brown’s laboratory, the addition of the second amine was tested on a single nucleoside as a model system. For the conditions to be applicable to oligonucleotides, the reaction with the amine needs to proceed to full conversion in mild aqueous conditions and allow for facile purification by size exclusion chromatography. These conditions were tested using the 5’-squaramide containing nucleoside 71 and analysed by TLC and NMR spectroscopy. Diethylamine was used for the optimisation (Scheme 3.7), as the desired product 72 was used for testing in Chapter 2 and data was therefore available for comparison.

![Scheme 3.7: Optimisation of reaction conditions for the substitution of squaryl monoamides with Et₂NH using the modified nucleoside 71 as a model system.](image)
An excess of amine (100 equivalents) was used to drive the reaction to completion, since it can easily be separated from the oligonucleotide product by size exclusion chromatography. These conditions led to full conversion using very mild conditions (Scheme 3.7). The difference in the alkoxy-substituent between the oligonucleotide (methoxy) and the nucleoside model (ethoxy) was not expected to make a difference to this reaction. Reaction with the primary amine methylamine was expected to proceed under the same conditions. Side reactions with the oligonucleotide are not expected as methylamine can be used to deprotect ultramild protecting groups of nucleobases.242

3.1.3.2 Synthesis on oligonucleotides

Following optimisation of the reaction conditions, the post solid-phase modification strategy was applied to oligonucleotides (Scheme 3.8). The first conjugation was carried out in borate buffer at pH 8.5 and gave oligonucleotides 94a-c in quantitative yield. A second coupling was carried out with diethylamine in water to give 92a-b and with methylamine in water to give 95a-b.

Scheme 3.8: Synthesis of squaramide-modified oligonucleotides 94a-c, 92a-b and 95a-b after solid-phase synthesis.

This strategy allows for the installation of squaryl monoamides as well as squaryl diamides and therefore for their comparison. Additionally, the introduction of a range of amines provides useful information on the steric and electronic requirements.
3.1.4 Design and synthesis of further squaramide-modified oligonucleotides

Based on the use of the post-solid phase synthesis for squaramides, further substituted squaramides were designed. The option of introducing one or two substituents on the nitrogen atom gives access to a wide range of potential compounds. Particularly the addition of chelating groups to bind both metal ions in the active site is expected to increase the potency of the modification.

3.1.4.1 Design of target structures

The introduction of an additional heteroatom in a position to form a chelate with one of the carbonyl oxygen atoms was anticipated to improve binding (Figure 3.7). As part of this series, oligonucleotides containing an ethanolamine substituted squaramide 96a-b were synthesised. Hydrolysis of the squarate ester to give the oligonucleotides 97a-b was also envisaged to increase binding through the increased negative charge on the squaramide moiety.

![Figure 3.7: Structures of nucleosides containing squaramides with an additional chelator or with a negative charge to improve metal binding.](image)

As for the other squaramides, the optimisation of the reaction conditions was carried out using the corresponding nucleoside as a model system.

3.1.4.2 Optimisation of reaction conditions

Test reactions showed that adding 330 equivalents of ethanolamine to nucleoside 71 with heating the reaction mixture to 40 °C for 19 h produced a mixture of compounds. Reducing the amount of ethanolamine to 30 equivalents and reducing the temperature gave the desired product 98 in quantitative yield (Scheme 3.9).
Scheme 3.9: Optimisation of reaction conditions for the substitution of squaryl monoamides with ethanolamine using the modified nucleoside 71 as a model system.

Hydrolysis of the squarate was also attempted to synthesise a negatively charged squaric acid derivative (Scheme 3.10).

Scheme 3.10: Attempted hydrolysis of squaryl monoamides using the modified nucleoside 71 as a model system.

Various concentrations of sodium borate buffer at pH 9.5 were tested, with multiple products observed by TLC analysis. NMR analysis was inconclusive due to the presence of salts. Extraction with an organic solvent was not a viable option due to the polarity of the products formed. As no suitable conditions could be identified, hydrolysis of the squarate ester was not attempted on the modified oligonucleotide.

3.1.4.3 Synthesis on oligonucleotides

The ethanolamine-modified oligonucleotides were synthesised from 5'-amino-oligonucleotides 93a-b using the optimised conditions (Scheme 3.11) and excess reagents were removed by size exclusion chromatography.

Scheme 3.11: Synthesis of squaramide-modified oligonucleotides 101a-b after solid-phase synthesis.
The ethanolamine-modified oligonucleotide 101a-b completed the panel of modified oligonucleotides which were examined as inhibitors for SNM1A.

### 3.2 Biological evaluation

Following solid-phase synthesis and post solid-phase synthesis, five modified oligonucleotides and two controls were obtained (Figure 3.8) and used for biological assays.

![Figure 3.8: Structures of modified oligonucleotides evaluated in biological assays.](image)

For the oligonucleotides to be tested as inhibitors, they need to be stable to digestion by SNM1A under the assay conditions. Following evaluation of their stability, the modified oligonucleotides were tested for their ability to compete with a 5'-phosphorylated oligonucleotide substrate.

#### 3.2.1 Stability to SNM1A

The modified oligonucleotides contain a 5'-modification that is designed to coordinate to the metal centre. The modifications are based on various heteroatoms that can act as a Lewis base, but this also makes them potential hydrogen bond acceptors. The 5'-phosphate group that is essential for SNM1A activity forms several hydrogen bonds with the enzyme\(^{50}\) and a similar interaction can be
envisioned for the metal-binding groups (Figure 3.9). Such binding could position the phosphodiester bond correctly for hydrolysis, leading to degradation of the oligonucleotide.

**Figure 3.9:** Schematic representation of hydrogen bonding between the metal-binding group and the enzyme, leading to hydrolysis of the oligonucleotide.

To establish whether this binding mode occurs for these metal-binding groups, the fluorescently-tagged modified oligonucleotides 90b, 92b, 94b, 95b and 101b were incubated with SNM1A under the conditions that were found to lead to maximum digestion of the phosphorylated substrate 82 (Figure 3.10).

**Figure 3.10:** Evaluation of the stability of modified oligonucleotides to SNM1A. Digestion of oligonucleotides 82, 90b, 92b, 94b, 95b and 101b (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) for 60 min at 37 °C, analysed by denaturing PAGE. ON = oligonucleotide, nt = nucleotides.

All oligonucleotides were incubated with or without the nuclease for 60 min. Analysis by denaturing gel electrophoresis shows that apart from the 5’-phosphorylated oligonucleotide 82 (Figure 3.10, lane 2), no oligonucleotide was digested in the presence of SNM1A (Figure 3.10, lanes 4, 6, 8, 10, 12). No degradation independent of SNM1A was observed during the incubation (Figure 3.10, lanes 1,
3, 5, 7, 9, 11). This experiment confirmed that the modified oligonucleotides are not recognised as substrates by SNM1A and are stable to the assay conditions. The modification does not appear to bind to the enzyme in a similar way to the 5'-phosphate group of natural substrates. These findings allow for the evaluation of the modified oligonucleotides as competitive inhibitors.

3.2.2 Binding to SNM1A

To assess binding of the modified oligonucleotides to SNM1A, nuclease activity was measured in the presence and absence of the modified oligonucleotides. In case of coordination of the metal-binding group to the metal centre, the active site is blocked and hydrolysis of the fluorescent 5'-phosphorylated substrate is impeded (Figure 3.11). The rate of hydrolysis is reflected in the size of the fragments after incubation, which was analysed using denaturing gel electrophoresis.

![Figure 3.11: Schematic representation of the metal-binding group of modified oligonucleotide binding to the active site metal centre, leading to inhibition.](image)

The binding between large molecules such as proteins and nucleic acids can also be analysed more directly using electrophoretic mobility shift assays, where the complex migrates as one during non-denaturing gel electrophoresis. The protein-nucleic acid complex has a larger molecular weight than the nucleic acid alone and typically moves through the gel at a slower rate. This method was investigated for the study of the modified oligonucleotides binding to SNM1A.
3.2.2.1 Gel-based inhibition assay

To investigate this binding mode, a competitive inhibition assay, similar to the one carried out with the nucleoside inhibitors, was performed. The unlabelled modified oligonucleotides were incubated with SNM1A and an equimolar amount of fluorescently tagged substrate 82 for one hour and the reaction mixture was separated using gel electrophoresis (Figure 3.12). The reactions were set up on ice and started by the addition of substrate 82 (Figure 3.12a) or the modified oligonucleotide and SNM1A were preincubated for 5 min prior to the addition of substrate 82 (Figure 3.12b). The non-modified oligonucleotide 81a was again used as a control to ensure that any observed difference is a result of the modification and not due to the presence of additional oligonucleotides.

**Figure 3.12:** Evaluation of modified oligonucleotides as inhibitors of SNM1A using the fluorescent substrate 82. a) Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and oligonucleotides 81a, 90a, 92a, 94a and 95a (1 eq. compared to substrate 82, 0.8 pmol, 80 nM) for 60 min at 37 °C, analysed by denaturing PAGE. b) Digestion of phosphorylated oligonucleotide 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and the modified oligonucleotides 95a and 101a (1 eq. compared to substrate 82, 0.8 pmol, 80 nM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified oligonucleotides 95a and 101a for 5 min at 37 °C prior to the addition of substrate 82. ON = oligonucleotide, nt = nucleotides.

The results show that the non-modified oligonucleotide 81a does not influence the hydrolysis reaction under the conditions used in the assay (Figure 3.12a, lane 2 vs lane 1). The oxyamide- and squaryl monoamide-modified oligonucleotides 90a (Figure 3.12a, lane 3) and 94a (Figure 3.12a, lane 4) also have no observable effect on the reaction. Surprisingly, the squaryl diamides 92a and 95a, an analogue of which was inactive as a nucleoside inhibitor, reduce the efficiency of the exonuclease, with larger oligonucleotide products remaining after the incubation.
Chapter 3 – Modified oligonucleotides for binding to SNM1A

(Figure 3.12a, lanes 5 and 6). This is particularly notable as the concentration of modified oligonucleotides used in this assay is approximately four orders of magnitude lower than that of the mononucleosides in the inhibitor assay. The oligonucleotide containing an ethanolamine substituted squaramide in the 5’-position was evaluated in a separate assay (Figure 3.12b). In this assay, preincubation of the modified oligonucleotide with SNM1A for 5 min was carried out to establish binding in the absence of the substrate. The substrate 82 was then added and its digestion after one hour was analysed by gel electrophoresis. While the preincubation enhances the inhibitory effect (Figure 3.12a, lane 5 vs Figure 3.12b, lane 2), the ethanolamine-substituted squaramide (Figure 3.12b, lane 2) was no more potent than the diethylamine substituted analogue (Figure 3.12b, lane 3) which was tested alongside it. The additional hydroxyl group appears not to contribute to metal coordination, possibly due to its insufficient acidity or the flexibility of the ethanolamine group. Rigidifying the group through an aromatic ring by using o-aminophenol instead of ethanolamine also lowers the pK_a of the hydroxyl group, but did not improve potency in work carried out by Werner Jauslin in the McGouran group.265 The ethanolamine-substituted oligonucleotide 101a was therefore not studied further.

To further explore the binding potency of the squaramides, a concentration-dependence study was carried out with oligonucleotides 92a (Figure 3.13a) and 95a (Figure 3.13b).

![Figure 3.13: Concentration dependence of inhibition of oligonucleotides 92a and 95a. Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and oligonucleotide 92a (a), 95a (b) or control 81a (10-0.3 eq. compared to substrate 82, 8-0.24 pmol, 800-24 nM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified oligonucleotides 92a, 95a and 81a for 5 min at 37 °C prior to the addition of substrate 82. ON = oligonucleotide. nt = nucleotides.](image)
The amount of modified oligonucleotide was varied from 10 to 0.3 equivalents relative to the substrate, which corresponds to 0.8 µM to 0.024 µM concentrations of the modified oligonucleotide. At concentrations above one equivalent, the presence of additional oligonucleotide had a notable effect on the hydrolysis reaction, with the unmodified oligonucleotide 81a inhibiting the reaction at these concentrations (Figure 3.13a-b, lanes 3 and 5). However, this effect was less pronounced than that observed with the modified oligonucleotides 92a (Figure 3.13a, lanes 4 and 6) or 95a (Figure 3.13b, lanes 4 and 6). The most pronounced difference after 60 min was observed at 3.3 equivalents of modified oligonucleotide (Figure 3.13a-b, lanes 4 and 6). Inhibition is not observed when the stoichiometry is below one equivalent (Figure 3.13a-b, lane 10). While this appears to be a high concentration of modified oligonucleotide, it is important to note that one equivalent corresponds to a concentration of only 80 nM.

The reaction progress was also analysed over time, showing that the reaction is near completion after 60 min (Figure 3.14a-b, lane 14).

![Table 3.1](image)

**Figure 3.14:** Time dependence of inhibition of oligonucleotides 92a and 95a. Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and oligonucleotide 92a (a), 95a (b) or control 81a (3.3 eq. compared to substrate 82, 2.6 pmol, 264 nM) or no additional oligonucleotide for 5-60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified oligonucleotides 92a, 95a and 82 for 5 min at 37 °C prior to the addition of substrate 82. ON = oligonucleotide. nt = nucleotides.
The rate of hydrolysis of 5’-phosphorylated oligonucleotide \(82\) is notably different between the different experiments. The addition of either oligonucleotide \(92a\) or \(95a\) slows down the reaction compared to reactions without any additional oligonucleotide, and this effect is visible from 10 min onwards (Figure 3.14a-b, lanes 5-19). However, the modified oligonucleotides \(92a\) and \(95a\) have a greater impact on the rate than the 5’-hydroxyl negative control \(81a\) between 20 and 60 min incubation (Figure 3.14a-b, lanes 8-16), with the biggest contrast after 60 min (Figure 3.14a-b, lanes 14-16). The same trends were observed for both squaramides \(92a\) and \(95a\), suggesting that there is no significant difference between the binding of the methylamine-substituted squaramide \(95a\) and the diethylamine-substituted squaramide \(92a\). The ethanolamine-substituted squaramide was not evaluated in this assay, as the hydroxyl group does not offer any advantage for binding based on the initial screening (Figure 3.12b).

To quantify their potency, one of the squaryl diamides (\(95a\)) was incubated with SNM1A and the substrate \(82\) and the reaction was stopped at a point where there was still full-length substrate remaining. The extent of inhibition was determined by comparing the amount of full-length substrate remaining in the different samples using the fluorescence intensity of the 21-nucleotide band after gel electrophoresis. This measures the efficiency of the removal of only the first nucleotide.

Experiments without enzyme, without modified oligonucleotide, and with the unmodified oligonucleotide \(81a\) were used as controls. Nine experiments were carried out in sets of three (Figure 3.15a).
Chapter 3 – Modified oligonucleotides for binding to SNM1A

Figure 3.15: Quantification of the inhibitory effect of modified oligonucleotide 95a. a) Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and oligonucleotide 95a, control 81a (3.3 eq. compared to substrate 82, 2.6 pmol, 264 nM) or no additional oligonucleotide for 10 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified oligonucleotides 95a and 81a for 5 min at 37 °C prior to the addition of substrate 82. b) Quantification of full-length substrate 82 remaining after experiments shown in a). Values represent the mean ±SD carried out for n = 9 experiments performed in sets of three. ON = oligonucleotide. nt = nucleotides.

The results show that in the absence of any additional oligonucleotide, 33% of the full-length substrate remained after the reaction. While the addition of unmodified oligonucleotide 81a increased this to 37%, the addition of 5'-modified oligonucleotide 95a showed a slightly stronger effect, with 45% of full-length substrate present after the reaction (Figure 3.15b). A paired t-test comparing the
non-modified oligonucleotide 81a and the squaramide oligonucleotide 95a gave a p-value of 0.019 which shows that the difference between the two is statistically significant.

This assay highlights again that while the squaramide modification confers some inhibitory potency, the binding is relatively weak. This is consistent with the observation that squaramide-modified nucleosides did not inhibit SNM1A.

3.2.2.2 Electrophoretic mobility shift assay

In order to probe the interaction between the nuclease and the squaramide-modified oligonucleotide further, an electrophoretic mobility shift assay (EMSA) was carried out. EMSA allows the visualisation of the enzyme-nucleic acid complex, as non-denaturing conditions are used and the complex is stabilised during gel electrophoresis.264 This method allows the calculation of affinity constants, but it requires the formation of a sufficiently strong interaction between the enzyme and the substrate.

An EMSA has been reported for SNM1A with a 32P-labelled 5'-phosphorylated 51mer substrate.50 The reported experiment was carried out at 1 nM concentrations of oligonucleotide and enzyme concentrations ranging from 0.1 nM to 50 nM. Due to the lower sensitivity of fluorescence detection methods, these conditions were adapted for the Cy3-labelled oligonucleotides and an oligonucleotide concentration of 0.4 μM and enzyme concentrations ranging from 2 μM to 20 μM were used. As the size of the oligonucleotide substrate influences the strength of the interaction, a Cy3-labelled 5'-phosphorylated 51mer was initially used to optimise the conditions (Figure 3.16a). Binding was observed with this substrate for all concentrations of SNM1A (Figure 3.16a, lanes 2-4). However, when using the shorter 21mer, no binding was observed with either the phosphorylated or modified oligonucleotide (Figure 3.16b). Instead, the 5'-phosphorylated oligonucleotide appeared to be digested (Figure 3.16b, lanes 2-4).
Figure 3.16: Electrophoretic mobility shift assay with various fluorescent oligonucleotides. 

a) Incubation of SNM1A (20-200 pmol, 2-20 µM) with a 5'-phosphorylated 51mer oligonucleotide (4 pmol, 0.4 µM) for 5 min at 37 °C before quenching on ice. Separation using non-denaturing PAGE. 
b) Incubation of SNM1A (20-200 pmol, 2-20 µM) with oligonucleotides 82, 95b and 81b (4 pmol, 0.4 µM) for 5 min at 37 °C before quenching on ice. Separation using non-denaturing PAGE. ON = oligonucleotide, nt = nucleotides.

These results show that EMSA also works at higher concentrations than those previously reported for SNM1A. However, the complex between 21mer oligonucleotides and SNM1A could not be observed in EMSA, regardless of whether the oligonucleotide was phosphorylated or bearing a squaramide modification (Figure 3.16, lanes 2-4 and lanes 6-8). This is valuable information for the design of ABPs, which may require an oligonucleotide scaffold longer than 21 nucleotides to achieve strong binding.

3.3 Squaramide-modified oligonucleotides bind to SNM1A

The synthesis of 5’-modified oligonucleotides through solid-phase synthesis proved challenging, with only one of eight potential modified nucleosides successfully incorporated in this way. However, an alternative synthetic strategy was developed for the synthesis of squaramide-modified oligonucleotides. This strategy permits the synthesis of squaryl monoamides, which were not accessible from solid-phase synthesis and allows for the introduction of a range of amines from a common precursor oligonucleotide. Reaction conditions for these late stage modifications were optimised using nucleosides as a model system and applied to the oligonucleotides, giving rise to four differently substituted squaramides.
Chapter 3 – Modified oligonucleotides for binding to SNM1A

Gel-based assays showed that all modified oligonucleotides were stable to the nuclease under the conditions used, and all squaryl diamide-containing oligonucleotides inhibited SNM1A to a similar degree. The introduction of an alcohol group as part of the squaramide functionality did not improve binding through additional metal coordination. Further analysis and quantification revealed that the binding interaction is weak, and the modified oligonucleotide cannot compete with the natural substrate in less than equimolar amounts. However, this is expected, given the lack of inhibitory activity of the corresponding modified nucleosides. EMSA was carried out with little success due to the size of the oligonucleotides, which were too short for strong binding. ABPs for SNM1A may require oligonucleotide scaffolds longer than 21 nucleotides to harness the electrostatic interactions between the enzyme surface and the phosphate backbone.

An important difference between the nucleoside and oligonucleotide assays is the concentration of the inhibitors. Due to the inherent ability of the oligonucleotide strand to bind to SNM1A, lower concentrations than with modified nucleosides were used. The highest concentration used for oligonucleotides is 0.8 µM, which is approximately 1000-fold lower than the highest concentration used for nucleosides. While at this concentration the unmodified oligonucleotide also partially inhibited digestion, the squaramide-modified oligonucleotide showed a stronger effect. The corresponding nucleoside inhibitor failed to show any effect at 1000-fold higher concentrations. This again highlights the increased binding in the active site resulting from the electrostatic interactions between the oligonucleotide backbone and the surface of the enzyme.

Overall, these results strongly support the hypothesis that the interaction of weak metal-binding groups can be enhanced by conjugation to an oligonucleotide. Squaramides were not found to bind to SNM1A as part of a nucleoside but bound to the enzyme once conjugated to an oligonucleotide. The introduction of the group after solid-phase synthesis and the scope for substitution makes it an attractive starting point for further investigations. The replacement of the carbonyl groups by thioketones was investigated by Werner Jauslin and Mark Berney in the McGouran group. The findings also illustrate that the incorporation of metal-binding groups into oligonucleotide scaffolds enhances their affinity for SNM1A and is a potential avenue to increase the probes’ selectivity for nucleases over other metalloenzymes.
4 Malonate-based mono- and dinucleoside inhibitors

A family of malonate-based nucleoside derivatives were designed to evaluate the potential of the malonate group as a metal-chelator in ABPs for SNM1A. The malonate group is a known phosphate isostere and can chelate metal ions. It also offers points for derivatisation as hydroxamic acids as well as multiple options for connection to nucleosides, making it an ideal candidate for metal-binding groups in ABPs targeting SNM1A.

In this chapter, malonate derivatives were installed in at the 5'-position, like in previous nucleoside inhibitors, but also at the 3'-position of a mononucleoside and as a linkage between two nucleosides. Comparison between these analogues was used to identify the site that leads to the strongest binding (Figure 4.1).

Figure 4.1: Anticipated binding of modified nucleosides to SNM1A compared to substrate binding.

Connectivity to the nucleoside via an amide linkage and alkylation of the α-carbon of the malonate group were both explored. For each nucleoside scaffold, methyl ester, sodium carboxylate and hydroxamic acid derivatives were designed and synthesised for comparison (Figure 4.2).
4.1 Design and synthesis of malonate-based nucleosides

In the evaluation of modified mononucleosides as inhibitors of SNM1A (Chapter 2), the hydroxamic acid group resulted in the strongest inhibitors. Therefore, further investigations into installing this group through a malonate scaffold were carried out in order to improve the potency. The malonate group adds further potential metal-binding sites and was therefore anticipated to enhance affinity. In several metallopeptidase inhibitors, the hydroxamic acid group is linked to the substituents

Twelve of these malonate-derived nucleosides and dinucleoside targets (Figure 4.2) were synthesised successfully and evaluated as inhibitors of SNM1A in gel-based assays. The concentration dependence of all active compounds was investigated. The results illustrate the potential of these compounds as metal-binding groups of ABPs for the nuclease SNM1A. They also provide insights into the binding mode of the modified nucleoside to the active site and the optimal positioning of malonate groups relative to the deoxyribose core. Results presented in this chapter were published in *Molecules* in 2021.

![Figure 4.2: Structures of target modified nucleosides and dinucleosides.](image)

102: \( R = \text{OMe}, R' = \text{ONa} 
103: \( R = \text{R} = \text{ONa} 
104: \( R = \text{OMe}, R' = \text{NHOH} 
105: \( R = \text{ONa}, R' = \text{NHOH} 
106: \( R = \text{R} = \text{NHOH} 
107: \( R = \text{OMe} 
108: \( R = \text{ONa} 
109: \( R = \text{NHOH} 
110: \( R = \text{OMe}, R' = \text{ONa} 
111: \( R = \text{R} = \text{ONa} 
112: \( R = \text{OMe}, R' = \text{NHOH} 
113: \( R = \text{ONa}, R' = \text{NHOH} 
114: \( R = \text{R} = \text{NHOH} 
115: \( R = \text{OMe} 
116: \( R = \text{ONa} 
117: \( R = \text{NHOH} 
118: \( R = \text{OMe} 
119: \( R = \text{ONa} 
120: \( R = \text{NHOH} 
121: \( R = \text{OMe} 
122: \( R = \text{ONa} 
123: \( R = \text{NHOH} 
124: \( R = \text{OMe} 
125: \( R = \text{ONa} 
126: \( R = \text{NHOH} 
127: \( R = \text{OMe} 
128: \( R = \text{ONa} 
129: \( R = \text{NHOH} \)
through a malonate scaffold.\textsuperscript{109,267-273} In addition, the malonate group itself can act as a phosphate bioisostere.\textsuperscript{187} These properties make combinations of malonate and hydroxamic acids a promising avenue to explore in the design of ABPs for SNM1A.

4.1.1 Design of malonate-based nucleosides

The malonate group has found application in inhibitors both as a scaffold for a hydroxamic acid group\textsuperscript{109,267-273} and as a phosphate bioisostere in many different contexts, including analogues of phosphosugars,\textsuperscript{199-202} nucleotides,\textsuperscript{190,203} phosphorylated amino acids\textsuperscript{204-207} and herbicides.\textsuperscript{208-210} The group’s suitability as a phosphate mimic arises from its doubly charged nature at physiological pH, which resembles the natural phosphate group (\textit{Figure 4.3a}).\textsuperscript{210} However, the malonate group occupies a \(~13\%\) larger volume than a phosphate group,\textsuperscript{274} so steric requirements of the binding site need to be considered. In the realm of nucleotides and oligonucleotides, the use of the malonate group has been limited, but the results are promising. A nucleotide triphosphate analogue bearing a malonic acid group in place of the pyrophosphate was recognised by HIV-1 reverse transcriptase.\textsuperscript{203} Malonic acid incorporated at the 5'-position of a siRNA strand as a phosphate mimic improved metabolic stability while maintaining the gene silencing activity.\textsuperscript{190} The malonate group was also found to coordinate to two metal ions in a ribozyme in a bridging manner (\textit{Figure 4.3b}),\textsuperscript{202} as well as chelating a single metal ion in recent crystal structures of SNM1A (\textit{Figure 4.3c}).\textsuperscript{54}

\textit{Figure 4.3}: a) Malonate group compared to phosphate group; b) bridging coordination of metal ions observed by Fei et al.;\textsuperscript{202} c) Chelation of Ni\textsuperscript{2+} by malonate anion in crystal structure of SNM1A.\textsuperscript{54}

By combining hydroxamic acid and malonic acid groups, an additional chelating moiety is introduced, and different binding modes to two metal ions can be envisaged (\textit{Figure 4.4}).
Figure 4.4: Potential metal-binding modes of hydroxamic acid malonates. a) Singly charged hydroxamic acid malonate. b) and c) Doubly charged hydroxamic acid malonate.

The binding mode shown in Figure 4.4a uses the singly charged metal-binding group and chelates through the deprotonated hydroxamic acid group and the two carbonyl groups. The other binding modes (Figure 4.4b-c) are doubly negatively charged. The pK\textsubscript{a} of the α-proton of diethyl malonate is 15.2 in water and 16.4 in DMSO\textsuperscript{275} and the pK\textsubscript{a} of alkyl-substituted dimethyl malonate in DMSO ranges from 15.9 to 18.5, depending on the substituents at the α-carbon.\textsuperscript{276} The metal-stabilised enolate forms shown in Figure 4.4b-c are therefore available for metal coordination under physiological conditions.

The malonate group offers several points for derivatisation, such as the introduction of substituents through amide couplings, α-alkylation or the introduction of hydroxamic acid groups. While the scope for derivatisation is an attractive aspect, alkylation at the α-carbon in combination with derivatisation of the carboxylic acid groups can produce a chiral centre at the α-carbon. However, the resulting stereoisomers are expected to interconvert readily in buffered solution due to the acidity of the α-proton. Additionally, in the enolate form that is anticipated to contribute to binding as shown in Figure 4.4, the stereochemistry at the α-carbon is lost. Therefore, several of the target compounds were synthesised and evaluated as a mixture of interconverting diastereomers.

The nucleobase used for this generation of modified nucleosides was thymine, and malonate-derived groups were installed at the 5’-position (Figure 4.5a), allowing for direct comparison with the previously evaluated compounds (Chapter 2). However, as the scissile phosphodiester bond links two nucleotides, a 5’-modified nucleoside only mimics part of this structure and other points of installation were explored. Malonate modifications were therefore also introduced at the 3’-position (Figure 4.5b) and as a linker between two nucleosides (Figure 4.5c). The altered position of the metal-binding group relative to the nucleoside leads to different
interactions between the nucleoside and the active site, which potentially influences binding. Some of the target nucleosides and dinucleosides have the 5’-position available for phosphorylation to potentially further enhance binding through the introduction of this important recognition element.\textsuperscript{28,37}

\textbf{Figure 4.5:} Installation of a metal-binding group at the 5’-position (a), 3’-position (b) or connecting two nucleosides (c).

Comparison of the 3’-analogue with the 5’-modified nucleoside allows for the evaluation of the interactions between the active site pocket and the nucleoside core. The second nucleoside of the dinucleoside targets is an additional recognition element and is expected to increase binding to the active site as well as specificity. However, due to the different linker between the nucleosides, steric clashes within the active site may occur and reduce binding affinity.

\subsection*{4.1.2 Synthesis of modified mononucleosides}

The linkage between the malonate group and the nucleoside was chosen to be either an amide bond or a carbon-carbon bond to the α-carbon of the malonate. The targets shown in \textbf{Figure 4.6} not only include carboxylic acids and hydroxamic acids, but also esters and amides. While esters and amides of malonic acid do not possess the charged carboxylate groups, malonamides have been demonstrated to bind metal ions in the active site of HIV integrase through their enol form.\textsuperscript{277} This provides the option of connecting the malonate group to the nucleoside through an amide linkage without the loss of a metal-binding site. Although ester analogues were found to be inactive compared to their carboxylic acid analogues,\textsuperscript{206} metal coordination of esters through the carbonyl carbon has been reported.\textsuperscript{277} The ester analogues of the malonate-modified nucleosides were therefore also included as targets.
Figure 4.6: Nucleosides containing malonate modifications in the 5'-position (a,b) or 3'-position (c,d).

As alkylation at the α-carbon leaves both carboxylic acid groups available for binding or derivatisation, the resulting nucleosides are promising targets (Figure 4.6a,c). Connection via amide linkage results in a different orientation of the malonate group. This connectivity gives the six modified nucleoside targets shown in Figure 4.6b,d. All malonic acid targets were designed as the sodium malonate salt, as this form is less prone to decarboxylation.278

The synthesis of these linkages exploits two reactive moieties of the malonate group, the carboxylic acid group and the acidity at the α-carbon (Scheme 4.1). The nucleosides were modified to contain either an amine or a leaving group (LG) in the desired positions.

Scheme 4.1: Two approaches to connect the malonate group to the nucleoside.
4.1.2.1 Synthesis of 5'-modified nucleosides

The synthesis of the 5’-malonate targets 102-106 in which the malonate group is connected to the nucleoside through a C-C bond was planned to proceed via the common intermediate 135 (Scheme 4.2).

![Scheme 4.2: Synthetic strategy for 5'-malonoyl nucleosides. Targets highlighted in green.](image)

Targets 104, 105 and 106 all contain hydroxamic acid groups that can be introduced via amide couplings with protected hydroxylamine. Therefore, all three hydroxamic acid targets derive from the common protected hydroxamic acids 133 or 134. The sodium malonate target 103 can be derived from ester 102, which is also an intermediate for all hydroxamic acid targets. This ester can be obtained from the protected common intermediate 135. Since this common intermediate 135 is similar in structure to a dimethyl malonate-substituted uridine that was reported by Zlatev et al., the synthesis of intermediate 135 was based on this reported synthesis (Scheme 4.3).
Scheme 4.3: Synthetic strategy for common intermediate 5'-malonyl nucleoside 135.

The malonate group can be installed in a substitution reaction with protected 5'-iodothymidine 137. The nucleobase of iodide 78 was protected using a benzyloxymethyl (BOM) group and the 3'-hydroxyl group of 5'-iodothymidine was masked by a TBDMS group to avoid undesired side reactions.

The synthesis of malonate ester 135 (Scheme 4.4) was carried out starting from iodothymidine 78, which was synthesised as an intermediate for previous targets (Chapter 2).

Scheme 4.4: Synthesis and deprotection of common intermediate 135 from 5'-iodothymidine.

Protection of the 3'-hydroxyl group of iodide 78 as the silyl ether to give iodide 138 proceeded in 87% yield using a modified literature procedure. BOM-protection of the imido group of thymidine afforded the bis-protected iodide 137 in 95% yield. This iodide was reacted with benzyl methyl malonate (136) under similar conditions to those reported by Zlatev to give the intermediate 135 in 66% yield. The introduction of the malonate ester results in the formation of two diastereoisomers which interconvert in solution and were thus not separated. The selective removal of the benzylation ester was achieved in 95% yield by hydrogenation with Pd/C, which also partially removes the BOM group, to give 139. The hydroxymethylene group
of the BOM-protecting group was not removed in this reaction. This hemiaminal was also observed by Pallan et al.\textsuperscript{231} but in their hands the desired product was obtained following treatment with Na\textsubscript{2}CO\textsubscript{3}. However, in this case, attempts to hydrolyse the hemiaminal of nucleoside 139 using NaHCO\textsubscript{3} in THF led to decomposition and the desired product 140 was not observed.

Reversing the order of deprotection was investigated to overcome the challenges associated with complete removal of the BOM group (Scheme 4.5).

**Scheme 4.5:** Synthesis of targets 102 and 103 via an alternative deprotection strategy.

The TBDMS-group of malonate ester 135 was removed using TBAF in 71\% yield, followed by catalytic hydrogenation of alcohol 141 to remove the benzyl ester and the BOM protecting group. Interestingly, the BOM protecting group of alcohol 141 was fully removed in this reaction, with no hemiaminal observed in the product 142, which served as an intermediate. For biological testing, ion exchange was carried out to obtain the more stable carboxylate salt 102, but contamination from the ion exchange resin was observed. Purification of a small amount of sodium salt 102 using reversed phase preparative TLC was successful in removing this impurity and afforded compound 102 in 22\% yield. For the synthesis of bis-carboxylate 103, the carboxylic acid 142 was used for ease of purification. Ester hydrolysis followed by treatment with ion exchange resin afforded target 103 in 55\% yield after purification by reversed phase preparative TLC.
Alternative protecting group strategies were explored for the synthesis of the other target compounds to circumvent problems with the partial deprotection, the challenging purification of nucleoside 141 and the relatively low yield of the hydrogenated product 142. For the synthesis of all targets, the hydroxyl group and the nucleobase need to be protected only for the alkylation of the malonate ester and are removed at the same stage, so no orthogonality is required. The global protection of iodothymidine 78 with BOM chloride as well as benzyl bromide was therefore attempted (Scheme 4.6).

Scheme 4.6: Exploration of global BOM-protection (a) or benzyl-protection (b) for iodothymidine 78.

The potential elimination of iodide and substitution by the chloride or bromide of the reagent was a concern, so mild conditions were attempted initially. Sodium iodide was added to the reaction with BOM chloride to form the more reactive iodide and to sequester the chloride ions as insoluble sodium chloride (Scheme 4.6a). While no substitution or elimination was observed under these conditions, the 3'-hydroxyl group of iodide 78 remained mostly unprotected, with the mono-protected product 144 obtained in 67 % yield and only a 21% yield of the desired product 143. The use of a stronger base and benzyl bromide (Scheme 4.6b) resulted in global protection, but both elimination and substitution of the iodide were observed, giving bromide 146 and alkene 147 as the major products and none of the desired product 145.

Returning to the original protecting group strategy, both the carboxylic acid containing the hemiaminal 139 and the carboxylic acid without hemiaminal group...
142 were used in amide couplings with protected hydroxylamine to form the protected hydroxamic acid groups (Scheme 4.7). The TBDMS group is used as a protecting group for the 2’-position in RNA solid-phase synthesis. Oligonucleotides are therefore stable to the deprotection conditions required to remove the group. This implies that nucleosides are also stable to the deprotection conditions and furthermore makes this route potentially applicable for incorporation into oligonucleotides. TBDMS-protected hydroxylamine is commercially available and has been employed in the synthesis of hydroxamic acids.

Schemes 4.7: Amide couplings between 5’-malonyl thymidine 139 or 142 and TBDMS-protected hydroxylamine (a) or benzyl-protected hydroxylamine (b,c).

When TBDMS-protected hydroxylamine was reacted with nucleoside 142, no product 148 was observed (Scheme 4.7a). Reaction of the same carboxylic acid with O-benzylhydroxylamine gave the desired product 149 in 14% yield (Scheme 4.7b). The reaction was also attempted with the 3’-protected carboxylic acid 139, and using DMAP in place of HOAt and DIPEA to lower the steric demand (Scheme 4.7c). Using catalytic amounts of DMAP resulted in no desired product 150, and the stoichiometric use of DMAP led to decarboxylation of starting material and loss of the hemiaminal to give ester 151.

While the protected hydroxamic acid 149 was obtained this way, the yield of 14% is very poor. As this compound is a synthetic intermediate for three target nucleosides,
higher yields were desirable. To avoid the loss of valuable material in the low-yielding amide couplings with protected hydroxylamine, an alternative synthetic strategy was developed (Scheme 4.8).

**Scheme 4.8:** Alternative synthetic strategy for hydroxamic acid targets 104, 105 and 106. Targets highlighted in green.

In this strategy (Scheme 4.8), the protected hydroxamic acid groups are introduced to the malonate core prior to the alkylation with iodothymidine. The final step in the synthesis of target 105 is deprotection of the ester in target 104. This target 104 can be obtained from protected precursor 152 by removing the protecting groups of the hydroxamic acid group and the nucleobase and deprotection of the 3'-hydroxyl group. The protected precursor 152 results from alkylation of protected iodothymidine 137 with a substituted malonate. The hydroxamic acid target 106 was also thought to be accessible through hydrogenation from its protected precursor 153. The malonate group of 153 can be installed in a substitution reaction with iodothymidine 137. This strategy moves the low yielding amide couplings with oxyamines to earlier in the synthesis prior to coupling to the nucleoside and therefore reduces the loss of valuable material.

For this alternative strategy, the substituted malonates were synthesised from commercially available monomethyl potassium malonate (154). Two different coupling reagents were compared to optimise the amide coupling (Scheme 4.9). Using EDC and HOAt resulted in a yield of only 14%, with the crude reaction mixture containing a range of different compounds. Using EDC with 0.1 equivalents of
DMAP not only resulted in the formation of less byproducts, but also increased the yield to 65%. This ester 155 is the substrate required for the synthesis of targets 104 and 105.

Scheme 4.9: Synthesis of O-benzyl hydroxamate malonate ester 155.

The alkylation reaction between iodothymidine 137 and malonate 155 resulted in the N-alkylated hydroxamate ester 156 instead of the desired C-alkylated product 152 (Table 4.1). This reactivity via the nitrogen has been described previously. Using THF as the solvent, the yield of nucleoside 156 was only 4% (Table 4.1, entry 1) despite the use of six equivalents of malonate relative to iodide 137 and heating to 70 °C for 30 h. Changing the solvent to 1,2-dimethoxyethane (DME) allowed for a higher reaction temperature of 85 °C and in combination with a longer reaction time of 50 h increased the yield of 156 from 4% to 28% (Table 4.1, entry 1 vs entry 2). Increasing the equivalents of sodium hydride from two to four equivalents relative to iodide 137 further increased the yield of 156 to 45% (Table 4.1, entry 2 vs entry 3). In all cases, none of the desired product 152 was isolated.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>Equivalents of NaH</th>
<th>Temperature</th>
<th>Time</th>
<th>Yield of 156</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>THF</td>
<td>2.0</td>
<td>70 °C</td>
<td>30 h</td>
<td>4%</td>
</tr>
<tr>
<td>2</td>
<td>DME</td>
<td>2.0</td>
<td>85 °C</td>
<td>50 h</td>
<td>28%</td>
</tr>
<tr>
<td>3</td>
<td>DME</td>
<td>4.0</td>
<td>85 °C</td>
<td>50 h</td>
<td>45%</td>
</tr>
</tbody>
</table>

Table 4.1: Alkylation of iodide 137 with O-benzyl hydroxamate malonate ester 155.
Despite alkylation occurring at the nitrogen of the protected hydroxamic acid instead of the α-carbon, the resulting nucleosides are still valuable synthetic targets. The deprotection of nucleoside 156 to afford the hydroxamic acid-modified nucleosides was therefore attempted (Scheme 4.10).

**Scheme 4.10:** Attempted synthesis of hydroxamic acid 158 from protected precursor 156.

The 3'-hydroxyl group was unmasked using TBAF in 51% yield to afford nucleoside 157 (Scheme 4.10). Deprotection of the hydroxamic acid group via hydrogenation proved challenging. Using palladium on charcoal appeared to only remove the benzyl group but not the BOM group, so the crude product was subjected to hydrogenation using palladium hydroxide, which resulted in the formation of several products. Due to the small amount of material and its poor solubility, purification of these compounds was unsuccessful and compound 158 was not obtained.

In an attempt to overcome the difficulties observed with the deprotection of the hydroxamic acid and the nucleobase, the sequence of deprotection was altered (Scheme 4.11).

**Scheme 4.11:** Attempted alternative deprotection strategy of protected hydroxamic acid 156.

Removal of the BOM-group and the benzyl group was attempted prior to TBDMS-deprotection. This change was anticipated to improve the solubility of the desired product of the hydrogenation 159 and therefore simplify purification if a mixture of products was obtained. Unfortunately, at least four new products were observed by TLC analysis of the reaction mixture and this route was not deemed viable.
In parallel, the synthesis of target 106 containing two hydroxamic acid groups was attempted (Scheme 4.12) through an analogous route to that used for targets 104 and 105.

**Scheme 4.12: Synthesis of bis-hydroxamate ester 161.**

To this end, the methyl ester of malonate 155 was hydrolysed to free the carboxylic acid. This was achieved using potassium hydroxide in methanol followed by ion exchange to obtain the sodium salt 160 in 98% yield. This salt was then coupled with O-benzylhydroxylamine in 49% yield, affording the bis-hydroxamate ester 161. This malonate derivative 161 was used in an alkylation reaction with iodothymidine 137 (Scheme 4.13).

**Scheme 4.13: Alkylation of iodide 137 with bis-hydroxamate ester 161 and attempted deprotection.**

Alkylation occurred at the nitrogen, giving product 162 in 31% yield and none of the desired product 153. No further nucleoside products other than starting material 137 were isolated from the reaction. While the positioning of the metal-binding group is different than in the desired product 162, the hydroxamic acid resulting from deprotection could also bind to the active site of SNM1A. Deprotection of the 3'-hydroxyl group was carried out in 49% yield using TBAF and the resulting alcohol 163 was hydrogenated using palladium on charcoal as the catalyst. The identity of
the product could not be confirmed conclusively due to broadened signals in the NMR spectrum and poor solubility in DMSO-d6. However, the presence of two methylene signals and signals in the aromatic region point towards the presence of a BOM-group. Further to this, the desired product 164 was not observed in MS, while a compound with a mass corresponding to the BOM-protected bis-hydroxamic acid was found.

Hydroxamic acids can be synthesised from esters by reacting the ester with hydroxylamine under basic conditions. This strategy is unsuitable for the synthesis of target 104 (Figure 4.6), which contains an ester as well as a hydroxamic acid group. However, it was expected to be applicable to the other two hydroxamic acid targets 105 and 106 (Figure 4.6).

Aminolysis of ester 142 to synthesise target 105 was carried out using Sharma’s conditions (Scheme 4.14). Following aminolysis, the product was eluted through ion exchange resin to obtain the carboxylate salt 105, which resulted in contamination. Purification by reversed phase preparative TLC successfully afforded compound 105 in 15% yield.

Scheme 4.14: Synthesis of hydroxamic acid 105 via aminolysis of ester 142 with hydroxylamine.

A similar approach was envisaged for target 106 (Scheme 4.15). Its synthesis requires replacing the benzyl ester with an ester that is stable to hydrogenation. Removal of the BOM-group prior to installation of the hydroxamic acid groups was expected to avoid the challenges observed with BOM-deprotection of hydroxamic acid containing compounds (Scheme 4.15).
Scheme 4.15: Alternative synthetic strategy for hydroxamic acid 106 via aminolysis.

The dimethyl ester 165 without any protecting groups was expected to be a suitable precursor for the bis-hydroxamic acid 106 (Scheme 4.15). It can be obtained from hydrogenation of nucleoside 166, which is accessible through alkylation of iodothymidine 137. However, to test whether the aminolysis of a malonate ester results in the presence of two hydroxamic acid groups, the available benzyl methyl ester 135 was used in a test reaction (Scheme 4.16).

Scheme 4.16: Attempted synthesis of bis-hydroxamic acid 167 via aminolysis of ester 135 with hydroxylamine.

The aminolysis of diester 135 as a model system for the dimethyl ester 165 showed that cyclisation occurred to give compound 168 rather than the desired product 167 (Scheme 4.16). The cyclisation reaction leading to nucleoside 168 has also been reported in the literature.292,293 NMR studies of compound 168 were carried out and showed that one of the carbonyl groups of the newly formed ring is present as its enol tautomer, but the exact structure of the tautomer could not be elucidated. While NMR studies carried out in deuterated methanol confirmed the cyclisation and presence of a quaternary carbon in the 6’-position, attempts to identify exchangeable protons through studies in deuterated acetone led to decomposition of the initial product and the formation of at least two new compounds.

With three of the five 5’-C-malonate targets in hand, the synthesis of the amide-linked 5’-modified nucleosides was planned. The synthesis of these nucleosides 107-109 was carried out in a divergent manner (Scheme 4.17).
Scheme 4.17: Synthesis of 5'-malonate modified nucleosides using an amide linkage.

5'-Aminothymidine 80 was obtained in 99% yield from catalytic hydrogenation of azide 79. Amide coupling with monomethyl potassium malonate (154) afforded ester 107 in 54% yield. This target served as an intermediate in the synthesis of target 108, which was obtained in 98% yield by ester hydrolysis followed by treatment with ion exchange resin. Reaction of ester 107 with hydroxylamine under basic conditions afforded target hydroxamic acid 109 in 83% yield.

These three targets complete the panel of 5’-modified nucleosides that were used for biological evaluation (Figure 4.7).

Figure 4.7: 5'-Malonyl compounds synthesised successfully.
4.1.2.2 Synthesis of 3’-modified nucleosides

The synthetic strategy for the 3’-modified targets 110-114 is shown in Scheme 4.18 and derives from a common intermediate. Deprotection of the corresponding benzyl-protected hydroxamic acid was chosen as the final step in the synthesis of hydroxamic acid derivatives 112, 113 and 114 as masking of the highly polar hydroxamic acid group was expected to aid solubility. Removing the protecting group as the final step was therefore anticipated to simplify purification of synthetic intermediates. When a carboxylate group is present in the final product such as in compounds 110, 111 and 113, ester hydrolysis was envisaged as the final step to minimise the risk of decarboxylation under acidic conditions required for deprotection of the 5’-hydroxyl group. All five target compounds arise from one common intermediate, the 3’-malonyl diester 169. The benzyl methyl malonate ester group of this common intermediate 169 was envisaged to be installed via nucleophilic substitution of a leaving group in the 3’-position of protected thymidine.

Scheme 4.18: Synthetic strategy for 3’-alkylated malonates from common intermediate 169. Targets highlighted in green, LG = leaving group.
To synthesise the common intermediate 169, the 5'-position of thymidine (1) was protected in 76% yield with DMTr chloride using a modified literature procedure (Scheme 4.19). The first leaving group that was explored for the alkylation with malonate ester 136 was a mesylate group. To retain the overall stereochemistry, inversion of the stereochemistry at the 3'-position of protected thymidine 170 was carried out in 90% yield by sequential mesylation and hydrolysis. The resulting alcohol 171 was then mesylated again to afford the substrate 172 in 90% yield.

The enolate ion of the malonate ester 136 was prepared in situ by reaction with sodium hydride, and reaction with the mesylate 172 to afford compound 169 was envisaged. However, the alkene 173, resulting from elimination of mesylate, was obtained in 46% yield and none of the desired product 169 was observed after 120 h at 100 °C. Interestingly, the nucleobase of alkene 173 was methylated during the reaction. Milder conditions (72 h at room temperature) were also attempted but only starting materials 172 and 136 were recovered.

Scheme 4.19: Attempted synthesis of 3'-malonyl thymidine 169 from mesylate 172 and malonate ester 136.

2,3'-Anhydrothymidine 174 was then explored as the electrophile, where the nucleobase acts as the leaving group in a ring opening reaction (Scheme 4.20). Anhydrothymidine 174 was synthesised from DMTr-protected thymidine 170 in 63% yield by mesylation of the 3'-hydroxyl group, followed by attack of the nucleobase at the 3'-position and loss of mesylate. Unfortunately, the reaction of anhydrothymidine 174 with the enolate ion of benzyl methyl malonate (136) did not afford the desired product 169.
A strategy to overcome the unreactive nature of 2,3'-anhydrothymidine was reported by Saha et al. in 1993.\textsuperscript{299} N-Alkylation of anhydrothymidine with alkyl triflates activates the sugar towards nucleophilic attack and this strategy was reported for a range of nucleophiles, including the dimethyl malonate enolate ion. The alkyl triflate used was methyl triflate in the majority of reactions, with one example reported with benzyl triflate. In this work, benzyl triflate was used initially (Scheme 4.21), as the benzyl group can be easily removed, and alkylation of the nucleobase is not desired for the target compounds \textit{110-114}. Benzyl triflate was generated \textit{in situ} from benzyl alcohol and triflic anhydride prior to the addition of anhydrothymidine \textit{174} and 2,6-di-\textit{tert}-butyl-4-methylpyridine to alkylate the anhydrosugar \textit{174}. Then, benzyl methyl malonate (\textit{136}), pre-treated with sodium hydride, was added to the reaction. A range of products that could not be identified were formed, with none of the desired product \textit{175} or anhydrosugar \textit{174} observed.

\textbf{Scheme 4.21:} Attempted activation of anhydrothymidine \textit{174} with benzyl triflate for reaction with benzyl methyl malonate.

As the above reaction is a sequence of several steps, a simpler system, which was part of the report by Saha \textit{et al.},\textsuperscript{299} was used to identify the cause of the failed reaction (Scheme 4.22).
Sodium azide was used as the nucleophile in place of the malonate enolate ion (Scheme 4.22a). Since the substitution with an azide does not lead to the formation of two diastereomers, as is the case with malonate ester 136, its use simplifies analysis of the reaction mixture. It is also less sterically hindered and has been reported to react with the activated sugar to form azide 176.299 However, no benzylated nucleoside was observed after the reaction of anhydrosugar 174 with benzyl triflate and sodium azide, which indicates that either benzyl triflate was not formed or it was unable to alkylate the nucleobase. To eliminate unsuccessful formation of the alkylating agent as a possible reason for the failed reaction, the activating agent was changed to trimethylsilyl triflate (Scheme 4.22b). The trimethylsilyl group can silylate either at oxygen or the nitrogen atom of the
nucleobase but was expected to be removed in a workup. However, no reaction was observed and the desired product 177 was not observed. A final attempt was made with methyl triflate and benzyl methyl malonate (Scheme 4.22c). In this case, alkylation of the nucleobase occurred, but the anhydrosugar was opened by water instead of the enolate to give nucleoside 179 as the major product in 31% yield.

These experiments indicate that while alkylation of the anhydrosugar 174 can be achieved with methyl triflate, the malonate enolate ion did not react with the activated structure under these conditions. In addition, analysis of the crude reaction mixtures show that elimination reactions compete with the substitution, and alkylation proved very challenging as a result. Targets requiring alkylation at the 3'-position were therefore not pursued further.

The remaining three target compounds 115-117 contain a malonate group that is connected via an amide linkage to the 3'-position of thymidine. All of these targets can be synthesised by coupling a substituted malonate salt 154 or 160 and 3'-aminothymidine 180 (Scheme 4.23). The hydroxamic acid target 117 can either be obtained from the corresponding ester 115 via aminolysis or by coupling of 3'-aminothymidine 180 with O-benzylhydroxamic acid substituted malonate 160. This sequence does not require the use of protecting groups on the nucleoside.

Scheme 4.23: Synthetic strategy for 3'-malonates 115-117. Targets highlighted in green.

The synthesis of the 3'-amino modified nucleoside 180 was achieved by the reduction of commercially available AZT (181) in 99% yield (Scheme 4.24).
malonate group was introduced in an EDC-mediated amide coupling between monomethyl potassium malonate (154) and 3’-aminothymidine 180 to give the methyl ester target 115 in 34% yield. Ester hydrolysis of the methyl ester of 115 afforded the carboxylate salt 116 in 82% yield.

Scheme 4.24: Synthesis of 3’-malonate ester 115 and carboxylate salt 116 from AZT (181).

The synthesis of the hydroxamic acid target 117 was first attempted via its benzylated precursor 182 (Scheme 4.25). Again, EDC-mediated amide coupling was used to install the malonate group at the 3’position of aminothymidine 180 to afford compound 182 in 65% yield. However, while following hydrogenation of compound 182, the desired product 117 was observed by NMR analysis alongside organic contaminants and decomposition products, the target nucleoside 117 was poorly soluble in many solvents and purification was unsuccessful.

Scheme 4.25: Attempted synthesis of 3’-hydroxamic acid malonate 117.

To circumvent hydrogenation as the final step in the synthesis of this target, an alternative strategy was employed. Aminolysis of the ester of compound 115 with
hydroxylamine in basic conditions\(^{291}\) afforded the target compound 117 in 70% yield (Scheme 4.26).

\[
\begin{align*}
\text{HO-} & \quad \text{O} \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{NH} \\
\text{Me} & \\
& \quad \text{H}_{2}\text{NOH-HCl, KOH, MeOH} \\
& \quad \text{r.t., 2.5 h} \\
& \quad 70\% \\
\end{align*}
\]

\text{Scheme 4.26: Synthesis of 3'-malonate hydroxamic acid 117 from malonate ester 115.}

This compound completes a series of analogues containing a carboxylate, a methyl ester or a hydroxamic acid group as part of a 3'-malonate modification (Figure 4.8). Comparison of the different groups is expected to provide insights into the suitability of the groups as metal-binding groups in ABPs for nucleases.

\[
\begin{align*}
\text{HO-} & \quad \text{O} \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{NH} \\
\text{Me} & \\
& \quad \text{HO-} \\
& \quad \text{OH} \\
\end{align*}
\]

\text{Figure 4.8: Successfully synthesised 3'-modified nucleosides.}

4.1.3 Synthesis of modified dinucleosides

A malonate-based metal-binding group is well suited for dinucleoside structures, as the group offers multiple points for attachment to a nucleoside. The α-carbon of the malonate group can be attached to the 3'-position or the 5'-position of the nucleoside, leaving the amide linkage for connection to the other nucleoside as in compounds 118-120 and 124-126 (Figure 4.9). Replacement of the amide by an oxyamide linkage, as in compounds 121-123 and 127-129 (Figure 4.9), allows for comparison of different linker lengths. The second carboxylic acid group remains available for derivatisation as a hydroxamic acid or ester, resulting in the twelve target dinucleosides shown in Figure 4.9.
Figure 4.9: Dinucleoside targets containing malonate-derived linkers.

As the dinucleoside targets are effectively combinations of two modified mononucleosides, the direct comparison of the 3’-modified nucleoside, 5’-modified nucleoside and dinucleoside for a given modification was expected to provide insights into points of beneficial and unfavourable interactions with the enzyme active site and aid in identifying the optimal sites for incorporating these metal-binding groups into nucleosides for ABPs.

Construction of dinucleosides 118-123 was planned via amide couplings with a 3’-malonyl nucleoside but as this modification could not be accessed (Section 4.1.2), these targets were not attainable. The synthesis of dinucleosides 124-126 was planned in an analogous manner, with an amide coupling between 3’-aminothymidine 183 and 5’-malonyl nucleoside 139 forming the dinucleoside scaffold (Scheme 4.27). Amine 183 is readily accessible from commercially available AZT (181) and intermediate 139 was synthesised successfully as part of the nucleoside series (Section 4.1.1).

Scheme 4.27: Synthetic strategy for dinucleoside targets 124-126 from amine 183 and 5’-malonyl nucleoside 139.
For the synthesis of the 5’-protected 3’-aminothymidine 183, AZT (181) was protected with DMTr chloride in 94% yield, followed by reduction of the protected azide 184 in 95% yield to afford the amine 183 (Scheme 4.28).

**Scheme 4.28:** Synthesis of protected 3’-amino thymidine 183.

An amide coupling between the two modified nucleosides 139 and 183 was carried out in 76% yield to afford the protected dinucleoside 185 (Scheme 4.29). Deprotection of the 3’-hydroxyl group proceeded in 92% yield to afford dinucleoside 186. Interestingly, the DMTr protecting group of a sample enriched in one diastereomer of 186 was removed fully during storage at -20 °C to afford dinucleoside 124, while a sample enriched in the other diastereomer proved stable under the same conditions. The DMTr group of the more stable diastereomer was removed in 92% yield using trifluoroacetic acid and the first target dinucleoside 124 was obtained as a mixture of interconverting diastereomers.

**Scheme 4.29:** Synthesis of dinucleoside target 124 from modified nucleosides 139 and 183.
Interconversion of the diastereomers of 186 was observed in an NMR sample in deuterated acetone over several days (Figure 4.10). A sample enriched in one diastereomer with ratio 3.2:1 (Figure 4.10a) changed to a 1.3:1 ratio over the course of twelve days at room temperature in deuterated acetone (Figure 4.10b).

Figure 4.10: NMR spectra of compound 186 twelve days apart. a) Initial NMR sample; b) NMR sample after twelve days.

Following the successful synthesis of the first target 124, its derivatisation to form the other targets 125-126 was attempted. The carboxylate target 125 was synthesised from the ester 124 in 89% yield via ester hydrolysis followed by treatment with ion exchange resin (Scheme 4.30).

Scheme 4.30: Synthesis of carboxylate dinucleoside 124 from ester 125.

Synthesis of the hydroxamic acid target 126 was attempted by two different routes. Amide coupling using protected hydroxylamine as described in Section 4.1.1 was attempted as well as aminolysis using hydroxylamine (Scheme 4.31). The synthetic
route shown in **Scheme 4.31a** uses the DMTr-protected dinucleoside **186** as its starting point to maintain solubility in organic solvents. The methyl ester of this dinucleoside **186** was hydrolysed by treatment with sodium hydroxide (**Scheme 4.31a**). Hydrolysis was carried out with one equivalent of hydroxide and due to the acid sensitive nature of the DMTr group, the resulting carboxylate could not be protonated and therefore could not be separated from the sodium salts. The crude product was used in the next step without purification or characterisation. However, the amide coupling was unsuccessful (**Scheme 4.31a**). While the lack of product is possibly due to the use of TBDMS-protected hydroxylamine, which was also unreactive in an amide coupling with a 5'-malonyl modified nucleoside (Section 4.1.1), the difficulty in purification of the carboxylate intermediate makes this reaction sequence challenging to optimise. Therefore, aminolysis of the ester of **124** was attempted as an alternative strategy (**Scheme 4.31b**). Treatment of ester **124** with hydroxylamine under basic conditions afforded target hydroxamic acid **126** in 74% yield.

![Scheme 4.31](image)

**Scheme 4.31**: a) Attempted synthesis of hydroxamic acid dinucleoside **187** from dinucleoside **186**. b) Aminolysis of dinucleoside **124** to make target **126**.

To construct the dinucleoside targets **127-129**, which all contain an oxyamide linkage, amide coupling was again used to connect the two nucleosides (**Scheme 4.32**). The oxyamine **188** was synthesised from thymidine, while the
malonate-containing nucleoside 139 was previously synthesised as an intermediate for other nucleosides.

Scheme 4.32: Synthetic strategy for dinucleoside targets 127-129 from oxyamine 188 and 5'-malonyl nucleoside 139.

For retention of the stereochemistry at the 3'-position, the previously synthesised alcohol 171 (Section 4.1.2) was used for installation of the oxyamine functionality (Scheme 4.33).

Scheme 4.33: Synthesis of 3'-oxyamine 188 from alcohol 171.

A Mitsunobu reaction between this alcohol 171 and N-hydroxyphthalimide afforded the protected oxyamine 189 in 46% yield (Scheme 4.33). Deprotection of the oxyamine group with hydrazine afforded nucleoside 188 in 80% yield.

The oxyamine 188 was then used in an amide coupling with 5'-malonyl nucleoside 139 (Scheme 4.34). Under similar conditions to the ones used for the construction of dinucleoside 185 (Scheme 4.29), no dinucleoside product 190 was observed. Instead, the α,β-unsaturated ester 191 was obtained in 54% yield and 68% of the oxyamine nucleoside 188 was recovered. The formation of the Michael acceptor 191 could have arisen from the following reaction sequence: loss of the hemiaminal group resulting from incomplete BOM-deprotection forms formaldehyde. If the malonate group underwent decarboxylation to form enolate 192, this enolate could
have reacted with formaldehyde in an aldol reaction, giving the alkoxide 193. Elimination of the alcohol of 193 via the enolate tautomer 194 would lead to the observed α,β-unsaturated ester 191. No other nucleoside products were isolated from the reaction, implying that the Michael acceptor 191 is not reactive towards the oxyamine 188.

Scheme 4.34: Attempted amide coupling of modified nucleosides 139 and 188, with a side reaction forming Michael acceptor 191.

As the oxyamine-linked dimers 127-129 appeared to not be accessible through this synthetic route, targets 127-129 were not synthesised. The three dinucleosides 124-126 that were obtained successfully (Figure 4.11) were evaluated for binding to SNM1A.

Figure 4.11: Dinucleosides containing malonate-based phosphodiester replacements.
4.2 Biological evaluation as inhibitors of SNM1A

The twelve malonate-based nucleosides shown in Figure 4.12a were evaluated for their ability to inhibit SNM1A. The same assay as described for 5'-modified nucleosides (Chapter 2, p. 54) was carried out. If the modified nucleosides bind to the metal centre, the fluorescently tagged substrate 82 cannot access the active site and its digestion is therefore inhibited (Figure 4.12b). SNM1A was treated with the fluorescently labelled oligonucleotide substrate 82 after preincubation with the modified nucleosides and dinucleosides, and the extent of digestion after a further 60-minute incubation was analysed using gel electrophoresis (Figure 4.12c).

Figure 4.12: Evaluation of modified nucleosides as inhibitors of SNM1A using fluorescent substrate 82. a) Panel of nucleosides, b) Schematic representation of assay, c) Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside inhibitors 102, 103, 105, 107-109, 115-117, 124-126 or thymidine (1) (1 mM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified nucleosides for 5 min at 37 °C prior to the addition of substrate 82. nt = nucleotides.
In addition to a control without SNM1A (Figure 4.12c, lane 1) and a control without any nucleoside (Figure 4.12c, lane 2), thymidine (1) was used as a control in place of a modified nucleoside (Figure 4.12c, lane 3) to ensure any effect was due to the modifications. Encouragingly, nine out of twelve malonate-based nucleosides showed some degree of inhibition at 1 mM concentration when incubated with SNM1A (2.5 nM) (Figure 4.12c). Only ester 107 (Figure 4.12c, lane 7), carboxylate salt 108 (Figure 4.12c, lane 8) and ester 115 (Figure 4.12c, lane 10) had no impact on the activity of SNM1A.

In this initial screen, compound 105 (Figure 4.12c, lane 6) emerges as the most potent inhibitor based on the size of the remaining oligonucleotides. Comparing the different positioning of the malonate group, it is apparent that installing the malonate group through the α-carbon as for compounds 102, 103 and 105 afforded the strongest inhibitors (Figure 4.12c, lanes 4-6). Connecting the malonate group in the 5′-position via an amide linker (compounds 107-109) resulted in the weakest inhibitors (Figure 4.12c, lanes 7-9). Installation of a malonate group in the 3′-position (compounds 115-117) led to similarly weak inhibitors as the amide linked 5′-compounds (Figure 4.12c, lanes 10-12 vs lanes 7-9). Connecting two nucleosides using a malonate-based linker (compounds 124-126, Figure 4.12c, lanes 13-15) produced inhibitors approaching the potency of the 5′-malonate series connected via the α-carbon (Figure 4.12c, lanes 4-6).

The concentration dependence of the nine compounds (102, 103, 105, 109, 116, 117, 124-126) that influenced the activity of SNM1A was explored. To this end, the assay was carried out with varying concentrations of inhibitor, ranging from 1 mM to 1 µM (Figures 4.13-4.16).
Figure 4.13: Concentration dependence of 5'-C-linked malonate nucleosides 102, 103 and 105. Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside 102, 103 or 105 (1000-1 µM) or thymidine (1) (1 mM) for 60 min at 37 °C, analysed by denaturing PAGE. SNM1A was preincubated with the modified nucleoside for 5 min at 37 °C prior to the addition of substrate 82. nt = nucleotides.

While at 1 mM concentration all three 5'-C-malonate compounds inhibit enzymatic activity to a similar extent (Figure 4.13a-c, lane 4), the difference in their potency is apparent at lower concentrations (Figure 4.13). Ester 102 (Figure 4.13a) and carboxylate salt 103 (Figure 4.13b) both show inhibition at 333 µM (Figure 4.13a-b, lane 5) but not at 100 µM or below (Figure 4.13a-b, lanes 6-10). In contrast, hydroxamic acid 105 inhibits SNM1A at concentrations as low as 33 µM (Figure 4.13c, lanes 4-7), but not at lower concentrations (Figure 4.13c, lanes 8-10).
Chapter 4 – Malonate-based mono- and dinucleoside inhibitors

Figure 4.14: Concentration dependence of 5′-amide-linked malonate nucleoside 109. Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside 109 (1000-1 µM) or thymidine (1) (1 mM) for 60 min at 37°C, analysed by denaturing PAGE. SNM1A was preincubated with the modified nucleoside for 5 min at 37 °C prior to the addition of substrate 82. nt = nucleotides.

The amide-linked 5′-malonate series afforded only one active compound, hydroxamic acid 109. While this compound impedes hydrolysis by SNM1A at concentrations of 1 mM and 333 µM (Figure 4.14, lanes 4-5), at lower concentrations no effect on the digestion of oligonucleotide 82 was observed (Figure 4.14, lanes 6-10).
Figure 4.15: Concentration dependence of 3'-malonate nucleosides 116-117. Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside 116 or 117 (1000-1 µM) or thymidine (1) (1 mM) for 60 min at 37°C, analysed by denaturing PAGE. SNM1A was preincubated with the modified nucleoside for 5 min at 37°C prior to the addition of substrate 82. nt = nucleotides.

The amide-linked 3'-malonate derivatives showed similar potency, with both carboxylate salt 116 and hydroxamic acid 117 inhibiting the enzyme at concentrations of 333 µM and above (Figure 4.15a-b, lanes 4-5). However, both compounds had no effect at 100 µM or below (Figure 4.15a-b, lanes 6-10).

The second most potent family of compounds that was evaluated, the dinucleoside series 124-126 (Figure 4.16), yielded similar results to the 3'-modified compounds (Figure 4.15). At the highest concentration however, addition of the dinucleosides 125 and 126 led to larger oligonucleotide products than the corresponding 3'-modified nucleosides 116 and 117.
Figure 4.16: Concentration dependence of malonate-containing dinucleosides 124-126. 
Digestion of fluorescent substrate 82 (0.8 pmol, 80 nM) after incubation with SNM1A (25 fmol, 2.5 nM) and nucleoside 124, 125 or 126 (1000-1 µM) or thymidine (1) (1 mM) for 60 min at 37°C, analysed by denaturing PAGE. SNM1A was preincubated with the modified nucleoside for 5 min at 37°C prior to the addition of substrate 82. nt = nucleotides.

Of the three dinucleosides 124-126, ester 124 is the weakest inhibitor and only affects SNM1A activity at 1 mM (Figure 4.16a, lane 4). Carboxylate salt 125 and hydroxamic acid 126 are more potent inhibitors, as they also inhibit SNM1A at 333 µM as well as 1 mM concentration (Figure 4.16b-c, lanes 4-5). None of the dinucleosides had an apparent impact at concentrations at or below 100 µM (Figure 4.16a-c, lanes 6-10).
The results from the concentration dependence experiments are summarised in Table 4.2. While these results are only qualitative in nature, some conclusions about the binding modes and requirements for metal-binding groups can be drawn.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Compound family</th>
<th>Substituent</th>
<th>Lowest inhibitory concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>102</td>
<td>5'-C Malonate</td>
<td>ONa, OMe</td>
<td>333</td>
</tr>
<tr>
<td>2</td>
<td>103</td>
<td>5'-C Malonate</td>
<td>ONa, ONa</td>
<td>333</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>5'-C Malonate</td>
<td>ONa, NHOH</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>107</td>
<td>5'-N-Malonamide</td>
<td>OMe</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>108</td>
<td>5'-N-Malonamide</td>
<td>ONa</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>109</td>
<td>5'-N-Malonamide</td>
<td>NHOH</td>
<td>333</td>
</tr>
<tr>
<td>7</td>
<td>115</td>
<td>3'-N-Malonamide</td>
<td>OMe</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>116</td>
<td>3'-N-Malonamide</td>
<td>ONa</td>
<td>333</td>
</tr>
<tr>
<td>9</td>
<td>117</td>
<td>3'-N-Malonamide</td>
<td>NHOH</td>
<td>333</td>
</tr>
<tr>
<td>10</td>
<td>124</td>
<td>Dinucleoside</td>
<td>OMe</td>
<td>1000</td>
</tr>
<tr>
<td>11</td>
<td>125</td>
<td>Dinucleoside</td>
<td>ONa</td>
<td>333</td>
</tr>
<tr>
<td>12</td>
<td>126</td>
<td>Dinucleoside</td>
<td>NHOH</td>
<td>333</td>
</tr>
</tbody>
</table>

Table 4.2: Lowest concentrations at which inhibition was observed in concentration dependence studies.

From the results presented in Table 4.2, it is apparent that in all series the ester analogue is the weakest inhibitor (Table 4.2, entries 4, 7, 10). This result can be explained by the lack of acidic proton compared to hydroxamic acids or the lack of negatively charged atoms compared to carboxylate salts, making chelation unfavourable. It implies that the malonate group is not coordinating exclusively through the carbonyl oxygen atoms but requires substituents with the potential to hold a negative charge to achieve chelation.

The finding that the 5'-C-linked malonate series (Table 4.2, entries 1-3) afforded the most potent inhibitors is not surprising given the additional carboxylate group that is
present compared to all other derivatives. It is therefore likely that this carboxylate participates in coordination to the metal centre.

In contrast to the C-linked malonates, of the N-linked 5'-modified nucleosides 107-109 (Table 4.2, entries 4-6) only hydroxamic acid 109 (Table 4.2, entry 6) inhibits SNM1A. Its lower activity compared to the corresponding C-linked hydroxamic acid 105 is possibly due to the increased distance of the hydroxamic acid group from the deoxyribose core in 109, and/or the absence of the additional carboxylate group. The 3'-modified nucleosides 115-117 (Table 4.2, entries 7-9) also contain a malonamide group, and both carboxylate 116 (Table 4.2, entry 8) and hydroxamic acid 117 (Table 4.2, entry 9) inhibit at 333 µM concentration. This implies that the relative positioning of the group compared to the deoxyribose core is a causative factor in the lack of potency of compound 108. A similar orientation of the metal-binding group compared to the native phosphodiester ensures the interactions between the enzyme and the substrate are replicated to achieve strong binding.

The dinucleosides 124-126 offer further insights into the importance of the connectivity between the malonate group and the deoxyribose core. Although the lowest concentrations at which inhibition is observed is the same for the dinucleosides and the 3'-modified nucleosides for the carboxylate salt (Table 4.2, entries 8 and 11) and the hydroxamic acid derivatives (Table 4.2, entries 9 and 12), the potency of the dinucleosides appears to be slightly higher based on the size of oligonucleotide products. This is confirmed by the dinucleoside ester derivative 124, which shows some inhibition at 1 mM concentration (Table 4.2, entry 10), while the corresponding 3'-derivative 115 is inactive (Table 4.2, entry 7). These results clearly point towards the importance of the second nucleoside as a recognition element. However, the potency of the strongest dinucleoside inhibitors was significantly lower than that of the strongest inhibitor 105 (Table 4.2, entry 3) which contained an additional carboxylate group. The benefits of a second nucleoside therefore do not counteract the negative effect of removing a carboxylate group.

Further evidence for the importance of the additional carboxylate group comes from comparing these results with those obtained in Chapter 2. While the strongest inhibitor of the malonate series, 105, appears to be slightly less potent than hydroxamic acid 163, which emerged as the best inhibitor in Chapter 2 (p. 53), it is
more potent than hydroxamic acid 67 (Figure 4.17). However, without IC\textsubscript{50} measurements, this comparison is only approximate. Malonate hydroxamic acid 105 is structurally very similar to hydroxamic acid 67 (Figure 4.17) but contains an additional carboxylate group, resulting in improved potency. This points towards the presence of a second metal ion, or alternatively hydrogen bonding interactions between the additional carboxylate group and the enzyme active site.

![Figure 4.17: Structures of compounds tested in Chapter 2 (63 and 67) and the strongest inhibitor described in this chapter (105) in order of potency.](image-url)

Assuming the presence of two metal ions, the results can be combined to propose a binding mode. As hydroxamic acid analogues were found to inhibit more strongly than their carboxylic acid or methyl ester analogues, it can be assumed that the hydroxamic acid group is involved in metal chelation. When comparing all malonate nucleosides with hydroxamic acid groups (105, 109, 117, 126), the strongest one (compound 105) also contains a carboxylate group, as discussed. This indicates that this group is also involved in binding. In recent crystal structures of SNM1A, a malonate anion was found chelating the single metal ion (Figure 4.18a).\textsuperscript{54} A similar binding mode can be envisaged for inhibitor 105, with the additional binding from the hydroxamic acid group (Figure 4.18b).

![Figure 4.18: Binding modes of malonate groups to SNM1A. a) Observed binding mode of malonate ion in crystal structure.\textsuperscript{54} b) Proposed binding mode of most potent inhibitor 105.](image-url)
The weaker inhibition of the carboxylate 103 compared to the hydroxamic acid 105 can be attributed to the strong chelating ability of the hydroxamic acid group resulting from the formation of a five-membered ring. For carboxylates, the ring resulting from chelation is four-membered, and as such this binding mode is less favourable.

While the binding mode shown in Figure 4.18b fits with the observed results, it is important to note that it assumes the presence of two metal ions, which is not confirmed. Similar trends in binding strength could also be a result of hydrogen bonding with the peptide backbone or a different metal cofactor, using an entirely different binding mode.

A further question is whether compound 105 binds in deprotonated form as the enolate ion. This is of importance as the compound was evaluated as a mixture of diastereomers. One diastereomer could bind preferentially or, if the compound is deprotonated once in the active site, both diastereomers could result in the same bound species. The introduction of a substituent at the α-carbon would give insights into whether deprotonation is required for binding, and if one diastereomer binds preferentially.

### 4.3 Malonate-modified nucleosides and dinucleosides bind to SNM1A

A family of sixteen nucleosides and twelve dinucleosides containing malonate-derived metal binding groups was designed. Synthesis of some of these targets proved challenging, and some compounds such as 1,3-dihydroxamic acid 106, 3’-C-linked nucleosides 110-114 and dinucleosides 118-123 and 127-129 were not accessible via the attempted strategies. For the twelve compounds that were synthesised successfully, the installation of the hydroxamic acid group was achieved by reaction of the corresponding ester with hydroxylamine. This strategy was adopted following unsuccessful attempts at amide coupling and deprotection with TBDMS- and benzyl-protected hydroxylamine.

Biological evaluation of the twelve nucleosides and dinucleosides revealed that nine of these compounds had some inhibitory effect on the activity of SNM1A. Concentration-dependence experiments revealed that a 5’-C-linked nucleoside containing both a hydroxamic acid group and a carboxylate group inhibits SNM1A at concentrations as low as 33 µM, while all other inhibitors showed no activity at
concentrations below 333 µM. The malonate-linked dinucleosides exhibited increased inhibition compared to the 3’-modified nucleosides containing very similar metal-binding groups, although they are weaker than their 5’-modified analogues which contain an additional carboxylate group. This finding suggests increased affinity resulting from a single additional nucleoside, and it is expected that incorporation into an oligonucleotide scaffold will increase the binding affinity of any modified nucleoside significantly, as observed in Chapter 3. In addition to incorporation into oligonucleotides, introduction of a phosphate group at the 5’-position is a potential route to increase binding even further, if a dinucleoside with a bridging malonate group is used.
Conclusions and future directions

This work details investigations into the design of ABPs targeting the nuclease SNM1A. As the active site of SNM1A contains a metal centre, such probes will combine metal-binding groups with an oligonucleotide scaffold. In this work, mononucleosides, dinucleosides and oligonucleotides were synthesised and evaluated for their ability to bind to SNM1A at its active site. The compounds found to inhibit SNM1A are shown in Figure 5.1.

Figure 5.1: Compounds found to inhibit SNM1A. a) Hydroxamic acid-modified nucleosides, b) Squaramide-modified oligonucleotides, c) Malonate-modified mono- and dinucleosides.

In the studies described in Chapter 2, a range of potential metal-binding groups were installed at the 5’-position of thymidine. These groups included hydroxamic acid and its derivatives as well as squaramides. Assays with recombinant SNM1A showed that the hydroxamic acid nucleosides 63 and 67 (Figure 5.1a) were the most potent inhibitors, and that the positioning relative to the deoxyribose core influences binding strength. The most potent inhibitor 63 was determined to have an IC$_{50}$ of 139 µM. A membrane permeability assay showed the limitations of the highly charged hydroxamic acid group for medicinal chemistry applications.

Work discussed in Chapter 3 aimed to investigate the effect of an oligonucleotide scaffold on binding strength. In this work, some of the modified nucleosides tested
in Chapter 2 were incorporated into oligonucleotides at the 5'-end. As part of this, a novel strategy to incorporate squaramide modifications at the 5'-end of oligonucleotides after solid-phase oligonucleotide synthesis was successfully employed. Biological evaluation showed that squaramide modified oligonucleotides 92b and 95b (Figure 5.1b) bind to SNM1A more strongly than non-modified ones, although they cannot compete with the natural substrate when present in sub-stoichiometric amounts. However, this finding is significant as the modified oligonucleotides were tested at 1000-fold lower concentrations than the modified nucleosides evaluated in Chapter 2. While the squaramide-modified nucleosides had no effect at this higher concentration, the corresponding oligonucleotides inhibited SNM1A activity. This illustrates how weak binding between a metal-binding group and the enzyme can be enhanced substantially when this group is part of an oligonucleotide. As such, the oligonucleotide scaffold of ABPs for SNM1A is an important factor in the binding strength of the probe.

In Chapter 4, the malonate group and its derivatives were explored as a metal-binding groups. Based on results detailed in Chapter 2, hydroxamic acid groups were incorporated as part of a malonate scaffold, alongside carboxylate and ester analogues. As the malonate group can be attached to nucleosides in various ways, a comparison between 5'-modified, 3'-modified and dinucleosides was carried out. Nine of twelve compounds (Figure 5.1c) lowered the activity of SNM1A at 1 mM concentration or below, and a 5'-modified nucleoside 105 containing both a hydroxamic acid and carboxylate group emerged as the most potent inhibitor. 5'-Modified analogues 102 and 103 showed lower activity, similar to that of dinucleoside inhibitors 125 and 126. The dinucleosides showed enhanced binding relative to their 3'-modified mononucleoside analogues 116 and 117. This finding shows that in addition to electrostatic interactions between the oligonucleotide backbone and SNM1A, interactions between the nucleosides and the enzyme are also of importance.

In conclusion, the results presented in this work offer insights into suitable metal-binding groups for probes targeting SNM1A. The hydroxamic acid group emerged as the most potent group. Additionally, this work confirms the enhanced binding affinity resulting from incorporating modified nucleosides into oligonucleotide scaffolds. Installation of hydroxamic acid groups in different positions has revealed that positioning it close to the deoxyribose core at the 5'-position of a nucleoside or
as part of a dinucleoside is the most promising approach for the synthesis of probes \((\text{Figure 5.2})\). While installation at the 5’-position afforded the most potent inhibitors 63 and 105 \((\text{Figure 5.1a,c})\), using it as part of a linker between two nucleosides allows for 5’-phosphorylation \((\text{Figure 5.2})\), a key factor in substrate recognition by SNM1A.²⁸,³⁷

\[ \text{Figure 5.2: Structures of three potential ABPs for SNM1A based on results presented in this work.} \]

Taken together, the results presented in this work suggest that the three structures shown in \text{Figure 5.2} will be potent probes for SNM1A. However, this hypothesis remains to be validated experimentally by synthesising and evaluating the probes shown in \text{Figure 5.2}. A crucial aspect in the successful synthesis of these structures will be the identification of protecting groups for hydroxamic acids that are compatible with solid-phase oligonucleotide synthesis. Further investigations into analogous structures using different nucleobases are an additional potential future direction of this work.
6  Experimental procedures

6.1  Synthesis of modified nucleosides and dinucleosides

$^1$H, $^{13}$C and $^{31}$P NMR spectra were recorded on Bruker 400 MHz or 600 MHz system spectrometers in DMSO-d$_6$, CDCl$_3$, CD$_3$OD, acetone-d$_6$ or D$_2$O relative to residual DMSO (δ$_H$ = 2.50 ppm, δ$_C$ = 39.52 ppm), CDCl$_3$, (δ$_H$ = 7.26 ppm, δ$_C$ = 77.16 ppm), CD$_3$OD (δ$_H$ = 3.31 ppm, δ$_C$ = 49.00 ppm), acetone-d$_6$ (δ$_H$ = 2.05 ppm, δ$_C$ = 29.84 ppm) or D$_2$O (δ$_H$ = 4.79 ppm). Chemical shifts are reported in ppm and coupling constants are reported in Hertz (Hz) accurate to 0.2 Hz. $^{13}$C NMR spectra are proton-decoupled. NMR spectra were assigned using HSQC, HMBC, DEPT and EXSY experiments. Modified nucleosides are numbered according to standard nucleoside convention. MS measurements were carried out on a Bruker ESI or APCI HRMS. Melting points were measured using a Griffin melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained on a Perkin Elmer spectrophotometer. 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 F$_{254}$ plates purchased from Merck and visualised by UV irradiation (254 nm), potassium permanganate stain (3 g KMnO$_4$, 20 g K$_2$CO$_3$, 300 mL H$_2$O), 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. H$_2$SO$_4$) and iodine. Ion exchange resin refers to Diaion WT01S(H) resin, which was purchased from Alfa Aesar and activated by consecutive washes with acetone, MeOH, 1 M NaOH (Na form only), H$_2$O and MeOH. Preparative reversed phase TLC was carried out on TLC silica gel 60 RP-18 F$_{254}$S plates purchased from Merck. THF and CH$_2$Cl$_2$ were dried using a PureSolv MD solvent purification system. Petroleum ether refers to the fraction of petroleum ether that boils at 40-60 °C. Chemicals were purchased from Acros Organics, Aldrich, Alfa Aesar, Carboxynth, Fisher Scientific, Fluorochem, Sigma Aldrich and Merck and were used as purchased without further purification.
6.1.1 Hydroxamic acid and squaramide-based nucleoside inhibitors

The procedures in this section refer to compounds described in Chapter 2.

6.1.1.1 Hydroxamic acid derivatives

**Synthesis of nucleosides 69 and 70 containing an N-oxyamide group**

![Chemical structures](image)

Nucleoside 73 was prepared according to a published procedure.\(^{251}\) Thymidine (1) (5.09 g, 21.0 mmol) was dissolved in dry DMF (55 mL) under argon and cooled to 0 °C. PPh\(_3\) (7.04 g, 26.8 mmol) and N-hydroxyphthalimide (4.49 g, 27.5 mmol) were added followed by the dropwise addition of DIAD (6.1 mL, 31.1 mmol) in DMF (10 mL). The reaction mixture was warmed to r.t. and stirred for 18 h. After this time, TLC analysis (CH\(_2\)Cl\(_2\)-MeOH, 9:1) showed the complete consumption of the starting material (R\(_t\) = 0.3) and the formation of the product (R\(_t\) = 0.7). The solution was concentrated, and the resulting oil dissolved in CH\(_2\)Cl\(_2\) (50 mL). The solution was poured into a mixture of H\(_2\)O-ice (250 mL) and stirred for 30 min. The resulting precipitate was collected by vacuum filtration and washed with cold EtOH (3 x 50 mL) to afford the desired product 73 as a white solid (6.11 g, 75%); mp 222 °C dec. (lit.\(^{251}\) 222 °C).
Experimental procedures

v\text{max}/\text{cm}^{-1} (neat) 3449 (OH, NH), 3234 (OH, NH), 3082 (CH), 2929 (CH), 1786 (C=O), 1728 (C=O), 1680 (C=O), 1648 (C=C), 1466 (CH), 1373 (OH), 1281, 1188, 1138, 1098 (C-O), 1058, 999, 880, 809, 784, 698.

$^1$H NMR (400 MHz, DMSO-d$_6$): δ = 1.80 (s, 3 H, CH$_3$), 2.10 - 2.14 (m, 2 H, H2'a, H2'b), 4.07-4.10 (m, 1 H, H4''), 4.36 (d, $J_{4',5'}$ = 4.4 Hz, 2 H, H5'a, H5'b), 4.39 (m, 1 H, H3''), 5.46 (d, $J_{3',OH}$ = 3.8 Hz, 1 H, OH), 6.19-6.23 (m, 1 H, H1''), 7.58 (s, 1 H, H6), 7.84 - 7.87 (m, 4 H, Phth), 11.29 (s, 1 H, NH) ppm.

$^{13}$C NMR (100 MHz, DMSO-d$_6$) δ = 12.1 (CH$_3$), 38.8 (C2''), 70.7 (C3''), 77.7 (C5''), 84.1 (C4''), 84.3 (C1''), 109.9 (C5), 123.3 (Phth), 128.6 (qC, Phth), 134.8 (Phth), 135.8 (C6), 150.4 (C2), 163.0 (CO Phth), 163.7 (C4) ppm.

HRMS (ESI$^+$): m/z calc. 410.0959 [M + Na]$^+$, found: 410.0958

The spectroscopic data are in agreement with those reported in the literature.$^{251}$

3'-O-(\textit{tert}-Butyldimethylsilyl)-5'-O-phthalimidothymidine (74)

Nucleoside 74 was prepared according to a published procedure.$^{251}$

5'-O-Phthalimidothymidine 73 (2.96 g, 7.7 mmol) was dissolved in dry DMF (15 mL) under argon and imidazole (1.37 g, 20.1 mmol) was added. A solution of TBDMS-Cl (1.52 g, 10.1 mmol) in dry DMF (15 mL) was added dropwise and the reaction mixture was stirred at r.t. for 18 h. After this time, TLC analysis (EtOAc) showed the consumption of the starting material ($R_l = 0.3$) and the formation of the product ($R_l = 0.8$). The solution was diluted with brine (150 mL) and extracted with EtOAc (200 mL). The organic layer was washed with brine (2 x 50 mL), dried over Na$_2$SO$_4$, filtered and concentrated. Purification by flash column chromatography (petroleum ether-EtOAc, 1:1) afforded the desired product 74 as a white solid (3.31 g, 86%); mp 185 °C (lit.$^{251}$ 201 °C).

v\text{max}/\text{cm}^{-1} (neat) 2930 (CH), 2856 (CH), 1790 (C=O), 1729 (C=O), 1701 (C=O), 1665 (C=O), 1472 (CH), 1359, 1252, 1187, 1133, 1108, 1080 (C-O), 1020, 875, 832, 776, 699.
\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 0.12\) (s, 6 H, 2 x CH\(_3\)TBDMS), 0.88 (s, 9 H, t-Bu\(^{\text{TBDMS}}\)), 1.80 (d, \(4J = 0.9\) Hz, 3 H, CH\(_3\)\(^T\)), 2.09 (ddd, \(J_{2a,3'} = 2.6\) Hz, \(J_{1'2a} = 6.1\) Hz, \(J_{2a,2b} = 13.6\) Hz, 1 H, H2'a), 2.25 (ddd, \(J_{2b,3'} = 6.0\) Hz, \(J_{1'2b} = 8.0\) Hz, \(J_{2a,2b} = 13.6\) Hz, 1 H, H2'b), 4.07-4.10 (m, 1 H, H4'), 4.35-4.37 (m, 2 H, H5'a, H5'b), 4.60-4.63 (m, 1 H, H3'), 6.19 (dd, \(J_{1'2a} = 6.1\) Hz, \(J_{1'2b} = 8.0\) Hz, 1 H, H1'), 7.56 (app. d, \(4J = 0.9\) Hz, 1 H, H6), 7.84-7.89 (m, 4 H, Phth), 11.30 (s, 1 H, NH) ppm.

\(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta = -4.88\) (CH\(_3\)TBDMS), -4.94 (CH\(_3\)TBDMS), 12.1 (CH\(_3\)\(^T\)), 17.7 (qC, t-Bu\(^{\text{TBDMS}}\)), 25.7 (t-Bu\(^{\text{TBDMS}}\)), 39.0 (C2'), 72.3 (C3'), 77.1 (C5'), 84.1 (C4'), 84.3 (C1'), 109.8 (C5), 123.3 (Phth), 128.5 (qC, Phth), 134.8 (Phth), 135.8 (C6), 150.4 (C2), 162.9 (CO Phth), 163.6 (C4) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 524.1823 [M + Na]\(^+\), found: 524.1825

The spectroscopic data are in agreement with those reported in the literature.\(^{251}\)

**5'-O-Amino-3'-O-(tert-butyldimethylsilyl)thymidine (75)**

Nucleoside 75 was prepared according to a published procedure.\(^{251}\)

Protected 5'-O-phthalimidothymidine 74 (2.00 g, 4.0 mmol) was suspended in MeOH (16 mL) and hydrazine hydrate solution (750 \(\mu\)L, 80\%, 12.3 mmol) was added. The reaction mixture became clear, followed by the formation of a precipitate. After 3.5 h stirring at r.t., TLC analysis (petroleum ether-EtOAc, 1:1) showed the complete consumption of the starting material (\(R_f = 0.4\)) and the formation of the product (\(R_f = 0.1\)). The suspension was diluted with Et\(_2\)O (80 mL) and washed with sat. aq. NaHCO\(_3\) (80 mL). The aq. layer was extracted with Et\(_2\)O (3 x 150 mL), dried over Na\(_2\)SO\(_4\) (80 mL). The crude product was carried forward without further purification; mp 120 °C (lit.\(^{251}\) 98 °C).

\(\nu_{\text{max}}/\text{cm}^{-1}\) (neat) 2955 (CH), 2928 (CH), 2859 (CH), 1711 (C=O), 1674 (C=O), 1472 (CH), 1278, 1253, 1080 (C-O), 1031, 1002, 955, 831, 774.

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 0.08\) (s, 6 H, 2 x CH\(_3\)TBDMS), 0.89 (s, 9 H, t-Bu\(^{\text{TBDMS}}\)), 1.93 (d, \(4J = 1.0\) Hz, 3 H, CH\(_3\)\(^T\)), 2.08 (app. dt, \(J = 6.5\) Hz, \(J = 13.4\) Hz, 1 H, H2'a), 2.26 (ddd, \(J_{2b,3'} = 3.7\) Hz, \(J_{1'2b} = 6.5\) Hz, \(J_{2a,2b} = 13.4\) Hz, 1 H, H2'b), 3.85 (dd, 

---

\(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta = -4.88\) (CH\(_3\)TBDMS), -4.94 (CH\(_3\)TBDMS), 12.1 (CH\(_3\)\(^T\)), 17.7 (qC, t-Bu\(^{\text{TBDMS}}\)), 25.7 (t-Bu\(^{\text{TBDMS}}\)), 39.0 (C2'), 72.3 (C3'), 77.1 (C5'), 84.1 (C4'), 84.3 (C1'), 109.8 (C5), 123.3 (Phth), 128.5 (qC, Phth), 134.8 (Phth), 135.8 (C6), 150.4 (C2), 162.9 (CO Phth), 163.6 (C4) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 524.1823 [M + Na]\(^+\), found: 524.1825
Experimental procedures

$J_{4',5'a} = 4.4$ Hz, $J_{5'a,5'b} = 11.0$ Hz, 1 H, H5'a), 3.96 (dd, $J_{4',5'b} = 3.1$ Hz, $J_{5'a,5'b} = 11.0$ Hz, 1 H, H5'b), 4.00-4.03 (m, 1 H, H4'), 4.37 (app. dt, $J = 3.7$ Hz, $J = 6.5$ Hz, 1 H, H3'), 6.24 (app. t, $J = 6.5$ Hz, 1 H, H1'), 7.38 (app. d, 1 H, J = 1.0 Hz, 1 H, H6), 8.53 (bs, 1 H, NH) ppm.

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = -4.7$ (CH$_3$$^{TBDMS}$), -4.6 (CH$_3$$^{TBDMT}$), 12.9 (CH$_3$), 18.1 (qC, t-Bu$^{TBDMT}$), 25.8 (t-Bu$^{TBDMT}$), 41.1 (C2'), 72.0 (C3'), 75.5 (C5'), 85.5 (C1'), 85.6 (C4'), 111.0 (C5), 135.9 (C6), 150.3 (C2), 163.7 (C4) ppm.

HRMS (ESI$^+$): m/z calc. 372.1949 [M + H]$^+$, found: 372.1940

The spectroscopic data are in agreement with those reported in the literature.$^{251}$

$N$-Acetyl-5'O-amino-3'O-(tert-butyldimethylsilyl)thymidine (76)

AcOH (57 µL, 1.00 mmol) was dissolved in dry DMF (10 mL) under argon and cooled to 0 °C. HOAt (166 mg, 1.22 mmol) and EDC (213 mg, 1.11 mmol) were added and the reaction mixture was stirred for 10 min. Amine 75 (350 mg, 0.94 mmol) was added and the reaction mixture was warmed to r.t. and stirred for 24 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 19:1) showed the consumption of the starting material (R$_f$ = 0.4) and the formation of the product (R$_f$ = 0.3). The reaction mixture was diluted with EtOAc (100 mL) and washed with sat. aq. NaHCO$_3$ (100 mL). The aq. layer was extracted with EtOAc (2 x 100 mL) and the combined organic extracts were washed with brine (100 mL), dried over Na$_2$SO$_4$, filtered and concentrated. Flash column chromatography (CH$_2$Cl$_2$-MeOH, 19:1) afforded the desired product 76 as a white solid (277 mg, 71%); mp 81-84 °C.

$\nu_{\text{max}}$/cm$^{-1}$ (CDCl$_3$) 3222 (NH), 2930 (CH), 2858 (CH), 1662 (C=O), 1471 (CH), 1368, 1274, 1198, 1079 (C-O), 1032, 910, 831, 777, 728.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta = 0.09$ (s, 6 H, 2 x CH$_3$$^{TBDMS}$), 0.89 (s, 9 H, t-Bu$^{TBDMT}$), 1.92 (s, 3 H, CH$_3$$^{Ac}$), 1.93 (s, 3 H, CH$_3$), 2.20-2.30 (m, 2 H, H2'a, H2'b), 4.01 (dd, $J_{3',4'} = 2.7$ Hz, $J_{4',5'a} = 3.5$ Hz, 1 H, H4'), 4.07 (dd, $J_{4',5'a} = 3.5$ Hz, $J_{5'a,5'b} = 10.9$ Hz, 1 H, H5'a), 4.16 (d, $J_{5'a,5'b} = 10.9$ Hz, 1 H, H5'b), 4.59 (m, 1 H, H3'), 6.15 (app. t, $J = 6.8$ Hz, 1 H, H1'), 7.45 (s, 1 H, H6), 8.58-8.72 (m, 2 H, NHT, CO-NHO) ppm.
Experimental procedures

\(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = -4.8\) (CH\(_3\)TBDMS), -4.6 (CH\(_3\)TBDMS), 12.5 (CH\(_3\)T), 18.0 (qC, t-BuTBDMS), 19.9 (CH\(_3\)Ac), 25.8 (t-BuTBDMS), 40.5 (C2'), 71.9 (C3'), 75.3 (C5'), 85.6 (C4'), 86.5 (C1'), 111.3 (C5), 137.1 (C6), 150.7 (C2), 164.5 (C4), 168.0 (CO-NHO) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 436.1874 [M + Na], found: 436.1864

\(5'-O\)-Amino-\(3'-O\)-(tert-butyldimethylsilyl)-\(N\)-formylthymidine (77)

Formic acid (39 µL, 0.94 mmol) was dissolved in dry DMF (10 mL) under argon and cooled to 0 °C. HOAt (167 mg, 1.22 mmol) and EDC (217 mg, 1.22 mmol) were added and the reaction mixture was stirred for 20 min. Amine 75 (350 mg, 0.94 mmol) was added and the reaction mixture was warmed to r.t. and stirred for 24 h. After this time, TLC analysis (CH\(_2\)Cl\(_2\)-MeOH, 19:1) showed the consumption of the starting material (R\(_f\) = 0.4) and the formation of the product (R\(_f\) = 0.1). The reaction mixture was diluted with EtOAc (50 mL), H\(_2\)O (20 mL) and sat. aq. NaHCO\(_3\) (20 mL). The layers were separated and the aq. layer was extracted with EtOAc (3 x 20 mL) and the combined organic extracts were dried over MgSO\(_4\), filtered and concentrated. Purification by flash column chromatography (CH\(_2\)Cl\(_2\)-MeOH, 19:1) afforded the desired product 77 as a white solid (100 mg, 27%); mp 164 °C.

\(\nu_{\text{max}}/\text{cm}^{-1}\) (CDCl\(_3\)) 3198 (OH, NH), 2955 (CH), 2928 (CH), 2856 (CH), 1690 (C=O), 1472 (CH), 1370 (CH), 1276, 1254, 1199, 1135, 1086 (C-O), 1032, 952, 836, 780.

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 0.09\) (s, 6 H, 2 x CH\(_3\)TBDMS), 0.89 (s, 9 H, t-BuTBDMS), 1.94 (s, 3 H, CH\(_3\)T), 2.23-2.33 (m, 2 H, H2'a, H2'b), 4.01-4.02 (m, 1 H, H4'), 4.13 (dd, \(J_{4',5'a} = 3.3\) Hz, \(J_{5'a,5'b} = 10.6\) Hz, 1 H, H5'a), 4.21 (dd, \(J_{4',5'b} = 2.1\) Hz, \(J_{5'a,5'b} = 10.6\) Hz, 1 H, H5'b), 4.59 (m, 1 H, H3'), 6.13 (app. t, \(J = 7.1\) Hz, 1 H, H1'), 7.40 (s, 1 H, H6), 7.99 (s, 1 H, HCO), 8.32 (s, 1 H, NH), 8.76 (s, 1 H, NH) ppm.

\(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = -4.7\) (CH\(_3\)TBDMS), -4.5 (CH\(_3\)TBDMS), 12.6 (CH\(_3\)T), 18.1 (qC, t-BuTBDMS), 25.8 (t-BuTBDMS), 40.5 (C2'), 71.6 (C3'), 75.8 (C5'), 85.6 (C4'), 86.9 (C1'), 111.6 (C5), 137.0 (C6), 150.4 (C2), 157.5 (HCO-NH), 163.6 (C4) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 422.1718 [M + Na], found: 422.1719
**Chapter 6 – Experimental procedures**

*N*-Acetyl-5'-*O*-aminothymidine (69)

Protected nucleoside 76 (200 mg, 48 µmol) was dissolved in THF (5.0 mL). TBAF (275 mg, 87 µmol) was added and the solution was stirred at r.t. for 17 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed the complete consumption of the starting material ($R_f = 0.4$) and the formation of the product ($R_f = 0.2$). The suspension was concentrated and purification by flash column chromatography (CH$_2$Cl$_2$-MeOH, 9:1) afforded the desired product 69 as a white solid (104 mg, 72%); mp 194-195 °C.

$\nu$$_{\text{max}}$/cm$^{-1}$(neat) 3263 (OH, NH), 3191 (OH, NH), 3022 (CH), 1647 (C=O), 1452 (CH), 1373 (C-O), 1270, 1106, 1049 (C-O), 968, 781.

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.73$ (s, 3 H, CH$_3$Ac), 1.80 (s, 3 H, CH$_3$), 2.06-2.08 (m, 2 H, H2’a, H2’b), 3.89 (dd, $J_{4',5'a} = 5.4$ Hz, $J_{5'a,5'b} = 10.0$ Hz, 1 H, H5’a), 3.92-3.94 (m, 1 H, H4’), 3.98 (dd, $J_{4',5'b} = 2.9$ Hz, $J_{5'a,5'b} = 10.0$ Hz, 1 H, H5’b), 4.26-4.28 (m, 1 H, H3’), 5.37 (bs, 1 H, OH), 6.19 (app. t, $J = 7.0$ Hz, 1 H, H1’), 7.65 (s, 1 H, H6), 11.23 (m, 2 H, NH$_2$, CO-NHO) ppm.

$^{13}$C NMR (151 MHz, DMSO-d$_6$): $\delta = 12.1$ (CH$_3$T), 19.6 (CH$_3$Ac), 39.1 (C2’), 70.7 (C3’), 75.7 (C5’), 84.0 (C4’), 84.1 (C1’), 109.8 (C5), 136.1 (C6), 150.5 (C2), 163.8 (C4), 166.6 (CO-NHO) ppm.

HRMS (ESI$^+$): $m/z$ calc. 322.1010 [M + Na]$^+$, found: 322.1011

5'-*O*-Amino-*N*-formylthymidine (70)

Protected nucleoside 77 (90 mg, 23 µmol) was dissolved in THF (2.5 mL). TBAF (128 mg, 41 µmol) was added and the solution was stirred at r.t. for 17 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed the complete consumption of the starting material ($R_f = 0.4$) and the formation of the product ($R_f = 0.2$). The
suspension was concentrated and purification by flash column chromatography (CH$_2$Cl$_2$-MeOH, 9:1) afforded the desired product 70 as a white solid (59 mg, 92%); mp 171-172 °C.

Compound 70 exhibits rotamers in NMR spectroscopy.

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3389 (OH, NH), 3181 (OH, NH), 3054 (CH), 2922 (CH), 2875 (CH), 1718 (C=O), 1655 (C=O), 1476 (CH), 1365, 1272, 1093 (C-O), 1078 (C-O), 874, 830.

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.79$ (d, $^4J = 0.9$ Hz, 3 H, CH$_3$), 2.06-2.09 (m, 2 H, H2'a, H2'b), 3.92-3.96 (m, 2 H, H4', H5'a), 4.00-4.03 (m, 1 H, H5'b), 4.26-4.28 (m, 1 H, H3'), 5.40 (bs, 1 H, OH), 6.19 (app. t, $^1J = 7.0$ Hz, 1 H, H1'), 7.45 (bs, 0.15 H, H6), 7.62 (app. d, $^4J = 0.9$ Hz, 0.85 H, H6), 7.88 (s, 0.8 H, HCO), 8.42 (s, 0.2 H, CHO), 11.28 (m, 2 H, NH$_\text{T}$, CO-NHO) ppm.

$^{13}$C NMR (151 MHz, DMSO-d$_6$): $\delta = 12.1$ (CH$_3$), 39.0 (C2'), 70.6 (C3'), 76.1 (C5'), 83.9 (C4'), 84.1 (C1'), 109.9 (C5), 136.0 (C6), 150.5 (C2), 157.4 (HCO), 163.8 (C4), 165.0 (HCO) ppm.

HRMS (ESI$^+$): $m/z$ calc. 308.0853 [M + Na]$^+$, found: 308.0856

### 6.1.1.2 Squaramide derivatives

**Synthesis of nucleosides 71 and 72 containing a squaramide group**
5'-Deoxy-5'-iodothymidine (78)

Nucleoside 78 was prepared according to a published procedure.\textsuperscript{253} Thymidine (1) (20.0 g, 82.6 mmol) was suspended in dry THF (300 mL) under argon. PPh\textsubscript{3} (26.0 g, 99.1 mmol) and imidazole (7.0 g, 102.8 mmol) were added and the reaction mixture was cooled to 0 °C. A solution of I\textsubscript{2} (23.0 g, 90.6 mmol) in dry THF (100 mL) was added dropwise and the reaction mixture was slowly warmed to r.t. and stirred for 18 h. After this time, TLC analysis (CH\textsubscript{2}Cl\textsubscript{2}-MeOH, 9:1) showed the complete consumption of the starting material (R\textsubscript{f} = 0.2) and the formation of the product (R\textsubscript{f} = 0.4). The reaction was quenched by the addition of H\textsubscript{2}O (100 mL). THF was removed \textit{in vacuo} and EtOAc (300 mL) was added. The desired product 78 was collected as a white precipitate by vacuum filtration. The layers of the filtrate were separated and the aq. layer was extracted with EtOAc (4 x 100 mL). The combined organic extracts were concentrated to approximately 100 mL to afford further product 78 as a white precipitate which was collected by vacuum filtration. The combined precipitates were recrystallized from EtOH to give the desired product 78 as a white crystalline solid (23.0 g, 81%); mp 166-168 °C dec. (EtOH) (lit.\textsuperscript{304} 170-173 °C).

\[ \nu_{\text{max}} / \text{cm}^{-1} \text{ (neat)} = 3462 (\text{OH, NH}), 3145 (\text{OH, NH}), 3018 (\text{CH}), 2816 (\text{CH}), 1698 (\text{C=O}), 1665 (\text{C=O}), 1479 (\text{CH}), 1412, 1258, 1073 (\text{C-O}), 1008, 957, 885, 820, 752. \]

\[ ^1H \text{ NMR (600 MHz, DMSO-d_6): } \delta = 1.80 (s, 3 H, CH_3), 2.08 (ddd, J_{2a,3'} = 3.1 Hz, J_{1',2'a} = 6.4 Hz, J_{2a,2'b} = 13.5 Hz, 1 H, H2'a), 2.29 (ddd, J_{2b,3'} = 6.4 Hz, J_{1',2'b} = 7.8 Hz, J_{2a,2'b} = 13.5 Hz, 1 H, H2'b), 3.39 (dd, J_{4',5'a} = 6.2 Hz, J_{5'a,5'b} = 10.4 Hz, 1 H, H5'a), 3.52 (dd, J_{4',5'b} = 6.2 Hz, J_{5'a,5'b} = 10.4 Hz, 1 H, H5'b), 3.81 (app. td, J = 3.0 Hz, J = 6.2 Hz, 1 H, H4'), 4.19 (app. dt, J = 3.0 Hz, J = 6.4 Hz, 1 H, H3'), 5.49 (bs, 1 H, OH), 6.22 (dd, J_{1',2'a} = 6.4 Hz, J_{1',2'b} = 7.8 Hz, 1 H, H1'), 7.52 (s, 1 H, H6), 11.33 (s, 1 H, NH) ppm. \]

\[ ^{13}C \text{ NMR (151 MHz, DMSO-d_6): } \delta = 7.9 (C5'), 12.2 (CH_3), 37.9 (C2'), 73.0 (C3'), 84.0 (C1'), 85.4 (C4'), 109.9 (C5), 136.2 (C6), 150.5 (C2), 163.7 (C4) ppm. \]

HRMS (APCI\textsuperscript{+}): m/z calc. 352.9998 [M + H]\textsuperscript{+}, found: 352.9992
The spectroscopic data are in agreement with those reported in the literature.253

5'-Azido-5'-deoxythymidine (79)

Nucleoside 79 was prepared according to a published procedure.254 Iodothymidine 78 (3.52 g, 10.0 mmol) was dissolved in dry DMF (50 mL) under argon. After NaN₃ (2.04 g, 31.4 mmol) was added, the reaction mixture was heated to 60 °C and stirred for 17 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 9:1) showed the complete consumption of the starting material (Rᵣ = 0.6) and the formation of the product (Rᵣ = 0.5). The reaction mixture was cooled to r.t., diluted with H₂O (30 mL) and extracted with EtOAc (12 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over MgSO₄, filtered and concentrated. The residue was recrystallised from MeOH to afford the desired product 79 as a white crystalline solid (2.39 g, 90%); mp 156-157 °C (MeOH) (lit.254 157-159 °C).

νmax/cm⁻¹ (neat) 3382 (OH, NH), 3184 (OH, NH), 3049 (CH), 2928 (CH), 2807, 2094 (N=N=N), 1716 (C=O), 1649 (C=O), 1475 (CH), 1270, 1199, 1065 (C-O), 960, 880, 860.

¹H NMR (600 MHz, DMSO-d₆): δ = 1.79 (d, 4 J = 1.2 Hz, 3 H, CH₃), 2.08 (ddd, J₂a,3' = 3.7 Hz, J₁;2a = 6.6 Hz, J₂a,2b = 13.6 Hz, 1 H, H2'a), 2.25 (app. dt, J = 7.0 Hz, J = 13.6 Hz, 1 H, H2'b), 3.55 (d, J₄;5' = 5.1 Hz, 2 H, H5'a, H5'b), 3.84 (app. dt, J = 3.7 Hz, J = 5.1 Hz, 1 H, H4'), 4.18-4.21 (m, 1 H, H3'), 5.40 (d, J₃;OH = 4.4 Hz, 1 H, OH), 6.20 (dd, J₁;2a = 6.6 Hz, J₁;2b = 7.0 Hz, 1 H, H1'), 7.49 (app. d, 4 J = 1.2 Hz, 1 H, H6), 11.32 (s, 1 H, NH) ppm.

¹³C NMR (151 MHz, DMSO-d₆): δ = 12.1 (CH₃), 38.1 (C2'), 51.7 (C5'), 70.7 (C3'), 83.9 (C1'), 84.6 (C4'), 109.8 (C5), 136.1 (C6), 150.5 (C2), 163.7 (C4) ppm.

HRMS (APCI⁺): m/z calc. 266.0894 [M - H]-, found: 266.0904

The spectroscopic data are in agreement with those reported in the literature.305
5'-Amino-5'-deoxythymidine (80)

\[ 
\begin{array}{c}
\text{H}_2\text{N} \text{O} \\
\text{O} \text{N} \\
\text{OH}
\end{array}
\]

Method A:
Azide 79 (1.68 g, 6.3 mmol) was dissolved in THF (45 mL) under argon. PPh\(_3\) (3.50 g, 13.4 mmol) was added, and the reaction mixture was stirred at r.t. for 10 min before H\(_2\)O (15 mL) was added. After stirring at r.t. for 4 h, TLC analysis showed the complete consumption of the starting material (EtOAc-MeOH, 9:1, R\(_f\) = 0.7) and the formation of the product (H\(_2\)O-i-PrOH-EtOAc, 1:2:2, R\(_f\) = 0.2). THF was removed \textit{in vacuo} and the remaining aq. mixture was extracted with CH\(_2\)Cl\(_2\) (5 x 30 mL). The organic layer was washed with H\(_2\)O (50 mL) and the combined aq. layers were concentrated to afford the desired product 80 as a white solid (1.47 g, 97%); mp 159-160 °C dec, (lit.\(^{305}\) 165-167 °C).

Method B (used in Chapter 4):
Azide 79 (572 mg, 2.14 mmol) was dissolved in MeOH (40 mL), added to a dried flask containing Pd/C (10%, 86 mg) and H\(_2\) was bubbled through the suspension while stirring at r.t. for 2 h. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material, (R\(_f\) = 0.5) and the formation of the product (R\(_f\) = 0.0). The reaction mixture was filtered through celite and the filtrate was concentrated to afford the desired product 80 as a white solid (513 mg, 99%).

\[\nu_{\text{max}}/\text{cm}^{-1}\] (neat) 3349 (OH, NH), 2930 (CH), 1658 (C=O), 1605 (C=O), 1440 (CH), 1255, 1192, 1134, 1072 (C-O), 1007, 942, 835, 768.

\(^1\text{H NMR}\) (400 MHz, DMSO-\text{d}_6): \(\delta = 1.78\) (d, \(^4J = 1.0\) Hz, 3 H, CH\(_3\)T), 2.03 (ddd, \(J_{2a,3'} = 3.5\) Hz, \(J_{1',2'a} = 6.3\) Hz, \(J_{2a,2b} = 13.4\) Hz, 1 H, H2'a), 2.13 (ddd, \(J_{2b,3'} = 6.4\) Hz, \(J_{1',2'b} = 7.5\) Hz, \(J_{2a,2b} = 13.4\) Hz, 1 H, H2'b), 2.68 - 2.76 (m, 2 H, H5'a, H5'b), 3.64 (app. dt, \(J = 3.4\) Hz, \(J = 5.2\) Hz, 1 H, H4'), 4.19 (app. dt, \(J = 3.5\) Hz, \(J = 6.4\) Hz, 1 H, H3'), 5.16 (bs, 3 H, NH2, OH), 6.13 (dd, \(J_{1',2'a} = 6.3\) Hz, \(J_{1',2'b} = 7.5\) Hz, 1 H, H1'), 7.64 (app. d, \(^4J = 1.0\) Hz, 1 H, H6) ppm.

\(^{13}\text{C NMR}\) (100 MHz, DMSO-\text{d}_6): \(\delta = 12.6\) (CH\(_3\)T), 39.3 (C2'), 44.1 (C5'), 71.2 (C3'), 83.8 (C1'), 88.4 (C4'), 110.0 (C5), 136.7 (C6), 151.0 (C2), 164.2 (C4) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 242.1135 [M - H]\(^+\), found: 242.1137
The spectroscopic data are in agreement with those reported in the literature.\(^{305}\)

**5'-Amino-5'-N(2-ethoxy-3,4-dioxocyclobuten-1-yl)-5'-deoxythymidine (71)**

Amine 80 (100 mg, 0.41 mmol) was dissolved in DMF (4.0 mL). DIPEA (36 µL, 0.21 mmol) and diethyl squarate (61 µL, 0.41 mmol) were added and the reaction mixture was stirred at r.t. for 24 h. After this time, TLC analysis showed the consumption of the starting material (H\(_2\)O-i-PrOH-EtOAc, 1:2:2, R\(_f\) = 0.2) and the formation of the product (CH\(_2\)Cl\(_2\)-MeOH, 9:1, R\(_f\) = 0.3). The reaction mixture was concentrated and purification by flash column chromatography (EtOAc-MeOH, 9:1) afforded the desired product 71 as a yellow solid (93 mg, 61%); mp 201-203 °C dec.

Compound 71 exhibits rotamers in NMR spectroscopy.

\(v_{\text{max}}/\text{cm}^{-1}\) (neat) 3450 (OH, NH), 3313 (OH, NH), 3191 (OH, NH), 3053 (CH), 2927 (CH), 1804 (C=O), 1685 (C=O), 1580 (C=C), 1490 (CH), 1440 (CH), 1411, 1077, 1038, 965, 855, 805.

\(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta = 1.32\) (t, \(J_{\text{CH2Et,CH3Et}} = 7.0\) Hz, 1.5 H, CH\(_3\)Et), 1.37 (t, \(J_{\text{CH2Et,CH3Et}} = 7.0\) Hz, 1.5 H, CH\(_3\)T), 2.04-2.10 (m, 1 H, H2'a), 2.12-2.22 (m, 1 H, H2'b), 3.44-3.48 (m, 0.5 H, H5'a), 3.55-3.58 (m, 0.5 H, H5'a), 3.70-3.73 (m, 1 H, H5'b), 3.76-3.82 (m, 1 H, H4'), 4.16-4.20 (m, 0.5 H, H3'), 4.22-4.26 (m, 0.5 H, H3'), 4.56-4.68 (m, 2 H, CH\(_2\)Et), 5.37 (d, \(J_{3',\text{OH}} = 4.6\) Hz, 0.5 H, OH), 5.38 (d, \(J_{3',\text{OH}} = 4.6\) Hz, 0.5 H, OH), 6.13-6.16 (m, 1 H, H1'), 7.33 (s, 0.5 H, H6), 7.36 (s, 0.5 H, H6), 8.75 (t, \(J_{\text{NHSq,5'a}} = 5.6\) Hz, \(J_{\text{NHSq,5'b}} = 5.6\) Hz, 0.5 H, NH\(_\text{Sq}\)), 11.31 (bs, 1 H, NH\(_T\)) ppm.

\(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)): \(\delta = 12.0\) (CH\(_3\)T), 12.1 (CH\(_3\)T), 15.6 (CH\(_3\)Et), 38.1 (C2'), 38.4 (C2'), 45.7 (C5'), 68.86 (CH\(_2\)Et), 68.90 (CH\(_2\)Et), 70.5 (C3'), 70.6 (C3'), 83.68 (C1'), 83.75 (C1'), 84.7 (C4'), 84.9 (C4'), 109.7 (C5), 109.8 (C5), 135.8 (C6), 136.0 (C6), 150.4 (C2), 163.7 (C4), 172.5 (CSq1), 173.2 (CSq1), 176.8 (CSq2), 177.3 (CSq2), 182.1 (CSq3), 182.4 (CSq3), 189.1 (CSq4), 189.4 (CSq4), ppm.

HRMS (APCI\(^+\)): m/z calc. 366.1296 [M + H]\(^+\), found: 366.1290
5'-Amino-5'-N-(2-diethylamino-3,4-dioxocyclobuten-1-yl)-5'-deoxythymidine (72)

Compound 72 was also synthesised using a different procedure that is compatible with oligonucleotides (see p. 147).

Squarylmonoamide 71 (100 mg, 0.27 mmol) was dissolved in a solution of Et₂NH (0.5 mL, 4.8 mmol) in MeCN (2.0 mL). The solution was heated to 55 °C and stirred for 1 h. After this time, TLC analysis (EtOAc-MeOH, 9:1) showed complete consumption of the starting material (Rf = 0.3) and formation of the product (Rf = 0.1). The orange solution was concentrated to afford the desired product 72 as a red solid (79 mg, 74%); mp 207-209 °C.

ν\text{max}/\text{cm}^{-1} (neat) 3438 (OH, NH), 3213 (OH, NH), 2978 (CH), 1790 (C=O), 1712 (C=O), 1651 (C=O), 1595 (C=C), 1517 (C=C), 1442 (CH), 1275, 1083 (C-O), 1063 (C-O).

1H NMR (600 MHz, DMSO-d₆): δ = 1.13 (t, J_{CH2Et,CH3Et} = 7.2 Hz, 6 H, 2 x CH₃Et), 1.78 (d, J = 1.0 Hz, 3 H, CH₃T), 2.04-2.11 (m, 2 H, H2'a, H2'b), 3.45-3.60 (m, 4 H, 2 x CH₂Et), 3.73 (app. dt, J = 5.1 Hz, J = 13.6 Hz, 1 H, H5'a), 3.87 (ddd, J₃',J₄' = 3.3 Hz, J₄',J₅' = 5.0 Hz, J₄',J₅'b = 7.1 Hz, 1 H, H4'), 3.93 (app. dt, J = 7.0 Hz, J = 13.6 Hz, 1 H, H5'b), 4.19-4.22 (m, 1 H, H3'), 5.35 (d, J₃',OH = 3.4 Hz, 1 H, OH), 6.14 (app. t, J = 7.0 Hz, 1 H, H1'), 7.42 (app. d, 4J = 1.0 Hz, 1 H, H6), 7.79 (dd, J₉NH₄,5'a = 5.2 Hz, 5NH, 5b's = 7.0 Hz, NH₄), 11.30 (s, 1 H, NH₃) ppm.

13C NMR (151 MHz, DMSO-d₆): δ = 12.4 (CH₃T'), 15.0 (2 x CH₃Et), 38.5 (C2'), 43.4 (2 x CH₂Et), 45.5 (C5'), 70.6 (C3'), 83.9 (C1'), 85.3 (C4'), 109.7 (C5), 135.8 (C6), 150.4 (C2), 163.7 (C4), 166.9 (CSq1), 167.0 (CSq2), 181.8 (CSq4), 182.5 (CSq3) ppm.

HRMS (ESI⁺): m/z calc. 393.1769 [M + H]⁺, found: 393.1756
6.1.2 Synthesis related to modified oligonucleotides

The procedures in this section refer to compounds described in Chapter 3.

6.1.2.1 Synthesis of phosphoramidites

\textbf{N- Acetyl-5'-O- aminothymidin-3'-yl 2-cyanoethyl diisopropylphosphoramidite (83)}

Alcohol 69 (71 mg, 273 µmol) was coevaporated with dry pyridine (3 x 4.0 mL), dried under high vacuum and dissolved in a mixture of dry CH$_2$Cl$_2$ (3.0 mL) and dry DMF (0.5 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (100 µL, 574 µmol). The flask was purged with argon and 2-cyanoethyl \(N,N\)-diisopropylchlorophosphoramidite (110 µL, 285 µmol) was added slowly in a dropwise manner and the reaction mixture was purged with argon. After stirring at r.t. for 2.5 h, TLC analysis (CH$_2$Cl$_2$-MeOH-pyridine, 90:10:0.5) showed the presence of starting material (R$_f$ = 0.2). Over the course of 2.5 h, more DIPEA (210 µL, 1.21 mmol) and 2-cyanoethyl \(N,N\)-diisopropylchlorophosphoramidite (180 µL, 807 µmol) were added in three portions. After a total reaction time of 6 h, TLC analysis showed the complete consumption of the starting material and the formation of the product (R$_f$ = 0.5). The reaction mixture was diluted with degassed EtOAc (30 mL) and washed with degassed sat. aq. KCl (20 mL). The aq. layer was extracted with degassed EtOAc (20 mL) and the combined organic layers were dried over Na$_2$SO$_4$ and concentrated. Purification by flash column chromatography (EtOAc-pyridine, 99.5:0.5) afforded the desired product 83 as an oil (118 mg, 98%). The formation of the phosphoramidite was confirmed by $^{31}$P NMR and the phosphoramidite was used in solid-phase synthesis without further characterisation.

$^{31}$P NMR (162 MHz, DMSO-$d_6$): $\delta$ = 147.6, 147.8 ppm.
**Chapter 6 – Experimental procedures**

5'-O-Amino-N-formylthymidin-3'-yl 2-cyanoethyl diisopropylphosphoramidite (84) (Attempted)

Alcohol 70 (174 mg, 610 µmol) was coevaporated with dry pyridine (3 x 3 mL), dried under high vacuum and dissolved in a mixture of dry CH₂Cl₂ (0.5 mL) and dry DMF (1 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (0.43 mL, 2.44 mmol). The flask was purged with argon and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.27 mL, 1.22 mmol) was added slowly in a dropwise manner and the reaction mixture was purged with argon. After stirring at r.t. for 1 h, TLC analysis (EtOAc-MeOH-pyridine, 90:10:0.5) showed the complete consumption of starting material (Rᵓ = 0.3) and the formation of products (Rᵓ = 0.0, Rᵓ = 0.1). The reaction mixture was diluted with degassed EtOAc (20 mL) and washed with degassed sat. aq. KCl (20 mL). The aq. layer was extracted with degassed EtOAc (20 mL) and the combined organic layers were dried over Na₂SO₄ and concentrated. ¹H and ³¹P NMR analysis of the crude reaction mixture showed decomposition of the starting material and no phosphoramidite. The desired product 84 was not obtained.

5'-Acetoxyamino-5'-deoxy-5'-oxothymidin-3'-yl 2-cyanoethyl diisopropyl phosphoramidite (85) (Attempted)

Alcohol 64 (64 mg, 204 µmol) was coevaporated with dry pyridine (3 x 3 mL), dried under high vacuum and suspended in dry THF (2 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (0.18 mL, 1.02 mmol). The flask was purged with argon and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.14 mL, 613 µmol) was added slowly in a dropwise manner and the reaction
mixture was purged with argon. After stirring at r.t. for 2 h, TLC analysis (EtOAc-MeOH-pyridine, 90:10:0.5) showed the consumption of starting material ($R_f = 0.5$) and the formation of several products ($R_f = 0.1, R_f = 0.7, R_f = 0.8, R_f = 0.9, R_f = 1.0$). The reaction mixture was diluted with degassed EtOAc (20 mL) and washed with degassed sat. aq. KCl (20 mL). The aq. layer was extracted with degassed EtOAc (20 mL) and the combined organic layers were dried over Na$_2$SO$_4$ and concentrated. $^1$H and $^{31}$P NMR analysis of the crude reaction mixture showed decomposition of the starting material and no phosphoramidite. The desired product 85 was not obtained.

5'-Deoxy-5'-methoxyamino-5'-oxothymidin-3'-yl 2-cyanoethyl diisopropyl phosphoramidite (86) (Attempted)

Alcohol 65 (81 mg, 284 µmol) was coevaporated with dry pyridine (3 x 3 mL), dried under high vacuum and dissolved in dry DMF (2 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (0.17 mL, 994 µmol). The flask was purged with argon and 2-cyanoethyl $N,N$-diisopropylchlorophosphoramidite (0.13 mL, 568 µmol) was added slowly in a dropwise manner and the reaction mixture was purged with argon. Over the course of 7 h stirring at r.t., further DIPEA (0.45 mL, 2.58 mmol) and 2-cyanoethyl $N,N$-diisopropylchlorophosphoramidite (0.39 mL, 1.75 mmol) were added in four portions, as TLC analysis (EtOAc-MeOH-pyridine, 95:5:0.5) showed the presence of starting material ($R_f = 0.2$). After 7.5 h, TLC analysis (EtOAc-MeOH-pyridine, 95:5:0.5) showed the formation of products ($R_f = 0.0, R_f = 0.5, R_f = 0.6$). The reaction mixture was diluted with degassed EtOAc (20 mL) and washed with degassed sat. aq. KCl (20 mL). The aq. layer was extracted with degassed EtOAc (20 mL) and the combined organic layers were dried over Na$_2$SO$_4$ and concentrated. $^{31}$P NMR analysis of the crude reaction mixture showed no phosphoramidite. The desired product 86 was not obtained.
Chapter 6 – Experimental procedures

5'-Amino-5'-N-(2-ethoxy-3,4-dioxocyclobuten-1-yl)-5'-deoxythymidin-3'-yl 2-cyanoethyl diisopropylphosphoramidite (87)

Alcohol 71 (90 mg, 246 µmol) was coevaporated with dry CH₂Cl₂ (3 x 2.0 mL), dried under high vacuum and suspended in dry THF (1.5 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (108 µL, 620 µmol). The flask was purged with argon and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (70 µL, 314 µmol) in dry THF (0.4 mL) was added dropwise and the reaction mixture was stirred for 2 h. After this time, TLC analysis (EtOAc-pyridine, 99.5:0.5) showed the consumption of the starting material (Rf = 0.1) and the formation of the product (Rf = 0.6). The solution was concentrated and dissolved in dry CHCl₃ (20 mL). The suspension was washed with sat. aq. KCl (10 mL), dried over Na₂SO₄ and concentrated. Purification by flash column chromatography (EtOAc-pyridine, 99.5:0.5) afforded the desired product 88 as an oil (98 mg, 70%). The formation of the phosphoramidite was confirmed by ³¹P NMR and the phosphoramidite was used in solid phase synthesis without further characterisation.

³¹P NMR (162 MHz, DMSO-d₆): 147.4, 147.56, 147.61, 147.64 ppm.
HRMS (ESI⁺): m/z calc. 588.2194 [M + Na]⁺, found: 588.2210

5'-Amino-5'-N-(2-diethylamino-3,4-dioxocyclobuten-1-yl)-5'-deoxythymidin-3'-yl 2-cyanoethyl diisopropylphosphoramidite (88) (Attempted)

Alcohol 72 (60 mg, 153 µmol) was coevaporated with dry CH₂Cl₂ (3 x 3 mL), dried under high vacuum and suspended in dry THF (1 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPEA (67 µL, 382 µmol). The flask was purged with argon and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite
(51 µL, 229 µmol) was added slowly in a dropwise manner and the reaction mixture was purged with argon. After stirring at r.t. for 2 h, TLC analysis (EtOAc-MeOH, 9:1) showed no consumption of starting material (R_f = 0.1) and no formation of products. The desired product 88 was not obtained.

6.1.2.2 Test reactions for synthesis of squaryl diamide oligonucleotides

5'-Amino-5'-N-(2-diethylamino-3,4-dioxocyclobuten-1-yl)-5'-deoxythymidine (72)

Compound 72 was initially synthesised using a different procedure (see p. 142).

Squarylmonoamide 71 (5 mg, 13.7 µmol) was dissolved in a solution of ethanolamine (27.5 µL, 411 µmol) in H_2O (45 mL). The solution was left at r.t. for 19 h. After this time, TLC analysis (EtOAc-MeOH, 17:3) showed complete consumption of the starting material (R_f = 0.5) and formation of the product (R_f = 0.1). The solution was concentrated to afford the desired product 72 as a white solid (5 mg, quant.).

5'-Amino-5'-N-(3,4-dioxo-2-(ethanolamino)cyclobuten-1-yl)-5'-deoxythymidine (98)

Squarylmonoamide 71 (5 mg, 13.7 µmol) was dissolved in a solution of ethanolamine (27.5 µL, 411 µmol) in H_2O (45 mL). The solution was left at r.t. for 19 h. After this time, TLC analysis (EtOAc-MeOH, 17:3) showed complete consumption of the starting material (R_f = 0.5) and formation of the product (R_f = 0.1). The solution was concentrated to afford the desired product 98 as a white solid (5 mg, quant.).
1H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.78$ (s, 3 H, CH$_3$T), 2.05-2.09 (m, 1 H, H2’a), 2.14-2.18 (m, 1 H, H2'b), 3.50 (bs, 2 H, CH$_2$OH), 3.55 (bs, 1 H, NCH$_2$CH$_2$), 3.69 (bs, 1 H, H5’a), 3.80-3.81 (m, 1 H, H4’), 3.87 (bs, 1 H, H5’b), 4.18-4.19 (m, 1 H, H3’), 5.94 (s, 1 H, OH), 6.18 (app. t, $J = 6.8$ Hz, 1 H, H1’), 7.41 (s, 1 H, H6), 8.05 (bs, 2 H, NH) ppm.

13C NMR (151 MHz, DMSO-d$_6$): $\delta = 12.0$ (CH$_3$T), 38.4 (C2’), 45.5 (C5’), 45.9 (NHCH$_2$CH$_2$), 60.8 (CH$_2$OH), 70.7 (C3’), 83.6 (C1’), 85.2 (C4’), 109.9 (C5), 135.9 (C6), 150.5 (C2), 163.7 (C4), 167.8 (qC$^\text{Sq}$), 168.1 (qC$^\text{Sq}$), 182.4 (CO$^\text{Sq}$), 182.7 (CO$^\text{Sq}$) ppm.

$N$-(5’-Amino-5’-deoxythymidin-5’-yl) 3,4-dioxo sodium cyclobut-1-en-1-olate (99) (Attempted)

Method A:
Squarylmonoamide 71 (5 mg, 13.7 µmol) was suspended in borate buffer (45 mL, adjusted to pH 9.5 with NaOH, 0.2 M). The solution was left at r.t. for 19 h. TLC analysis and NMR analysis were inconclusive and the desired product 99 was not obtained.

Method B:
Squarylmonoamide 71 (5 mg, 13.7 µmol) was suspended in borate buffer (45 mL, adjusted to pH 9.6 with NaOH, 0.01 M). The solution was left at r.t. for 15 h. NMR analysis showed a mixture of compounds.

Method C:
Squarylmonoamide 71 (3 mg, 8.2 µmol) was suspended in borate buffer (10 mL, adjusted to pH 9.6 with NaOH, 0.01 M). The solution was left at r.t. for 24 h. NMR analysis showed a mixture of compounds.
6.1.3 Synthesis of malonate-based mono- and dinucleosides

The procedures in this section refer to compounds described in Chapter 4.

6.1.3.1 Synthesis of 5’-modified nucleosides

Synthesis and deprotection of common intermediate 135 from iodothymidine

Nucleoside 138 was prepared according to a modified procedure. Iodide 78 (10.0 g, 28.4 mmol) was dissolved in dry DMF (20 mL) under argon and cooled to 0 °C. Imidazole (2.51 g, 36.9 mmol) and TBDMS-Cl (5.56 g, 36.9 mmol) were added and the reaction mixture was stirred at 0 °C for 6 h. After this time, TLC analysis (EtOAc) showed the consumption of starting material (Rf = 0.5) and the formation of the product (Rf = 0.8). The reaction was quenched by the addition of MeOH (5 mL), diluted with EtOAc (250 mL) and H2O (100 mL). The layers were separated and the organic layer was washed with brine (100 mL), dried over MgSO4, filtered and concentrated. The residue was recrystallized from EtOH to afford the desired product 138 as a white crystalline solid (11.59 g, 87%), mp 156-159 °C.
ν_{max}/\text{cm}^{-1} \text{ (neat)} 3155 (NH), 3024 (CH), 2930 (CH), 2892 (CH), 2857 (CH), 1691 (C=O), 1655 (C=O), 1470 (CH), 1426, 1402, 1369, 1291, 1274, 1197, 1130, 1100 (C-O), 1051, 1039, 829, 813, 777.

^1\text{H} \text{ NMR (400 MHz, CDCl}_3\): } \delta = 0.11 \text{ (s, 3 H, CH}_3\text{TBDMS)}, 0.12 \text{ (s, 3 H, CH}_3\text{TBDMS)}, 0.90 \text{ (s, 9 H, t-BuTBDMS)}, 1.95 \text{ (s, 3 H, CH}_3\text{T)}, 2.15-2.22 \text{ (m, 1 H, H}_2\text{a)}, 2.29 \text{ (ddd, } J_{2b,3} = 3.9 \text{ Hz, } J_{1',2b} = 6.6 \text{ Hz, } J_{2a,2b} = 13.7 \text{ Hz, 1 H, H}_2\text{b)}, 3.39 \text{ (dd, } J_{1',5a} = 4.6 \text{ Hz, } J_{5a,5b} = 11.0 \text{ Hz, 1 H, H}_5\text{a)}, 3.47 \text{ (dd, } J_{1',5b} = 3.7 \text{ Hz, } J_{5a,5b} = 11.0 \text{ Hz, 1 H, H}_5\text{b)}, 3.67-3.70 \text{ (m, 1 H, H}_4'), 4.26 \text{ (app. dt, } J = 3.9 \text{ Hz, } J = 7.0 \text{ Hz, 1 H, H}_3'), 6.27 \text{ (app. t, } J = 6.6 \text{ Hz, 1 H, H}_1'), 7.47 \text{ (s, 1 H, H}_6), 8.49 \text{ (bs, 1 H, NH) ppm.}

^13\text{C} \text{ NMR (100 MHz, CDCl}_3\): } \delta = -4.5 \text{ (CH}_3\text{TBDMS)}, -4.4 \text{ (CH}_3\text{TBDMS)}, 7.3 \text{ (C}_5\text{)}, 12.8 \text{ (CH}_3\text{T)}, 18.0 \text{ (qC, t-BuTBDMS)}, 25.8 \text{ (t-BuTBDMS)}, 40.6 \text{ (C}_2'), 75.2 \text{ (C}_3'), 84.3 \text{ (C}_4'), 84.8 \text{ (C}_1'), 111.4 \text{ (C}_5), 136.0 \text{ (C}_6), 150.1 \text{ (C}_2), 163.6 \text{ (C}_4) \text{ ppm.}

HRMS (ESI\): m/z calc. 465.0712 [M - H], found: 465.0704

The spectroscopic data are in agreement with those reported in the literature.\textsuperscript{279}

\textbf{N-Benzylmethoxymethyl-3'-O-(tert-butyldimethylsilyl)-5'-deoxy-5'-iodothymidine (137)}

TBDMS-protected iodide \textbf{138} (9.33 g, 20.0 mmol) was dissolved in dry DMF (80 mL) under argon and cooled to 0 °C. DIPEA (17.5 mL, 97.6 mmol) was added, followed by the dropwise addition of benzyl chloromethyl ether (8.4 mL, 57.7 mmol). The reaction mixture was stirred at 0 °C for 4 h. After this time, TLC analysis (petroleum ether-EtOAc, 2:1) showed the consumption of starting material (R\textsubscript{f} = 0.1) and the formation of the product (R\textsubscript{f} = 0.4). The reaction was quenched by the addition of MeOH (10 mL) followed by H\textsubscript{2}O (100 mL). The mixture was extracted with Et\textsubscript{2}O (2 x 100 mL) and the combined organic extracts were dried over MgSO\textsubscript{4}, filtered, concentrated and coevaporated with toluene. Purification by flash column chromatography (EtOAc-petroleum ether, 3:1→2:1) afforded the desired product \textbf{137} as a yellow oil (1.16 g, 95%).
1H NMR (400 MHz, CDCl3): δ = 0.11 (s, 3 H, CH3[TBDMS]), 0.12 (s, 3 H, CH3[TBDMS]), 0.90 (s, 9 H, t-Bu[TBDMS]), 1.95 (d, 4J = 1.1 Hz, 3 H, CH3[T]), 2.13 (app. dt, J = 7.0 Hz, J = 13.7 Hz, 1 H, H2'a), 2.31 (ddd, J2b,3a = 4.1 Hz, J1'a,2b = 6.4 Hz, J2a,2b = 13.7 Hz, 1 H, H2'b), 3.38 (dd, J4'a,5a = 4.1 Hz, J5a,5b = 10.8 Hz, 1 H, H5'a), 3.46 (dd, J4'a,5b = 4.6 Hz, J5a,5b = 10.8 Hz, 1 H, H5'b), 3.70 (app. q, J = 4.1 Hz, 1 H, H3'), 4.25 (app. dt, J = 4.1 Hz, J = 1.1 Hz, 1 H, H6) ppm.

13C NMR (100 MHz, CDCl3): δ = -4.5 (CH3[TBDMS]), -4.4 (CH3[TBDMS]), 7.2 (C5'), 13.5 (CH3[T]), 18.0 (qC, t-Bu[TBDMS]), 25.8 (t-Bu[TBDMS]), 40.7 (C2'), 70.7 (N-CH2-O), 72.4 (O-CH2-Ph), 75.2 (C3'), 84.3 (C4'), 85.4 (C1'), 110.6 (C5), 127.77 (Ph), 127.81 (Ph), 128.4 (Ph), 134.7 (C6), 138.1 (qC, Ph), 150.9 (C2), 163.5 (C4) ppm.

HRMS (APCI⁺): m/z calc. 587.1433 [M + H]+, found: 587.1421

C-(N³-Benzylxoyxymethyl-3'-O-(tert-butyldimethylsilyl)-5'-deoxythymidin-5'-yl) benzyl methyl malonate (135)

NaH (60% dispersion in mineral oil, 0.153 g, 3.82 mmol) was suspended in dry DMF (10 mL) under argon and benzyl methyl malonate (136) (2.1 mL, 11.7 mmol) was added. BOM-protected iodide 137 (1.117 g, 1.91 mmol) was dissolved in dry DMF (10 mL) and added dropwise, the reaction mixture was heated to 100 °C and stirred for 24 h. After this time, TLC analysis (CH2Cl2-EtOAc, 19:1) showed the consumption of starting material (Rf = 0.4) and the formation of the product (Rf = 0.3). The reaction was cooled to r.t., diluted with EtOAc (40 mL) and washed with H2O (20 mL). The aq. layer was extracted with EtOAc (2 x 40 mL) and the combined organic extracts were dried over MgSO4, filtered and concentrated. Purification by flash column chromatography (CH2Cl2-EtOAc, 19:1) afforded the desired product 135 as a yellow oil (0.838 g, 66%).

Compound 135 was isolated as a 1:1 mixture of interconverting diastereoisomers.
Chapter 6 – Experimental procedures

$\nu_{\text{max}}/\text{cm}^{-1}$ (neat) 2953 (CH), 2930 (CH), 2857 (CH), 1734 (C=O), 1710 (C=O), 1656 (C=O), 1464 (CH), 1361, 1151, 1087 (C-O), 1046 (C-O), 833, 773, 735, 696.

$^1$H NMR (600 MHz, CDCl$_3$): $\delta = 0.07-0.08$ (m, 12 H, 4 x CH$_3$TBDMS), 0.88-0.89 (2 x s, 18 H, t-Bu(TBDMS)), 1.91 (d, $^4J = 1.1$ Hz, 3 H, CH$_3$T), 1.95 (d, $^4J = 1.1$ Hz, 3 H, CH$_3$T), 1.97-2.02 (m, 2 H, H2'a), 2.11-2.17 (m, 2 H, H5'a), 2.22-2.26 (m, 2 H, H2'b), 2.36-2.40 (m, 2 H, H5'b), 3.62-3.66 (m, 2 H, H6'), 3.68 (s, 3 H, CO$_2$CH$_3$), 3.73 (s, 3 H, CO$_2$CH$_3$), 3.80-3.84 (m, 2 H, H4'), 4.05-4.08 (m, 2 H, H3'), 4.70 (s, 4 H, 2 x O-CH$_2$-PhBOM), 5.12-5.19 (m, 4 H, 2 x CO$_2$CH$_2$Bn), 5.47 (s, 2 H, N-CH$_2$-O), 5.48 (s, 2 H, N-CH$_2$-O), 6.14 (app. t, $^1J = 6.5$ Hz, 1 H, H1'), 6.18 (app. t, $^1J = 6.6$ Hz, 1 H, H1'), 7.04 (app. d, $^1J = 1.1$ Hz, 1 H, H6), 7.10 (app. d, $^1J = 1.1$ Hz, 1 H, H6), 7.24-7.38 (m, 20 H, 4 x Ph) ppm.

$^{13}$C NMR (151 MHz, CDCl$_3$): $\delta = -4.8$ (CH$_3$TBDMS), -4.63 (CH$_3$TBDMS), -4.61 (CH$_3$TBDMS), 13.16 (CH$_3$T), 13.20 (CH$_3$T), 17.9 (qC, t-Bu(TBDMS)), 25.7 (t-Bu(TBDMS)), 32.59 (C5'), 32.61 (C5'), 40.48 (C2'), 40.55 (C2'), 48.9 (C6'), 49.1 (C6'), 52.72 (CO$_2$CH$_3$), 52.78 (CO$_2$CH$_3$), 67.3 (CO$_2$CH$_2$Bn), 67.4 (CO$_2$CH$_2$Bn), 70.5 (N-CH$_2$-O), 70.6 (N-CH$_2$-O), 72.2 (O-CH$_2$-PhBOM), 74.95 (C3'), 75.03 (C3'), 84.3 (C4'), 84.5 (C4'), 85.62 (C1'), 85.64 (C1'), 110.3 (C5), 110.4 (C5), 127.6 (Ph), 127.7 (Ph), 127.9 (Ph), 128.1 (Ph), 128.3 (Ph), 128.4 (Ph), 128.47 (Ph), 128.48 (Ph), 128.6 (Ph), 134.03 (C6), 134.05 (C6), 135.23 (qC, CO$_2$Bn), 135.24 (qC, CO$_2$Bn), 138.0 (qC, O$\text{BnBOM}$), 150.77 (C2), 150.80 (C2), 163.37 (C4), 163.39 (C4), 168.5 (CO$_2$Bn), 169.1 (CO$_2$CH$_3$), 169.3 (CO$_2$Bn), 169.8 (CO$_2$CH$_3$) ppm.

HRMS (APCI$^+$): m/z calc. 667.3045 [M + H]$^+$, found: 667.3054

**C-(3'-O-(tert-Butyldimethylsilyl-5'-deoxy-\$^{N^3}\$-hydroxymethyl)thymidin-5'-yl) malonic acid monomethyl ester (139)**

![Diagram](image)

Fully protected malonate 135 (550 mg, 825 µmol) was dissolved in MeOH (20 mL) and added to a dried flask containing Pd/C (10%, 104 mg) and H$_2$ was bubbled through the suspension while stirring at r.t. for 1.5 h. After this time, TLC analysis showed the complete consumption of starting material (CH$_2$Cl$_2$-EtOAc, 4:1, $R_f = 0.8$) and the formation of the product (CH$_2$Cl$_2$-MeOH, 4:1, $R_f = 0.5$). The reaction mixture
was filtered through celite and the filtrate was concentrated to afford the desired product 139 as a yellow foam (380 mg, 95%).

Compound 139 was isolated as a 1:1 mixture of interconverting diastereoisomers.

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3188 (OH), 2955 (CH), 2929 (CH), 2858 (CH), 1705 (C=O), 1473 (CH), 1437, 1275, 1048 (C-O), 837, 779.

$^1$H NMR (600 MHz, acetone-d$_6$): $\delta = 0.13$-$0.14$ (m, 12 H, 4 x CH$_3$TBDMS), 0.918-0.922 (2 x s, 18 H, t-BuTBDMS), 1.86 (s, 3 H, CH$_3$T), 1.88 (s, 3 H, CH$_3$T), 2.15-2.25 (m, 4 H, H2’a, H5’a), 2.33-2.41 (m, 4 H, H2’b, H5’b), 3.60-3.63 (m, 2 H, H6’), 3.67 (s, 3 H, CO$_2$CH$_3$), 3.70 (s, 3 H, CO$_2$CH$_3$), 3.83-3.86 (m, 1 H, H4’), 3.88-3.91 (m, 1 H, H4’), 4.38 (m, 2 H, H3’), 5.38 (s, 4 H, N-CH$_2$-O), 6.27-6.30 (m, 2 H, H1’), 7.47 (s, 1 H, H6), 7.49 (s, 1 H, H6) ppm.

$^{13}$C NMR (151 MHz, acetone-d$_6$): $\delta = -4.7$ (CH$_3$TBDMS), -4.5 (CH$_3$TBDMS), 13.0 (CH$_3$T), 18.48 (qC, t-BuTBDMS), 18.49 (qC, t-BuTBDMS), 26.1 (t-BuTBDMS), 33.3 (C5’), 33.4 (C5’), 40.3 (C2’), 40.4 (C2’), 49.3 (C6’), 49.5 (C6’), 52.60 (CO$_2$CH$_3$), 52.64 (CO$_2$CH$_3$), 65.4 (N-CH$_2$-O), 76.0 (C3’), 76.1 (C3’), 85.1 (C4’), 85.4 (C4’), 86.1 (C1’), 110.5 (C5), 110.6 (C5), 135.9 (C6), 151.5 (C2), 163.6 (C4), 170.3 (CO$_2$CH$_3$), 170.4 (CO$_2$H), 170.90 (CO$_2$CH$_3$), 170.93 (CO$_2$H) ppm.

HRMS (ESI$^+$): m/z calc. 509.1926 [M + Na]$^+$, found: 509.1928

**C-(3’-O-(tert-Butyldimethylsilyl)-5’-deoxythymidin-5’-yl) malonic acid monomethyl ester (140) (Attempted)**

Alcohol 139 (130 mg, 267 µmol) was dissolved in THF (10 mL), NaHCO$_3$ (100 mg, 1.19 mmol) was added and the reaction was stirred at r.t. for 4 h. After this time, TLC analysis showed the complete consumption of starting material (CH$_2$Cl$_2$-EtOAc, 4:1, R$_f$ = 0.8) and the formation of a product (CH$_2$Cl$_2$-MeOH, 4:1, R$_f$ = 0.5). The reaction mixture was filtered and the filtrate was concentrated to a yellow oil. The desired product 140 was not obtained.
Synthesis of targets 102 and 103 using an alternative protecting group strategy

Protected malonate 135 (100 mg, 150 µmol) was dissolved in THF (1.5 mL). TBAF (85 mg, 270 µmol) was added and the solution was stirred at r.t. for 1 h. After this time, TLC analysis (CH$_2$Cl$_2$-EtOAc, 9:1) showed the complete consumption of the starting material ($R_f$ = 0.7) and product formation ($R_f$ = 0.2). The yellow solution was concentrated and purification by flash column chromatography (CH$_2$Cl$_2$-MeOH, 99:1→19:1) afforded the desired product 141 as a colourless wax (59 mg, 71%).

Compound 141 was isolated as a 1:1 mixture of interconverting diastereoisomers.

$\nu_{\text{max}}/\text{cm}^{-1}$ (CH$_2$Cl$_2$): 3458 (OH), 3033 (CH), 2955 (CH), 2928 (CH), 1732 (C=O), 1705 (C=O), 1641 (C=O), 1467 (CH), 1454 (CH), 1438, 1361, 1273, 1222, 1153, 1088 (C-O), 1074 (C-O), 1048, 1028, 774, 737, 697.

$^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ = 1.83 (d, $^4J$ = 1.1 Hz, 3 H, CH$_3^\beta$), 1.87 (d, $^4J$ = 1.1 Hz, 3 H, CH$_3^\gamma$), 2.17-2.33 (m, 6 H, H2'α, H2'β, H5'a), 2.38 (ddd, $J$ = 3.8 Hz, $J = 7.8$ Hz, $J = 14.2$ Hz, 2 H, H5'b), 3.65 (s, 3 H, CO$_2$CH$_3$), 3.69 (s, 3 H, CO$_2$CH$_3$), 3.69-3.74 (m, 2 H, H6'), 3.83-3.89 (m, 2 H, H4'), 4.25-4.30 (m, 2 H, H3').
\[ J = 4.4 \text{ Hz}, 1 \text{ H, OH}, \ 4.54 \ (d, \ J = 4.4 \text{ Hz}, 1 \text{ H, OH}), \ 4.65 \ (s, \ 2 \text{ H, O-CH}_2-\text{Ph}^{BOM}), \ 4.66 \ (s, \ 2 \text{ H, O-CH}_2-\text{Ph}^{BOM}), \ 5.16 \ (d, \ J = 3.5 \text{ Hz}, 2 \text{ H, CO}_2\text{CH}_2^{Bn}), \ 5.20 \ (d, \ J = 3.7 \text{ Hz}, 2 \text{ H, CO}_2\text{CH}_2^{Bn}), \ 5.44 \ (m, \ 2 \text{ H, N-CH}_2-O), \ 5.45 \ (s, \ 2 \text{ H, N-CH}_2-O), \ 6.26 \ (\text{app. t, } J = 6.8 \text{ Hz}, 1 \text{ H, H1'}), \ 6.28 \ (\text{app. t, } J = 6.8 \text{ Hz}, 1 \text{ H, H1'}), \ 7.22-7.38 \ (m, 21 \text{ H, 4 x Ph, H6}), \ 7.41 \ (\text{app. d, } 4 J = 1.1 \text{ Hz}, 1 \text{ H, H6}) \text{ ppm.}

^{13}C \text{ NMR (100 MHz, acetone-d}_6): \ \delta = 13.1 \ (\text{CH}_3^{T}), \ 33.4 \ (\text{C5'}), \ 39.9 \ (\text{C2'}), \ 40.0 \ (\text{C2'}), \ 49.6 \ (\text{C6'}), \ 49.7 \ (\text{C6'}), \ 52.8 \ (\text{CO}_2\text{CH}_3), \ 67.5 \ (\text{CO}_2\text{CH}_2^{Bn}), \ 71.26 \ (\text{N-CH}_2-O), \ 71.28 \ (\text{N-CH}_2-O), \ 72.4 \ (\text{O-CH}_2-\text{Ph}^{BOM}), \ 74.79 \ (\text{C3'}), \ 74.85 \ (\text{C3'}), \ 84.9 \ (\text{C4'}), \ 85.1 \ (\text{C4'}), \ 86.1 \ (\text{C1'}), \ 110.35 \ (\text{C5}), \ 110.43 \ (\text{C5}), \ 128.2 \ (\text{Ph}), \ 128.68 \ (\text{Ph}), \ 128.73 \ (\text{Ph}), \ 128.9 \ (\text{Ph}), \ 129.0 \ (\text{Ph}), \ 129.2 \ (\text{Ph}), \ 129.3 \ (\text{Ph}), \ 135.84 \ (\text{C6}), \ 135.88 \ (\text{C6}), \ 136.90 \ (\text{qC, CO}_2\text{Bn}), \ 136.92 \ (\text{qC, CO}_2\text{Bn}), \ 139.6 \ (\text{qC, OBn}^{BOM}), \ 151.79 \ (\text{C2}), \ 151.81 \ (\text{C2}), \ 163.79 \ (\text{C4}), \ 163.81 \ (\text{C4}), \ 169.4 \ (\text{CO}_2\text{Bn}), \ 169.91 \ (\text{CO}_2\text{CH}_3), \ 169.94 \ (\text{CO}_2\text{Bn}), \ 170.4 \ (\text{CO}_2\text{CH}_3) \text{ ppm.}

HRMS (APCI\(+\): m/z calc. 575.2000 [M + Na]\(^+\), found: 575.2015

C-(5'-Deoxythymidin-5'-yl) malonic acid monomethyl ester (142)

Malonate ester 141 (115 mg, 208 µmol) was dissolved in MeOH (10 mL) and added to a dried flask containing Pd/C (10%, 17 mg) and H\(_2\) was bubbled through the suspension while stirring at r.t. for 28 h. After this time, TLC analysis (CH\(_2\)Cl\(_2\)-MeOH, 9:1) showed the complete consumption of starting material (R\(_f\) = 0.6) and the formation of the product (R\(_f\) = 0.2). The reaction mixture was filtered through celite and the filtrate was concentrated to a colourless film. Purification by flash column chromatography (CH\(_2\)Cl\(_2\)-MeOH, 19:1→MeOH) afforded the desired product 142 as a white foam (61 mg, 86%).

Compound 142 was isolated as a 1:1 mixture of interconverting diastereoisomers.

\( \nu_{\text{max}} / \text{cm}^{-1} \) (neat) 3375 (OH, NH), 2955 (CH), 1686 (C=O), 1665 (C=O), 1588 (C=O), 1474 (CH), 1437 (OH), 1371, 1269, 1198, 1168, 1086 (CO), 1049, 767.

\(^1\text{H NMR (600 MHz, DMSO-d}_6): \ \delta = 1.80 \ (2 x s, 6 \text{ H, CH}_3^{T}), \ 1.89-1.95 \ (m, \ 1 \text{ H, H5'}a), \ 1.98-2.06 \ (m, \ 4 \text{ H, H2'a, H5'}a, H5'b), \ 2.08-2.16 \ (m, \ 3 \text{ H, H2'b, H5'}b), \ 3.06 \ (\text{app. t,} \)
$J = 6.9$ Hz, $1$ H, H6'), 3.13-3.15 (m, 1 H, H6'), 3.49 (s, 3 H, CO$_2$CH$_3$), 3.53 (s, 3 H, CO$_2$CH$_3$), 3.59-3.62 (m, 1 H, H4'), 3.68-3.71 (m, 1 H, H4'), 4.00-4.02 (m, 1 H, H3'), 4.03-4.06 (m, 1 H, H3'), 4.14 (bs, 2 H, OH), 5.53 (bs, 2 H, OH), 6.05 (app. t, J = 6.9 Hz, 1 H, H1'), 6.09 (app. t, J = 6.9 Hz, 1 H, H1'), 7.36 (s, 1 H, H6), 7.42 (s, 1 H, H6), 11.27 (bs, 2 H, NH) ppm.

$^{13}$C NMR (150 MHz, DMSO-d$_6$): $\delta = 12.12$ (CH$_3$), 12.13 (CH$_3$), 33.4 (C5'), 33.8 (C5'), 38.7 (C2'), 50.9 (CO$_2$CH$_3$), 51.4 (C6'), 52.1 (C6'), 73.1 (C3'), 73.4 (C3'), 83.38 (C1'), 83.42 (C1'), 84.2 (C4'), 85.4 (C4'), 109.5 (C5), 109.9 (C5), 136.0 (C6), 150.4 (C2), 150.5 (C2), 163.8 (C4), 172.3 (CO$_2$), 172.8 (CO$_2$) ppm.

HRMS (APCI?): m/z calc. 341.0990 [M - H], found: 341.0990

C-(5'-Deoxythymidin-5'-yl) monomethyl sodium malonate (102)

Malonate ester 141 (250 mg, 452 µmol) was dissolved in MeOH (15 mL) and added to a dried flask containing Pd/C (10%, 50 mg) and H$_2$ was bubbled through the suspension while stirring at r.t. for 7 h. After this time, TLC analysis (EtOAc-MeOH, 2:1) showed the complete consumption of starting material ($R_f = 0.9$) and the formation of the product ($R_f = 0.2$). The reaction mixture was filtered through celite and the filtrate was concentrated to a colourless film. Purification by flash column chromatography (EtOAc-MeOH, 3:1→2:1) was followed by elution through Diaion resin WT01S(H) (Na form) in MeOH. The resulting white solid was purified by preparative reversed phase TLC (H$_2$O-MeCN, 1:1), extracted with MeOH and concentrated to afford the desired product 102 as a white solid (37 mg, 22%).

Compound 102 was isolated as a 1:1 mixture of interconverting diastereomers.

$\nu_{\text{max}}/\text{cm}^{-1}$ (neat) 3435 (OH, NH), 3210 (CH), 2951 (CH), 1665 (C=O), 1594 (C=O), 1473 (CH), 1436, 1369, 1269, 1195, 1165, 1084 (C-O), 1048, 768.

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.80$ (d, $^4J = 0.9$ Hz, 6 H, CH$_3$), 1.93-2.17 (m, 8 H, H2'a, H2'b, H5'a, H5'b), 3.18 (app. t, J = 6.9 Hz, 1 H, H6'), 3.23 (app. t, J = 9.4 Hz, 1 H, H6'), 3.52 (s, 3 H, CO$_2$CH$_3$), 3.56 (s, 3 H, CO$_2$CH$_3$), 3.59-3.62 (m, 1 H, H4'), 3.65-3.68 (m, 1 H, H4'), 4.02-4.07 (m, 2 H, H3'), 6.07 (app. t, J = 6.8 Hz, J = 6.9 Hz, 1 H, H6').
1H, H1'), 6.10 (app. t, J = 6.9 Hz, 1H, H1'), 7.37 (app. d, 'J = 0.9 Hz, 1H, H6), 7.42 (app. d, J = 0.9 Hz, 1H, H6), 11.27 (bs, 2H, NH) ppm.

13C NMR (150 MHz, DMSO-d6): δ = 12.1 (CH3), 33.0 (C5'), 33.3 (C5'), 38.5 (C2'), 38.6 (C2'), 50.4 (C6'), 50.8 (C6'), 51.3 (CO2CH3), 73.1 (C3'), 73.3 (C3'), 83.4 (C1'), 84.0 (C4'), 84.9 (C4'), 109.6 (C5), 109.9 (C5), 136.05 (C6), 136.10 (C6), 150.42 (C2), 150.45 (C2), 163.7 (C4), 170.1 (CO2Na), 170.3 (CO2Na), 171.5 (CO2CH3), 171.8 (CO2CH3) ppm.

HRMS (ESI+): m/z calc. 373.0618 [M + H]+, found: 373.0618

C-(5'-Deoxythymidin-5'-yl) disodium malonate (103)

KOH (20 mg, 356 µmol) was dissolved in MeOH (0.5 mL) and cooled to 0 °C. Malonic ester 142 (30 mg, 88 µmol) was added and the reaction was slowly warmed to r.t. and stirred for 3 h. A further portion of KOH (20 mg, 356 µmol) in MeOH (0.5 mL) was added and the reaction was stirred for a further 4 h. After this time, TLC analysis (H2O-i-PrOH-EtOAc, 1:2:2) showed the consumption of starting material (Rf = 0.7) and the formation of a product (Rf = 0.6). The reaction mixture was concentrated and the residue was taken up in H2O and eluted through Diaion resin WT01S(H) (H form) followed by elution through Diaion resin WT01S(H) (Na form). The white solid was purified by preparative reversed phase TLC (H2O-MeCN, 1:1), extracted with H2O and concentrated to afford the desired product 103 as a white solid (18 mg, 55%); mp 166-170 °C dec.

νmax/cm−1 (neat) 3316 (OH, NH), 2937 (CH), 2822 (CH), 1686 (C=O), 1587 (C=O), 1475 (CH), 1420, 1365, 1271, 1082 (C=O), 1024, 767.

1H NMR (600 MHz, D2O): δ = 1.92 (s, 3H, CH3), 2.17-2.23 (m, 1H, H5'a), 2.29-2.41 (m, 3H, H2'a, H2'b, H5'b), 3.32-3.40 (m, 1H, H6'), 3.97 (bs, 1H, H4'), 4.34-4.36 (m, 1H, H3'), 6.24-6.26 (m, 1H, H1'), 7.51 (s, 1H, H6) ppm.

13C NMR (150 MHz, D2O): δ = 11.5 (CH3), 33.2 (C5'), 37.8 (C2'), 55.2 (C6'), 73.6 (C3'), 84.8 (C1'), 85.0 (C4'), 111.5 (C5), 137.4 (C6), 151.7 (C2), 166.5 (C4), 176.5 (CO2), 176.6 (CO2) ppm.

HRMS (ESI+): m/z calc. 373.0618 [M + H]+, found: 373.0618
Exploration of global BOM-protection or benzyl-protection for iodothymidine 78

a) BOM-Cl, NaI, DIPEA, acetone

\[ \text{BOM-} \text{Cl, NaI, DIPEA, acetone} \]

\[ 0 \degree C \text{ to r.t., 22 h} \]

\[ \text{78} \]

\[ \text{143} \quad 21\% \]

\[ \text{144} \quad 67\% \]

b) BnBr, NaH, DMF

\[ \text{BnBr, NaH, DMF} \]

\[ 0 \degree C \text{ to r.t., 22 h} \]

\[ \text{78} \]

\[ \text{145} \quad 0\% \]

\[ \text{146} \quad (53\%) \]

\[ \text{147} \quad (37\%) \]

\[ N^2,3'-O-(\text{Di(benzyloxymethyl)})-5'-\text{deoxy-5'}-\text{iodothymidine (143)} \]

5'-Iodothymidine 78 (300 mg, 0.85 mmol) was dissolved in dry acetone (10 mL) under argon. Sodium iodide (390 mg, 2.60 mmol) was added and the solution was cooled to 0 °C. DIPEA (740 µL, 4.25 mmol) was added, followed by the dropwise addition of benzyl chloromethyl ether (360 µL, 2.60 mmol). The reaction was slowly warmed to r.t. and stirred for 17 h. After this time, TLC analysis (EtOAc-petroleum ether, 2:1) showed partial consumption of the starting material (Rf = 0.1) and formation of an intermediate (Rf = 0.4) and the desired product (Rf = 0.7). The suspension was cooled to 0 °C and further portions of sodium iodide (195 mg, 1.30 mmol), DIPEA (300 µL, 1.72 mmol) and benzyl chloromethyl ether (180 µL, 1.30 mmol) were added. The mixture was slowly warmed to r.t. and stirred for a further 84 h. After this time, the suspension was diluted with EtOAc (100 mL), washed with H2O (100 mL), brine (50 mL), dried over MgSO4, filtered and concentrated to a brown oil. Purification by flash column chromatography (petroleum ether-EtOAc, 3:1→1:2) afforded the desired product 143 as a yellow oil (105 mg, 21%).
ν\textsubscript{max}/cm\textsuperscript{-1} (neat) 3064 (CH), 3031 (CH), 2954 (CH), 2927 (CH), 1709 (C=O), 1650 (C=O), 1464 (CH), 1453 (CH), 1361, 1276, 1245, 1199, 1157, 1071 (C-O), 1025, 964, 907, 772, 731, 697.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ = 1.93 (app. s, 3 H, CH\textsubscript{3}T), 2.10-2.19 (m, 1 H, H2’a), 2.44 (ddd, J = 3.0 Hz, J = 6.0 Hz, J = 14.0 Hz, 1 H, H2’b), 3.38 (dd, J = 4.0 Hz, J\textsubscript{5’a,5’b} = 11.0 Hz, 1 H, H5’a), 3.43 (dd, J = 4.7 Hz, J\textsubscript{5’a,5’b} = 11.0 Hz, 1 H, H5’b), 3.88 (app. q, J = 4.1 Hz, 1 H, H4’), 4.23-4.26 (m, 1 H, H3’), 4.63-4.64 (m, 2 H, O-CH\textsubscript{2}-O-CH\textsubscript{2}-Ph), 4.70 (s, 2 H, N-CH\textsubscript{2}-O-CH\textsubscript{2}-Ph), 4.80-4.84 (m, 2 H, O-CH\textsubscript{2}-O), 5.49 (s, 2 H, N-CH\textsubscript{2}-O), 6.28-6.32 (m, 1 H, H1’), 7.25-7.38 (m, 10 H, 2 x Ph), 7.42 (app. d, J = 1.0 Hz, 1 H, H6) ppm.

\textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): δ = 7.6 (C5’), 13.5 (CH\textsubscript{3}T), 38.0 (C2’), 70.4 (O-CH\textsubscript{2}-O-CH\textsubscript{2}-Ph), 70.7 (N-CH\textsubscript{2}-O), 72.4 (N-CH\textsubscript{2}-O-CH\textsubscript{2}-Ph), 80.1 (C3’), 82.6 (C4’), 85.5 (C1’), 94.3 (O-CH\textsubscript{2}-O), 110.8 (C5), 127.8 (Ph), 128.1 (Ph), 128.2 (Ph), 128.4 (Ph), 128.7 (Ph), 134.6 (C6), 137.4 (qC, Ph), 138.1 (qC, Ph), 151.0 (C2), 163.5 (C4) ppm.

HRMS (APCI\textsuperscript{+}): m/z calc. 615.0963 [M + Na]\textsuperscript{+}, found: 615.0953

\textit{N\textsuperscript{3}-Benzyloxymethyl-5’-deoxy-5’-iodothymidine (144)}

Alcohol \textbf{144} was isolated as a yellow oil (264 mg, 66%) as a byproduct in the synthesis of compound \textbf{143}.

ν\textsubscript{max}/cm\textsuperscript{-1} (neat) 3428 (OH), 3065 (CH), 3031 (CH), 2958 (CH), 2929 (CH), 1704 (C=O), 1638 (C=O), 1466 (CH), 1453, 1361, 1273, 1196, 1072 (C-O), 1027, 966, 907, 773, 731, 697.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ = 1.92 (s, 3 H, CH\textsubscript{3}T), 2.14-2.21 (m, 1 H, H2’a), 2.36 (ddd, J = 3.3 Hz, J = 6.1 Hz, J = 13.9 Hz, 1 H, H2’b), 3.37-3.44 (m, 2 H, H5’a, H5’b), 3.81-3.84 (m, 1 H, H4’), 4.29-4.32 (m, 1 H, H3’), 4.67 (s, 2 H, O-CH\textsubscript{2}-Ph), 5.47 (s, 2 H, N-CH\textsubscript{2}-O), 6.27 (app. t, J = 6.9 Hz, 1 H, H1’), 7.23-7.35 (m, 10 H, 2 x Ph), 7.40 (s, 1 H, H6) ppm.
13C NMR (100 MHz, CDCl3): δ = 7.2 (C5'), 13.5 (CH3T), 40.4 (C2'), 70.7 (N-CH2-O), 72.4 (O-CH2-Ph), 75.0 (C3'), 84.3 (C4'), 85.6 (C1'), 110.7 (C5), 127.8 (Ph), 128.4 (Ph), 134.8 (C6), 138.0 (qC, Ph), 150.9 (C2), 163.5 (C4) ppm.

HRMS (APCI+): m/z calc. 495.0387 [M + Na]+, found: 495.0378

\(N^3,3'O\)-Dibenzyl-5'-deoxy-5'-idothymidine (145) (Attempted)

\[ \text{NaH (60\% dispersion in mineral oil, 57 mg, 1.42 mmol) was suspended in dry DMF (5 mL) under argon and cooled to 0 °C. 5'-Iodothymidine 78 (200 mg, 0.57 mmol) was added and benzyl bromide (170 µL, 1.43 mmol) was added dropwise. The reaction was stirred at 0 °C for 2 h and then slowly warmed to r.t. and stirred for 18 h. After this time, TLC analysis (EtOAc-petroleum ether, 2:1) showed complete consumption of the starting material (Rf = 0.1) and formation of an intermediate (Rf = 0.5) and product (Rf = 0.7). Further portions of NaH (60\% dispersion in mineral oil, 34 mg, 0.85 mmol) and benzyl bromide (100 µL, 0.84 mmol) were added. The mixture was stirred for a further 4 h, when TLC analysis ((EtOAc-petroleum ether, 2:1) showed complete consumption of the intermediate (Rf = 0.5) and formation of a product (Rf = 0.7). After this time, the reaction was quenched with H2O (0.5 mL), diluted with EtOAc (100 mL), and washed with H2O (50 mL) and brine (50 mL). The organic layer was dried over MgSO4, filtered and concentrated to a yellow oil. Purification by flash column chromatography (petroleum ether-EtOAc, 3:1→2:1) did not afford the desired product 145.}

A 2:3 mixture of by-products bromide 146 and alkene 147 was isolated as a colourless oil (232 mg, 90%).

5'-Bromo-5'-deoxy-\(N^3,3'O\)-dibenzylthymidine (146)
Chapter 6 – Experimental procedures

1H NMR (600 MHz, CDCl₃): δ = 1.95 (s, 3 H, CH₃), 2.07-2.13 (m, 1 H, H2'a), 2.49 (ddd, J = 2.4 Hz, J₁',2'b = 5.9 Hz, J = 13.8 Hz, 1 H, H2'b), 3.57 (dd, J = 3.4 Hz, J₅'a,₅'b = 11.1 Hz, 1 H, H5'a), 3.63 (dd, J = 4.4 Hz, J₅'a,₅'b = 11.1 Hz, 1 H, H5'b), 4.17-4.19 (m, 1 H, H3'), 4.26-4.28 (m, 1 H, H4'), 4.51 (d, J₉a,H₉b = 11.6 Hz, 1 H, O-CHaHB-Ph), 4.59 (d, J₉a,H₉b = 11.6 Hz, 1 H, O-CHaHB-Ph), 5.11-5.12 (m, 2 H, N-CH₂-Ph), 6.34 (dd, J₁',2'b = 5.9 Hz, J = 8.1 Hz, 1 H, H1'), 7.25-7.37 (m, 8 H, 2 x Ph), 7.41 (s, 1 H, H6), 7.47-7.49 (m, 2 H, 2 x Ph) ppm.

13C NMR (100 MHz, CDCl₃): δ = 13.6 (CH₃), 33.7 (C5'), 37.6 (C2'), 44.7 (N-CH₂-Ph), 71.9 (O-CH₂-Ph), 80.3 (C3'), 82.7 (C4'), 85.8 (C1'), 110.8 (C5), 127.7 (Ph), 127.9 (Ph), 128.2 (Ph), 128.5 (Ph), 128.7 (Ph), 129.3 (Ph), 133.6 (C6), 137.0 (qC, Ph), 151.0 (C2), 163.4 (C4) ppm.

HRMS (APCI⁺): m/z calc. 507.0890 [M + Na]⁺, found: 507.0889

5'-Deoxy-N⁳,3'-O-dibenzyl-4',5'-didehydrothymidine (147)

1H NMR (600 MHz, CDCl₃): δ = 1.95 (s, 3 H, CH₃), 2.10-2.15 (m, 1 H, H2'a), 2.60 (ddd, J₂b,₃' = 1.3 Hz, J₁',2'b = 5.8 Hz, J = 13.7 Hz, 1 H, H2'b), 4.31 (d, J₅'a,₅'b = 1.9 Hz, 1 H, H5'a), 4.45 (dd, J₂b,₃' = 1.3 Hz, J = 5.5 Hz, 1 H, H3'), 4.47 (d, J₉a,H₉b = 11.7 Hz, 1 H, O-CHaHB-Ph), 4.66 (d, J₅'a,₅'b = 1.9 Hz, 1 H, H5'b), 4.68 (d, J₉a,H₉b = 11.7 Hz, 1 H, O-CHaHB-Ph), 5.11-5.12 (m, 2 H, N-CH₂-Ph), 6.67 (dd, J₁',2'b = 5.8 Hz, J = 7.7 Hz, 1 H, H1'), 6.96 (s, 1 H, H6), 7.25-7.37 (m, 8 H, 2 x Ph), 7.47-7.49 (m, 2 H, 2 x Ph) ppm.

13C NMR (100 MHz, CDCl₃): δ = 13.6 (CH₃), 38.4 (C2'), 44.7 (N-CH₂-Ph), 70.4 (O-CH₂-Ph), 76.8 (C3'), 87.1 (C1'), 87.4 (C5'), 110.8 (C5), 127.7 (Ph), 127.9 (Ph), 128.2 (Ph), 128.5 (Ph), 128.7 (Ph), 129.3 (Ph), 132.6 (C6), 137.0 (qC, Ph), 151.0 (C2), 159.1 (C4'), 163.4 (C4) ppm.

HRMS (APCI⁺): m/z calc. 427.1628 [M + Na]⁺, found: 427.1617

167
Attempted amide couplings between 5’-malonyl thymidine and protected hydroxylamine

**a)**

![Diagram a](image)

**b)**

![Diagram b](image)

**c)**

![Diagram c](image)

\( C\text{-}(5'\text{-Deoxythymidin-5'\text{-yl})} \quad O\text{-}(\text{tert-butyl}d\text{imethylsilyl})\text{oxyamido} \quad \text{methyl malonate} \) (148) (Attempted)

Malonate ester 142 (42 mg, 123 µmol) was dissolved in dry DMF (2 mL) under argon and cooled to 0 °C. EDC (35 mg, 183 µmol) and HOAt (25 mg, 184 µmol) were added followed by DIPEA (32 µL, 184 µmol). The solution was stirred at 0 °C for 30 min. \( O\text{-}(\text{tert-Butyl}d\text{imethylsilyl})\text{hydroxylamine} \) (28 mg, 190 µmol) was added and the reaction was slowly warmed to r.t. and stirred for 65 h. After this time, TLC analysis (CH\(_2\)Cl\(_2\)-MeOH, 9:1) showed the consumption of malonate 142 \( (R_f = 0.3) \) and the formation of products \( (R_f = 0.8, R_f = 0.2) \). The reaction mixture was diluted with EtOAc (20 mL), washed with H\(_2\)O (20 mL) and the aq. layer was extracted with EtOAc (20 mL). The combined organic layers were washed with H\(_2\)O (20 mL) and brine (20 mL). The combined aq. layers were extracted with EtOAc (3 x 20 mL), dried over MgSO\(_4\), filtered and concentrated to a colourless glassy solid. Purification
by flash column chromatography (CH₂Cl₂-MeOH, 9:1) afforded three compounds that were not identified, but not the desired product 148.

**C-(5'-Deoxythymidin-5'-yl) (benzyloxy)amido methyl malonate (149)**

![Chemical structure of compound 149](image)

Malonate ester 142 (99 mg, 266 μmol) was dissolved in dry DMF (4 mL) under argon and cooled to 0 °C. EDC (66 mg, 344 μmol) and HOAt (47 mg, 345 μmol) were added and the solution was stirred at 0 °C for 1 h. O-Benzylhydroxylamine hydrochloride (55 mg, 345 μmol) was added and the reaction was slowly warmed to r.t. and stirred for 22 h. DIPEA (93 μL, 534 μmol) was added and the reaction was stirred for a further 24 h. After this time, TLC analysis showed the complete consumption of malonate ester 142 (CH₂Cl₂-MeOH, 19:1, Rᵣ = 0.2) and the formation of a product (EtOAc-petroleum ether, 4:1, Rᵣ = 0.1). The reaction mixture was diluted with EtOAc (20 mL), washed with H₂O (20 mL) and the aq. layer was extracted with EtOAc (20 mL). The combined organic layers were washed with brine (20 mL), dried over MgSO₄, filtered and concentrated to a colourless glassy solid. Purification by flash column chromatography (EtOAc-petroleum ether, 4:1) afforded the desired product 149 as a white solid (42 mg, 35%).

Compound 149 was isolated as a 1:1 mixture of interconverting diastereomers.

ν<sub>max</sub>/cm<sup>-1</sup> (CH₂Cl₂) 3402 (OH, NH), 3269 (OH, NH), 3081 (CH), 2954 (CH), 2925 (CH), 2857 (CH), 1726 (C=O), 1653 (C=O), 1478 (CH), 1438 (CH), 1364, 1294, 1278, 1240, 1167, 1086 (C-O), 1026, 986, 958, 747.

¹H NMR (600 MHz, DMSO-d₆): δ = 1.79-1.80 (2 x s, 6 H, CH₃), 1.89-1.95 (m, 1 H, H5’a), 1.99-2.04 (m, 3 H, H2’a, H2’b), 2.15-2.25 (m, 4 H, H2’b, H5’a, H5’b), 3.22-3.27 (m, 2 H, H6’), 3.61-3.62 (2 x s, 6 H, CO₂CH₃), 3.65-3.66 (m, 2 H, H4’), 4.04-4.11 (m, 2 H, H3’), 4.69 (d, J<sub>Hₐ,Hₜ</sub> = 10.7 Hz, 1 H, O-CH₃Hₕ-PH), 4.75 (d, J<sub>Hₐ,Hₜ</sub> = 10.7 Hz, 1 H, O-CH₃Hₕ-PH), 4.77-4.81 (m, 2 H, O-CH₂-PH), 5.31-5.34 (m, 2 H, OH), 6.09-6.13 (m, 2 H, H1’), 7.33-7.40 (m, 12 H, H6, CH₆P), 11.25-11.29 (2 x bs, 2 H, NHₗ), 11.39-11.42 (2 x bs, 2 H, CONH) ppm.
Chapter 6 – Experimental procedures

$^{13}$C NMR (150 MHz, DMSO-$d_6$): $\delta = 12.07$ (CH$_3^T$), 12.10 (CH$_3^T$), 31.9 (C5’), 32.1 (C5’), 38.1 (C2’), 38.2 (C2’), 45.8 (C6’), 46.4 (C6’), 52.2 (CO$_2$CH$_3$), 67.2 (O-CH$_2$-Ph), 73.2 (C3’), 73.3 (C3’), 76.6 (O-CH$_2$-Ph), 76.8 (O-CH$_2$-Ph), 83.5 (C1’), 83.7 (C4’), 84.0 (C4’), 109.7 (C5), 109.9 (C5), 128.26 (Ph), 128.30 (Ph), 128.8 (Ph), 129.0 (Ph), 135.7 (qC, Ph), 136.2 (C6), 136.3 (C6), 150.4 (C2), 150.5 (C2), 163.68 (C4), 163.70 (C4), 164.4 (CONHOBn), 165.0 (CONHOBn), 169.5 (CO$_2$CH$_3$), 169.9 (CO$_2$CH$_3$) ppm.

HRMS (APCI$^+$): $m/z$ calc. 448.1714 [M + H]$^+$, found: 448.1720

$C$-($3'$-O-($tert$-Butyldimethylsilyl$)-5'$-deoxythymidin$-5'$-yl) (benzyloxy)amido methyl malonate (150) (Attempted)

O-Benzylhydroxylamine hydrochloride (25 mg, 157 µmol) was suspended in dry DMF (1 mL) under argon and NaHCO$_3$ (13 mg, 155 µmol) was added and the suspension was stirred at r.t for 1 h. Carboxylic acid 139 (84 mg, 173 µmol) was dissolved in dry DMF (1 mL) under argon and cooled to 0 °C. EDC (33 mg, 172 µmol) was added and the solution was stirred at 0 °C for 1 h. The hydroxylamine solution was added followed by 4-dimethylaminopyridine (21 mg, 172 µmol) and the reaction was slowly warmed to r.t. and stirred for 22 h. After this time, TLC analysis showed the consumption of carboxylic acid 139 (CH$_2$Cl$_2$-MeOH, 98:2, $R_f$ = 0.1) and the formation of a product (CH$_2$Cl$_2$-MeOH, 98:2, $R_f$ = 0.3). The reaction mixture was concentrated and coevaporated with toluene (50 mL). Purification by flash column chromatography (CH$_2$Cl$_2$-MeOH, 98:2→95:5) did not afford the desired product 150.

$3'$-O-($tert$-Butyldimethylsilyl$)-5'$-deoxy-6'$-methoxy-6'$-oxo-thymidine (151)

Ester 151 was isolated as a colourless film (6 mg, 9%) as a byproduct in the synthesis of compound 150.
ν<sub>max</sub>/cm<sup>-1</sup> (CH<sub>2</sub>Cl<sub>2</sub>) 3193 (NH), 3035 (CH), 2953 (CH), 2929 (CH), 2856 (CH), 1686 (C=O), 1471 (CH), 1438, 1363, 1274, 1253, 1197, 1100 (C-O), 1059, 833, 777.

<sup>1</sup>H NMR (600 MHz, acetone-d<sub>6</sub>): δ = 0.12 (s, 3 H, CH<sub>3</sub>TBDMS), 0.13 (s, 3 H, CH<sub>3</sub>TBDMS), 0.92 (s, 9 H, t-BuTBDMS), 1.83 (s, 3 H, CH<sub>3</sub>), 1.89-1.95 (m, 1 H, H5′a), 2.01-2.05 (m, 1 H, H5′b), 2.19 (dd, J = 3.8 Hz, J = 6.4 Hz, J = 13.5 Hz, 1 H, H2′a), 2.32-2.37 (m, 1 H, H2′b), 2.44-2.50 (H6′), 3.62 (s, 3 H, CO<sub>2</sub>CH<sub>3</sub>), 3.79-3.82 (m, 1 H, H4′), 4.34 (app. dt, J = 3.9 Hz, J = 6.5 Hz, 1 H, H3′), 6.23-6.25 (m, 1 H, H1′), 7.43 (s, 1 H, H6), 9.95-10.06 (m, 1 H, NH<sub>3</sub>) ppm.

<sup>13</sup>C NMR (150 MHz, acetone-d<sub>6</sub>): δ = -4.7 (CH<sub>3</sub>TBDMS), -4.5 (CH<sub>3</sub>TBDMS), 12.5 (CH<sub>3</sub>), 18.5 (qC, t-BuTBDMS), 26.1 (t-BuTBDMS), 29.3 (C5′), 30.9 (C6′), 40.4 (C2′), 51.7 (CO<sub>2</sub>CH<sub>3</sub>), 75.9 (C3′), 85.1 (C1′), 86.3 (C4′), 111.0 (C5), 136.7 (C6), 151.2 (C2), 164.2 (C4), 173.8 (CO<sub>2</sub>CH<sub>3</sub>) ppm.


**Alkylation of iodide 78 with O-benzyl hydroxamate malonate ester and optimisation of conditions**

![Diagram of alkylation reaction]

(Benzyloxy)amido methyl malonate (155)

Method A:

O-Benzylhydroxylamine hydrochloride (40 mg, 251 µmol) was suspended in dry DMF (1 mL) under argon and NaHCO<sub>3</sub> (21 mg, 250 µmol) was added and the suspension was stirred at r.t. for 1 h. Monomethyl potassium malonate (154) (43 mg, 275 µmol) was suspended in dry DMF (1.5 mL) under argon and cooled to 0 °C.
EDC (53 mg, 276 µmol) and HOAt (38 mg, 276 µmol) were added and the solution was stirred at 0 °C for 1 h. The hydroxylamine solution was added and the reaction was slowly warmed to r.t. and stirred for 23 h. After this time, TLC analysis showed the complete consumption of malonate 154 (CH₂Cl₂-MeOH, 19:1, Rᵣ = 0.5) and the formation of a product (petroleum ether-ΣOAc, 1:1, Rᵣ = 0.5). The reaction mixture was diluted with EtOAc (10 mL), washed with H₂O (10 mL), brine (10 mL), dried over MgSO₄, filtered and concentrated to a yellow oil. Purification by flash column chromatography (petroleum ether-ΣOAc, 3:1→1:1) afforded the desired product 155 as a colourless solid (8 mg, 14%); mp 71-73 °C.

Method B:
O-Benzylhydroxylamine hydrochloride (160 mg, 1.00 mmol) was suspended in dry DMF (5 mL) under argon and NaHCO₃ (84 mg, 1.00 mmol) was added and the suspension was stirred at r.t. EDC (211 mg, 1.10 mmol) was suspended in dry DMF (5 mL) under argon and cooled to 0 °C. Monomethyl potassium malonate (154) (172 mg, 1.10 mmol) was added and the solution was stirred at 0 °C for 1 h. The hydroxylamine solution was added followed by 4-dimethylaminopyridine (12 mg, 0.10 mmol) and the reaction was slowly warmed to r.t. and stirred for 23 h. After this time, TLC analysis showed the complete consumption of malonate 154 (CH₂Cl₂-MeOH, 19:1, Rᵣ = 0.5) and the formation of a product (petroleum ether-ΣOAc, 1:1, Rᵣ = 0.5). The reaction mixture was diluted with EtOAc (50 mL) and washed with H₂O (50 mL) and brine (50 mL). The aq. layers were extracted with EtOAc (3 x 20 mL) and the combined organic extracts were dried over MgSO₄, filtered and concentrated to a colourless oil. Purification by flash column chromatography (petroleum ether-ΣOAc, 3:1→1:1) followed by recrystallisation from CH₂Cl₂ afforded the desired product 155 as colourless crystals (145 mg, 65%); mp (CH₂Cl₂) 71-73 °C.

νmax/cm⁻¹ (CH₂Cl₂) 3151 (NH), 2994 (CH), 2976 (CH), 2953 (CH), 2941 (CH), 2832 (CH), 1734 (C=O), 1672 (C=O), 1649 (C=O), 1531, 1496 (CH), 1454, 1432, 1415, 1367, 1345, 1196, 1177, 1170, 1061 (C-O), 1008, 967, 949, 909, 792, 727.

¹H NMR (600 MHz, CDCl₃): δ = 3.30 (s, 2 H, α-CH₂), 3.72 (s, 3 H, OCH₃), 4.93 (s, 2 H, OCH₂Ph), 7.37-7.39 (m, 5 H, Ph), 9.33 (bs, 1 H, NH) ppm.

¹³C NMR (150 MHz, CDCl₃): δ = 39.9 (α-C), 52.8 (OCH₃), 78.4 (OCH₂Ph), 128.8 (Ph), 129.0 (Ph), 129.3 (Ph), 135.2 (qC, Ph), 162.9 (CONH), 169.0 (CO₂) ppm.
HRMS (APCI\(^+\)): \(m/z\) calc. 224.0917 [M + H]\(^+\), found: 224.0914

\(C(N^3\text{-Benzyloxymethyl}-3'-O(\text{tert-butyldimethylsilyl})-5'\text{-deoxythymidin}-5'\text{-yl}) (\text{benzyloxy})\text{amido methyl malonate (152) (Attempted)}\)

Method A:
Oxyamide 155 (1.14 g, 5.11 mmol) was suspended in dry THF (3 mL) under argon and NaH (60% dispersion in mineral oil, 0.14 g, 3.40 mmol) was added. Protected iodide 137 (0.50 g, 0.85 mmol) was dissolved in dry THF (2 mL) under argon and was added dropwise to the enolate. The reaction was heated to reflux at 70 °C for 30 h. After this time, TLC analysis (petroleum ether-EtOAc, 1:1) showed the consumption of iodide 137 (\(R_f = 0.9\)) and the formation of a product (\(R_f = 0.7\)). The reaction was cooled to r.t., quenched by the addition of MeOH (1 mL) and concentrated. Purification by flash column chromatography (petroleum ether-EtOAc, 2:1→1:1) afforded starting materials 137 (348 mg, 70%) and malonate 155 (1.00 g, 88%), and byproduct 156 but not the desired product 152.

Method B:
Oxyamide 155 (114 mg, 511 µmol) was suspended in dry DME (0.5 mL) under argon and NaH (60% dispersion in mineral oil, 7 mg, 175 µmol) was added. Protected iodide 137 (50 mg, 85 µmol) was dissolved in dry DME (0.5 mL) under argon and was added dropwise to the enolate. The reaction was heated to reflux at 85 °C for 50 h. After this time, TLC analysis (petroleum ether-EtOAc, 2:1) showed the consumption of iodide 137 (\(R_f = 0.5\)) and the formation of a product (\(R_f = 0.1\)). The reaction was cooled to r.t., quenched by the addition of MeOH (1 mL) and concentrated to an orange oil. Purification by flash column chromatography (petroleum ether-EtOAc, 2:1→1:1) afforded starting materials 137 (36 mg, 72%) and malonate 155 (72 mg, 63%), and byproduct 156 but not the desired product 152.

Method C:
Oxyamide 155 (1.14 g, 5.11 mmol) was suspended in dry DME (3 mL) under argon and NaH (60% dispersion in mineral oil, 0.14 g, 3.40 mmol) was added. Protected iodide 137 (0.50 g, 0.85 mmol) was dissolved in dry DME (2 mL) under argon and
was added dropwise to the enolate. The reaction was heated to reflux at 85 °C for 50 h. After this time, TLC analysis (petroleum ether-EtOAc, 1:1) showed the consumption of iodide 137 (R<sub>f</sub> = 0.9) and the formation of a product (R<sub>f</sub> = 0.7). The reaction was cooled to r.t., quenched by the addition of MeOH (1 mL) and concentrated to an orange oil. Purification by flash column chromatography (petroleum ether-EtOAc, 2:1→1:1) afforded starting materials 137 (250 mg, 50%) and malonate 155 (580 mg, 51%), and byproduct 156 but not the desired product 152.

\[ N(N^2\text{-Benzyloxymethyl}-3'-O\text{-}(\text{tert-butyldimethyl}silyl)-5'\text{-deoxythymidin-5'\text{-yl})} \]

\[ \text{benzylcyanoamido methyl malonate (156)} \]

The N-alkylated product 156 was obtained from the attempted syntheses of compound 152 as a colourless solid (Method A: 24 mg, 4%; Method B: 16 mg, 28%; Method C: 262 mg, 45%); mp 166-170 °C dec.

\[ \nu_{\text{max}}/\text{cm}^{-1} \] (neat) 3033 (CH), 2953 (CH), 2930 (CH), 2887 (CH), 2857 (CH), 1744 (C=O), 1708 (C=O), 1656 (C=O), 1464 (CH), 1453 (CH), 1437, 1361, 1277, 1252, 1070 (C-O), 1028, 832, 774, 735, 697.

\[ ^1H \text{ NMR (600 MHz, CDCl}_3): \delta = 0.06-0.07 \text{ (m, 6 H, 2 x CH}_3^{\text{TBDMS}}), 0.89 \text{ (s, 9 H, t-Bu}^{\text{TBDMS}}), 1.84 \text{ (s, 3 H, CH}_3^{\text{T}}), 1.97-2.02 \text{ (m, 1 H, H}_2'^{a}), 2.25-2.28 \text{ (m, 1 H, H}_2'^{b}), 3.50 \text{ (s, 2 H, CO-CH}_2^{\text{-CO}}), 3.64 \text{ (dd, } J_{4',5'a} = 4.0 \text{ Hz, } J_{5a,5'b} = 14.6 \text{ Hz, 1 H, H}_5'^{a}), 3.68 \text{ (s, 3 H, OCH}_3), 4.05-4.09 \text{ (m, 1 H, H}_5'^{b}), 4.16-4.18 \text{ (m, 1 H, H}_4'), 4.26-4.27 \text{ (m, 1 H, H}_3'), 4.69 \text{ (s, 2 H, O-CH}_2^{\text{-Ph}^{\text{BOM}}}), 4.87-4.91 \text{ (m, 2 H, N-O-CH}_2^{\text{-Ph}), 5.46-5.50 \text{ (m, 2 H, N-CH}_2^{\text{-O})}, 6.29 \text{ (dd, } J = 5.5 \text{ Hz, } J = 7.7 \text{ Hz, 1 H, H}_1'), 7.23-7.39 \text{ (m, 11 H, Ph}^{\text{BOM}}, \text{Ph}^{\text{Bn}}, \text{H}_6 \text{ ppm).} \]

\[ ^{13}C \text{ NMR (150 MHz, CDCl}_3): \delta = -4.7 \text{ (CH}_3^{\text{TBDMS}}), -4.6 \text{ (CH}_3^{\text{TBDMS}}), 13.0 \text{ (CH}_3^{T}), 18.0 \text{ (C}^{\text{TBDMS}}, 25.8 \text{ (t-Bu}^{\text{TBDMS}}), 40.3 \text{ (C}_2^{2}, \text{CO-CH}_2^{\text{-CO}}, 47.6 \text{ (C}_5^{5}), 52.5 \text{ (OCH}_3), 70.7 \text{ (N-CH}_2^{\text{-O}), 72.3 \text{ (O-CH}_2^{\text{-Ph}^{\text{BOM}}}), 73.2 \text{ (C}_3^{3}), 77.3 \text{ (O-CH}_2^{\text{-Ph}^{\text{Bn}})}, 83.8 \text{ (C}_4^{4}), 86.2 \text{ (C}_1^{1}), 110.5 \text{ (C}_5), 127.7 \text{ (Ph), 127.8 \text{ (Ph), 129.0 \text{ (Ph), 129.49 \text{ (Ph), 129.54 \text{ (Ph), 133.8 \text{ (qC, Ph), 134.6 \text{ (C}_6), 138.1 \text{ (qC, Ph), 151.1 \text{ (C}_2), 163.6 \text{ (C}_4), 167.7 \text{ (COCH}_3), 168.5 \text{ (CONOBn) ppm.}}} \]

174
HRMS (APCI\(^+\)): \(m/z\) calc. 682.3154 [M + H]\(^+\), found: 682.3151

**Attempted deprotection of protected hydroxamic acid 156**

Protected nucleoside 156 (100 mg, 147 µmol) was dissolved THF (1.5 mL), TBAF (69 mg, 219 µmol) was added and the reaction was stirred at r.t. for 80 min. After this time, TLC analysis (petroleum ether-EtOAc, 1:1) showed the complete consumption of starting material (R\(_f\) = 0.5) and the formation of the product (R\(_f\) = 0.1). The reaction was concentrated to a yellow oil. Purification by flash column chromatography (petroleum ether-EtOAc, 3:2→9:1) afforded a white film. The product was taken up in MeCN (10 mL) and extracted with hexane (3 x 10 mL). After concentration of the MeCN layer the desired product 157 was obtained as a colourless oil (42 mg, 51%).

\(\nu_{\text{max}}/\text{cm}^{-1}\) (CH\(_2\)Cl\(_2\)) 3452 (OH), 3063 (CH), 3033 (CH), 2953 (CH), 1742 (C=O), 1706 (C=O), 1647 (C=O), 1466 (CH), 1453 (CH), 1437, 1362, 1329, 1269, 1212, 1087 (C-O), 1073 (C-O), 1027, 967, 774, 732, 698.

\(^1\text{H NMR (600 MHz, acetone-d6):}\) \(\delta = 1.80\) (s, 3 H, CH\(_3\)), 2.22 (ddd, \(J_{2'a,3'} = 5.9\) Hz, \(J_{1',2'a} = 7.7\) Hz, \(J_{2'a,2'b} = 13.6\) Hz, 1 H, H2'a), 2.29 (ddd, \(J_{2b,3'} = 3.0\) Hz, \(J_{1',2'a} = 6.0\) Hz, ...
$J_{2'a,2'b} = 13.6$ Hz, 1 H, H2'b), 3.52 (d, $J_{H_a,H_b} = 15.9$ Hz, 1 H, CO-CH$_3$H$_b$-CO), 3.56 (d, $J_{H_a,H_b} = 15.9$ Hz, 1 H, CO-CH$_3$H$_b$-CO), 3.61 (s, 3 H, CO$_2$CH$_3$), 3.95 (dd, $J_{4',5'a} = 4.6$ Hz, $J_{5'a,5'b} = 14.8$ Hz, 1 H, H5'a), 4.10 (dd, $J_{4',5'b} = 7.8$ Hz, $J_{5'a,5'b} = 14.8$ Hz, 1 H, OH), 4.24-4.26 (m, 1 H, H4'), 4.43-4.46 (m, 1 H, H3'), 4.47-4.48 (m, 1 H, OH), 4.65 (s, 2 H, O-CH$_2$-PhBOM), 5.01 (d, $J_{H_a,H_b} = 10.0$ Hz, 1 H, N-O-CH$_2$H$_b$-Ph), 5.07 (d, $J_{H_a,H_b} = 10.0$ Hz, 1 H, N-O-CH$_2$H$_b$-Ph), 5.43-5.46 (m, 2 H, N-CH$_2$O), 6.33 (dd, $J_{1',2'a} = 6.0$ Hz, $J_{1',2'a} = 7.7$ Hz, 1 H, H1'), 7.23-7.25 (m, 1 H, PhBOM), 7.29-7.34 (m, 4 H, PhBOM), 7.39-7.41 (m, 3 H, PhBn), 7.47-7.49 (m, 2 H, PhBOM), 7.55 (s, 1 H, H6) ppm.

$^{13}$C NMR (150 MHz, acetone-d$_6$): $\delta = 12.9$ (CH$_3$), 39.8 (C2'), 40.9 (CO-CH$_2$-CO), 48.3 (C5'), 52.2 (CO$_2$CH$_3$), 71.2 (N-CH$_2$-O), 72.4 (O-CH$_2$-PhBOM), 73.0 (C3'), 77.1 (O-CH$_2$-PhBn), 84.3 (C4'), 86.6 (C1'), 110.3 (C5), 128.17 (PhBOM), 128.19 (PhBOM), 129.0 (PhBOM), 129.4 (PhBn), 129.7 (PhBn), 130.4 (PhBn), 135.5 (qC, PhBn), 136.0 (C6), 139.6 (qC, PhBOM), 151.8 (C2), 163.8 (C4), 168.5 (COCH$_3$), 168.9 (CONOBn) ppm.

HRMS (ESI$^+$): $m/z$ calc. 590.2109 [M + Na]$^+$, found: 590.2111

$N$-($5'$-deoxythymidin-$5'$-yl) methyl $N$-hydroxymalonamide (158) (Attempted)

Protected hydroxamic acid 157 (58 mg, 102 µmol) was dissolved in MeOH (2 mL) and added to a dried flask containing Pd/C (10%, 9 mg) and H$_2$ was bubbled through the suspension while stirring at r.t. for 8 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed the complete consumption of starting material ($R_f = 0.6$) and the formation of a product ($R_f = 0.5$). The reaction mixture was filtered through celite and the filtrate was concentrated to a brown film. NMR analysis showed the presence of the BOM-group. The crude material was dissolved in MeOH (2 mL), the solution was purged with argon, and Pd(OH)$_2$/C (9 mg, unreduced, 20% Pd, 60% moisture) was added. H$_2$ was bubbled through the suspension while stirring at r.t. for 4 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 4:1) showed the consumption of the intermediate ($R_f = 0.9$) and the formation of various products ($R_f = 0.2$, $R_f = 0.5$, $R_f = 0.9$). The reaction mixture was filtered through celite and the
filtrate was concentrated to a brown solid. Purification by flash column chromatography (toluene-acetone 3:2 → acetone) was unsuccessful and the desired product 158 was not isolated.

*N-(3'-O-(tert-butyldimethylsilyl)-5'-deoxythymidin-5'-yl) methyl N-hydroxy malonamide* (159) (Attempted)

Protected hydroxamic acid 156 (100 mg, 147 µmol) was dissolved in MeOH (3 mL) and added to a dried flask containing Pd/C (10%, 20 mg) and H₂ was bubbled through the suspension while stirring at r.t. for 30 h. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material (R_f = 0.5) and the formation of several products (R_f = 0.1, R_f = 0.3, R_f = 0.7, R_f = 0.8, R_f = 0.9). The reaction mixture was filtered through celite and the filtrate was concentrated to a brown film. The desired product 159 was not isolated.

**Synthesis of bis-hydroxamate ester** 161

Sodium 3-((benzyloxy)amino)-3-oxopropanoate (160)

KOH (0.75 g, 13.4 mmol) was dissolved in MeOH (20 mL) and cooled to 0 °C. Ester 155 (1.00 g, 4.48 mmol) was added and the reaction was stirred at 0 °C for 6 h. After this time, TLC analysis (petroleum ether-EtOAc, 1:1) showed the consumption of starting material (R_f = 0.3) and the formation of a product (R_f = 0.0). The reaction mixture was concentrated to a white solid, the residue was taken up in H₂O (100 mL) and extracted with CH₂Cl₂ (50 mL). The organic layer was discarded and the aq. layer was acidified to pH 2 using HCl and extracted with EtOAc (3 x 100 mL). The EtOAc extracts were concentrated and the residue was taken up in MeOH, eluted
through Diaion resin WT01S(H) (Na form) and concentrated to afford the desired product 160 as a white foam (1.02 g, 98%).

\( \nu_{\text{max}}/\text{cm}^{-1} \) (neat) 3162 (NH), 2940 (CH), 1655 (C=O), 1591 (C=O), 1378 (C=O), 1054 (C-O), 982, 912, 838, 738, 694.

\(^1\)H NMR (600 MHz, DMSO-\(d_6\)): \( \delta = 2.72 \) (s, 2 H, \( \alpha-\text{CH}_2 \)), 4.77 (s, 2 H, \( \text{OCH}_2\text{Ph} \)), 7.31-7.39 (m, 5 H, Ph) ppm.

\(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)): \( \delta = 43.6 \) (\( \alpha-\text{C} \)), 76.9 (\( \text{OCH}_2\text{Ph} \)), 128.5 (Ph), 128.7 (Ph), 129.0 (Ph), 137.1 (qC, Ph), 167.3 (CONH), 170.2 (CO\(_2\)) ppm.

HRMS (ESI\(^+\)): \( m/z \) calc. 254.0400 [M + Na]\(^+\), found: 254.0409

\( N'\),\( N''\)-bis(benzyloxy)malonamide (161)

O-Benzylhydroxylamine hydrochloride (25 mg, 157 \( \mu \)mol) was suspended in dry DMF (0.5 mL) under argon and NaHCO\(_3\) (13 mg, 155 \( \mu \)mol) was added and the suspension was stirred at r.t. for 1 h. EDC (30 mg, 156 \( \mu \)mol) was suspended in dry DMF (1 mL) under argon and cooled to 0 °C. Carboxylate salt 160 (36 mg, 156 \( \mu \)mol) was added to the EDC suspension and the resulting solution was stirred at 0 °C for 1 h. The hydroxylamine suspension was added to the activated ester followed by 4-dimethylaminopyridine (2 mg, 16 \( \mu \)mol), and the reaction was slowly warmed to r.t. and stirred for 17 h. After this time, TLC analysis (petroleum ether-EtOAc, 1:1) showed the consumption of \( O\)-benzylhydroxylamine (\( R_f = 0.4 \)) and the formation of a product (\( R_f = 0.5 \)). The reaction mixture was diluted with EtOAc (10 mL) and washed with H\(_2\)O (10 mL) and brine (10 mL). The combined organic extracts were dried over MgSO\(_4\), filtered and concentrated to a colourless oil. Purification by flash column chromatography (\( \text{CH}_2\text{Cl}_2\)-MeOH, 49:1→4:1) afforded the desired product 161 as a colourless solid (24 mg, 49%); mp 122-124 °C.

\( \nu_{\text{max}}/\text{cm}^{-1} \) (neat) 3197 (NH), 3062 (CH), 3010 (CH), 2869 (CH), 1646 (C=O), 1519 (C=O), 1498, 1453 (CH), 1361, 1232, 1163, 1070 (C-O), 1027, 970, 908, 836, 751, 731, 695.

\(^1\)H NMR (600 MHz, DMSO-\(d_6\)): \( \delta = 2.81 \) (s, 2 H, \( \alpha-\text{CH}_2 \)), 4.80 (s, 4 H, \( \text{OCH}_2\text{Ph} \)), 7.35-7.41 (m, 10 H, Ph), 11.17 (s, 2 H, NH) ppm.

\(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)): \( \delta = 38.2 \) (\( \alpha-\text{C} \)), 76.9 (\( \text{OCH}_2\text{Ph} \)), 128.29 (Ph), 128.33 (Ph), 128.8 (Ph), 129.2 (Ph), 135.9 (qC, Ph), 163.3 (CONH) ppm.
HRMS (ESI\(^+\)): \(m/z\) calc. 337.1159 [M + Na]\(^+\), found: 337.1164

Alkylation of iodide 137 with bis-hydroxamate ester 161 and attempted deprotection

![Reaction Scheme](image)

\(C-(N^2\)-Benzyloxymethyl-3'-O-(tert-butyltrimethylsilyl)-5'-deoxythymidin-5'-yl)\n\(N',N^2\)-bis(benzyloxy)malonamide (153) (Attempted)

Malonate oxyamide 161 (444 mg, 1.41 mmol) was suspended in dry dimethoxyethane (1 mL) under argon and NaH (60% dispersion in mineral oil, 38 mg, 0.95 mmol) was added. Protected iodide 137 (138 mg, 0.24 mmol) was dissolved in dry DME (0.5 mL) under argon and was added dropwise to the enolate. The reaction was heated to reflux at 90 °C for 72 h. After this time, TLC analysis (hexane-EtOAc, 1:1) showed the consumption of iodide 137 (\(R_f = 0.7\)) and the formation of a product (\(R_f = 0.2\)). The reaction was cooled to r.t., quenched by the addition of MeOH (1 mL) and concentrated to an orange oil. Purification by flash column chromatography (hexane-EtOAc, 2:1→1:2, then \(\text{CH}_2\text{Cl}_2\)-MeOH, 99:1→97:3, then toluene-acetone, 4:1) followed by coevaporation with MeOH and \(\text{CH}_2\text{Cl}_2\) did not afford the desired product 153 but the \(N\)-alkylated byproduct 162.
\[ N'-(N^2\text{-Benzyloxymethyl}-3'-O-(\text{tert-butyldimethylsilyl})-5'-deoxythymidin-5'-yl) \]

\[ N',N'\text{-bis(benzyloxy)malonamide (162)} \]

The \(N\)-alkylated product 162 was obtained from the attempted synthesis of compound 153 as a yellow crystalline solid (57 mg, 31%); mp 46-50 °C (CH\(_2\)Cl\(_2\)).

\( \nu_{\text{max}}/\text{cm}^{-1} \) (CH\(_2\)Cl\(_2\)) 3244 (NH), 3065 (CH), 3033 (CH), 2952 (CH), 2929 (CH), 2856 (CH), 1706 (C=O), 1654 (C=O), 1465 (CH), 1454, 1279, 1252, 1071 (C-O), 834, 775, 739, 698.

\(^1\text{H NMR (600 MHz, acetone-\text{d}_6)}: \delta = 0.12-0.13 (2 \times s, 6 \text{ H}, 2 \times \text{CH}_3^{\text{TBDMS}}), 0.92 (s, 9 \text{ H}, t\text{-Bu}^{\text{TBDMS}}), 1.80 (s, 3 \text{ H}, \text{CH}_3^T), 2.21-2.23 (m, 1 \text{ H}, \text{H2'a}), 2.28-2.31 (m, 1 \text{ H}, \text{H2'b}), 3.43 (bs, 2 \text{ H}, \text{CO-CH}_2-\text{CO}), 3.87 (dd, J_{4';5'a} = 4.1 \text{ Hz}, J_{5'a,5'b} = 14.6 \text{ Hz}, 1 \text{ H}, \text{H5'a}), 4.13-4.17 (m, 1 \text{ H}, \text{H5'b}), 4.19-4.21 (m, 1 \text{ H}, \text{H4'}), 4.55-4.56 (m, 1 \text{ H}, \text{H3'}), 4.65 (s, 2 \text{ H}, \text{O-CH}_2-\text{Ph}^{\text{BOM}}), 4.86 (s, 2 \text{ H}, \text{HN-O-CH}_2-\text{Ph}), 5.05-5.09 (m, 2 \text{ H}, \text{N-O-CH}_2-\text{Ph}), 5.41-5.44 (m, 2 \text{ H}, \text{N-CH}_2-\text{O}), 6.32-6.34 (m, 1 \text{ H}, \text{H1'}), 7.23-7.25 (m, 1 \text{ H}, \text{Ph}^{\text{BOM}}), 7.29-7.37 (m, 7 \text{ H}, \text{Ph}^{\text{BOM}}, \text{Ph}^{\text{Bn}}), 7.39-7.42 (m, 5 \text{ H}, \text{Ph}^{\text{Bn}}), 7.48-7.49 (m, 2 \text{ H}, \text{Ph}^{\text{BOM}}), 7.55 (s, 1 \text{ H}, \text{H6}), 10.19 (bs, 1 \text{ H}, \text{CNHO}) \text{ ppm.} \]

\(^{13}\text{C NMR (150 MHz, acetone-\text{d}_6)}: \delta = -4.60 (\text{CH}_3^{\text{TBDMS}}), -4.57 (\text{CH}_3^{\text{TBDMS}}), 13.0 (\text{CH}_3^T), 18.5 (\text{qCH}_2^{\text{TBDMS}}), 26.2 (t\text{-Bu}^{\text{TBDMS}}), 40.2 (\text{C2'}), 39.2 (\text{CO-CH}_2-\text{CO}), 48.2 (\text{C5'}), 71.2 (\text{N-CH}_2-\text{O}), 72.4 (\text{O-CH}_2-\text{Ph}^{\text{BOM}}), 74.3 (\text{C3'}), 77.4 (\text{N-O-CH}_2-\text{Ph}^{\text{Bn}}), 78.4 (\text{HN-O-CH}_2-\text{Ph}^{\text{Bn}}), 84.9 (\text{C4'}), 86.7 (\text{C1'}), 110.5 (\text{C5}), 128.18 (\text{Ph}^{\text{BOM}}), 128.20 (\text{Ph}^{\text{BOM}}), 129.0 (\text{Ph}^{\text{BOM}}), 129.2 (\text{Ph}^{\text{Bn}}), 129.5 (\text{Ph}^{\text{Bn}}), 129.8 (\text{Ph}^{\text{Bn}}), 129.9 (\text{Ph}^{\text{Bn}}), 130.3 (\text{Ph}^{\text{Bn}}), 130.4 (\text{Ph}^{\text{Bn}}), 135.6 (\text{qC}, \text{Ph}^{\text{Bn}}), 136.1 (\text{C6}), 137.1 (\text{qC}, \text{Ph}^{\text{Bn}}), 139.6 (\text{qC}, \text{Ph}^{\text{BOM}}), 151.9 (\text{C2}), 163.8 (\text{C4}), 164.5 (\text{CONHOBn}), 169.6 (\text{CONOBn}) \text{ ppm.} \]

HRMS (ESI\(^+\)): \(m/z\) calc. 795.3396 [M + Na]\(^+\), found: 795.3396
*N*-(*N*-Benzyloxymethyl-5'-deoxythymidin-5'-yl) *N*,*N*-bis(benzyloxy) malonamid (163)

Nucleoside 162 (50 mg, 65 µmol) was dissolved THF (1 mL), TBAF (31 mg, 98 µmol) was added and the reaction was stirred at r.t. for 100 min. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material (Rf = 0.9) and the formation of the product (Rf = 0.4). The reaction was concentrated to a yellow oil. Purification by flash column chromatography (EtOAc) afforded a white amorphous solid. The product was taken up in MeCN (20 mL) and extracted with hexane (3 x 10 mL). After concentration of the MeCN layer the desired product 163 was obtained as a colourless film (21 mg, 49%).

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3430 (OH, NH), 3247 (OH, NH), 3064 (CH), 3032 (CH), 2931 (CH), 2879 (CH), 1703 (C=O), 1639 (C=O), 1466 (CH), 1453 (CH), 1361, 1274, 1071 (C-O), 1028, 967, 733, 697.

$^1$H NMR (600 MHz, acetone-d$_6$): $\delta$ = 1.82 (s, 3 H, CH$_3$T), 2.25-2.28 (m, 2 H, H2'a, H2'b), 3.38-3.44 (m, 2 H, CO-CH$_2$-CO), 3.95 (dd, J = 4.8 Hz, $J_{5'a,5'b}$ = 14.8 Hz, 1 H, H5'a), 4.10 (dd, J = 7.3 Hz, $J_{5'a,5'b}$ = 14.8 Hz, 1 H, H5'b), 4.22-4.24 (m, 1 H, H4'), 4.47 (bs, 1 H, H3'), 4.57 (bs, 1 H, OH), 4.65 (s, 2 H, O-CH$_2$-Ph$_\text{BOM}$), 4.84 (s, 2 H, HN-O-CH$_2$-Ph), 5.03-5.08 (m, 2 H, N-O-CH$_2$-Ph), 5.43 (s, 2 H, N-CH$_2$-O), 6.34 (app. t, J = 6.7 Hz, 1 H, H1'), 7.23-7.25 (m, 1 H, Ph$_\text{BOM}$), 7.29-7.41 (m, 12 H, Ph$_\text{BOM}$, Ph$_\text{Bn}$), 7.47-7.50 (m, 2 H, Ph$_\text{BOM}$), 7.55 (s, 1 H, H6), 10.27 (bs, 1 H, CNHO) ppm.

$^{13}$C NMR (150 MHz, acetone-d$_6$): $\delta$ = 13.0 (CH$_3$T), 39.4 (CO-CH$_2$-CO), 39.7 (C2'), 48.2 (C5'), 71.2 (N-CH$_2$-O), 72.4 (O-CH$_2$-Ph$_\text{BOM}$), 73.0 (C3'), 77.2 (N-O-CH$_2$-Ph$_\text{Bn}$), 78.4 (HN-O-CH$_2$-Ph$_\text{Bn}$), 84.3 (C4'), 86.4 (C1'), 110.5 (C5), 128.18 (Ph$_\text{BOM}$), 128.20 (Ph$_\text{BOM}$), 129.0 (Ph$_\text{BOM}$), 129.2 (Ph$_\text{Bn}$), 129.4 (Ph$_\text{Bn}$), 129.7 (Ph$_\text{Bn}$), 129.9 (Ph$_\text{Bn}$), 130.4 (Ph$_\text{Bn}$), 135.6 (qC, Ph$_\text{Bn}$), 136.1 (C6), 137.0 (qC, Ph$_\text{Bn}$), 139.6 (qC, Ph$_\text{BOM}$), 151.9 (C2), 163.9 (C4), 164.7 (CONHOBn), 169.7 (CONOBn) ppm.

HRMS (APCI$^+$): m/z calc. 659.2712 [M + H]$^+$, found: 659.2727
N-(5'-deoxythymidin-5'-yl) N',N³-dihydroxymalonamide (164) (Attempted)

Protected hydroxamic acid 163 (19 mg, 29 µmol) was dissolved in MeOH (2 mL) and added to a dried flask containing Pd/C (10%, 4 mg) and H₂ was bubbled through the suspension while stirring at r.t. for 8 h. After this time, TLC analysis (EtOAc-MeOH, 9:1) showed the complete consumption of starting material (R_f = 0.7) and the formation of two products (R_f = 0.1, R_f = 0.2). The reaction mixture was filtered through celite and the filtrate was concentrated to a brown solid. The desired product 164 was not obtained.

Synthesis of hydroxamic acid 105 via aminolysis of ester 142

C-(5'-deoxythymidin-5'-yl) sodium N-hydroxymalonamide (105)

Hydroxylamine hydrochloride (426 mg, 6.13 mmol) was suspended in MeOH (6 mL) and KOH (430 mg, 7.66 mmol) was added. The suspension was warmed to 40 °C to aid dissolution and the precipitate was removed by filtration. Ester 142 (93 mg, 272 µmol) was dissolved in the filtrate and the reaction was stirred at r.t. for 5 h. After this time, TLC analysis showed the consumption of starting material (CH₂Cl₂-MeOH, 4:1, R_f = 0.8) and the formation of a product (H₂O-⁻PrOH-EtOAc, 1:2:2, R_f = 0.3). The pH was adjusted to pH 7 using 1 M aq. HCl to quench the reaction. The product was purified by flash column chromatography (CH₂Cl₂-MeOH, 4:1→MeOH followed by H₂O-⁻PrOH-EtOAc, 1:5:4→1:2:2), followed by elution
Chapter 6 – Experimental procedures

through Diaion resin WT01S(H) (Na form). The resulting white solid was purified by preparative reversed phase TLC (H₂O-MeCN, 1:1), extracted with MeOH and concentrated to afford the desired product 105 as a white solid (15 mg, 15%).

Compound 105 was isolated as a 1:1 mixture of interconverting diastereomers.

\[ \nu_{\text{max}}/\text{cm}^{-1} \text{ (neat)}: 3424 \text{ (OH, NH)}, 3213 \text{ (CH)}, 3045 \text{ (CH)}, 2936 \text{ (CH)}, 2818 \text{ (CH)}, 1655 \text{ (C=O)}, 1604 \text{ (C=O)}, 1579 \text{ (C=O)}, 1515, 1474 \text{ (CH)}, 1447, 1369 \text{ (C=O)}, 1272 \text{ (CO)}, 1126 \text{ (C-O)}, 1083 \text{ (C-O)}, 958, 770. \]

\[ ^1H \text{ NMR} (600 \text{ MHz, D}_2\text{O}): \delta = 1.92 \text{ (s, 3 H, CH}_3\text{T)}, 1.93 \text{ (s, 3 H, CH}_3\text{T)}, 2.08-2.15 \text{ (m, 2 H, H5'a)}, 2.27-2.33 \text{ (m, 2 H, H5'b)}, 2.34-2.42 \text{ (m, 4 H, H2'a, H2'b)}, 3.19 \text{ (app. t, J = 7.2 Hz, 1 H, H6')}, 3.23 \text{ (dd, J = 4.6 Hz, J = 10.4 Hz, 1 H, H6')}, 3.82 \text{ (app. dt, J = 4.0 Hz, J = 9.6 Hz, 1 H, H4')}, 4.31-4.34 \text{ (m, 2 H, H3')}, 6.22-6.27 \text{ (m, 2 H, H1')}, 7.48 \text{ (s, 1 H, H6)}, 7.50 \text{ (s, 1 H, H6)} \text{ ppm.} \]

\[ ^{13}C \text{ NMR} (150 \text{ MHz, D}_2\text{O}): \delta = 11.48 \text{ (CH}_3\text{T)}, 11.52 \text{ (CH}_3\text{T)}, 32.7 \text{ (C5')}, 32.9 \text{ (C5')}, 37.7 \text{ (C2')}, 37.8 \text{ (C2')}, 49.3 \text{ (C6')}, 50.0 \text{ (C6')}, 73.5 \text{ (C3')}, 73.7 \text{ (C3')}, 83.9 \text{ (C4')}, 84.8 \text{ (C1')}, 84.9 \text{ (C1')}, 85.1 \text{ (C4')}, 111.5 \text{ (C5)}, 111.7 \text{ (C5)}, 137.3 \text{ (C6)}, 137.4 \text{ (C6)}, 151.69 \text{ (C2)}, 151.71 \text{ (C2)}, 166.5 \text{ (C4)}, 166.6 \text{ (C4)}, 170.0 \text{ (CONHO)}, 170.4 \text{ (CONHO)}, 175.5 \text{ (CO}_2\text{Na)}, 175.8 \text{ (CO}_2\text{Na)} \text{ ppm.} \]

HRMS (ESI⁺): \( m/z \) calc. 388.0727 [M + Na]⁺, found: 388.0731

Attempted synthesis of bis-hydroxamic acid 167 via aminolysis of ester 135

\[ C-(R^3-\text{Benzyloxymethyl-3'}-O-(\text{tert-butylimethylsilyl})-5'-\text{deoxythymidin-5'-yl}) \]

\[ N',N^2-\text{dihydroxymalonamide (167) (Attempted)} \]

Hydroxylamine hydrochloride (208 mg, 3.00 mmol) was suspended in MeOH (3 mL) and KOH (210 mg, 3.75 mmol) was added. The suspension was warmed to 40 °C
to aid dissolution and the precipitate was removed by filtration. Diester 135 (100 mg, 150 µmol) was dissolved in the filtrate and the reaction was stirred at r.t. for 4.5 h. After this time, TLC analysis showed the complete consumption of starting material (CH2Cl2-EtOAc, 9:1, Rf = 0.7) and the formation of a product (CH2Cl2-MeOH, 4:1, Rf = 0.3). The reaction was poured into H2O (10 mL) and the pH was adjusted to pH 6 using 1 M aq. HCl. The resulting suspension was extracted with EtOAc (3 x 10 mL) and the organic layer was washed with brine (10 mL), dried over MgSO4 and concentrated. Purification by flash column chromatography (CH2Cl2-MeOH, 4:1) did not afford the desired product 167.

C-(N2-Benzoyloxymethyl-3'-O-(tert-butyldimethylsilyl)-5'-deoxythymidin-5'-yl) hydroxyisoxazolone (168)

Compound 168 was obtained from the attempted synthesis of compound 167 as a white solid (22 mg, 26%). The compound decomposed in acetone-d6; it could therefore not be determined which tautomer of the heterocycle was present.

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3217 (OH, NH), 2956 (CH), 2928 (CH), 2857 (CH), 1707 (C=O), 1645 (C=O), 1586 (C=O), 1469 (CH), 1280, 1253, 1072 (C-O), 1029, 837, 777.

$^1$H NMR (600 MHz, CD$_3$OD): $\delta = 0.07$-0.08 (2 x s, 6 H, 2 x CH$_3$TBDMS), 0.90 (s, 9 H, t-BuTBDMS), 1.92 (s, 3 H, CH$_3$T), 2.06-2.11 (m, 1 H, H2'a), 2.15 (dd, $J_{1',2'b} = 5.2$ Hz, $J = 13.2$ Hz, 1 H, H2'b), 2.24 (dd, $J_{4',5'a} = 9.5$ Hz, $J_{5'a,5'b} = 14.1$ Hz, 1 H, H5'a), 2.42 (dd, $J_{4',5'b} = 4.7$ Hz, $J_{5'a,5'b} = 14.1$ Hz, 1 H, H5'b), 4.08 (dd, $J_{4',5'b} = 4.7$ Hz, $J_{4',5'a} = 9.5$ Hz, 1 H, H4'), 4.47 (app. d, $J = 4.1$ Hz, 1 H, H3'), 4.66 (s, 2 H, O-CH$_2$-PhBOM), 5.49 (s, 2 H, N-CH$_2$-O), 6.24 (dd, $J_{1',2'b} = 5.2$ Hz, $J = 9.0$ Hz, 1 H, H1'), 7.22-7.25 (m, 1 H, PhBOM), 7.28-7.32 (m, 4 H, PhBOM), 7.56 (s, 1 H, H6) ppm.

$^{13}$C NMR (150 MHz, CD$_3$OD): $\delta = -4.8$ (CH$_3$TBDMS), -4.6 (CH$_3$TBDMS), 13.1 (CH$_3$T), 18.8 (qC$_{\text{TBDMS}}$), 26.28 (t-BuTBDMS), 26.31 (t-BuTBDMS), 26.6 (C5'), 40.5 (C2'), 71.9 (N-CH$_2$-O), 73.2 (O-CH$_2$-PhBOM), 74.3 (C6'), 76.2 (C3'), 87.6 (C1'), 89.4 (C4'), 111.0 (C5), 128.6 (PhBOM), 128.7 (PhBOM), 129.3 (PhBOM), 136.5 (C6), 139.6 (qC, PhBOM), 152.6 (C2), 165.4 (C4), 164.7, 169.7 ppm.

HRMS (ESI): m/z calc. 558.2277 [M - H], found: 558.2278
Synthesis of 5’-malonate nucleosides using an amide linkage

Aminothymidine 80 (500 mg, 2.07 mmol) was dissolved in dry DMF (15 mL), under argon. EDC (437 mg, 2.28 mmol) was suspended in dry DMF (20 mL) under argon and cooled to 0 °C. Monomethyl potassium malonate (356 mg, 2.28 mmol) was added and the suspension was stirred at 0 °C for 1 h. After this time, the aminothymidine solution was added followed by 4-dimethylaminopyridine (25 mg, 0.21 mmol). The reaction was slowly warmed to r.t. and stirred for 20 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 4:1) showed the consumption of amine starting material 80 (Rᵣ = 0.0) and the formation of the product (Rᵣ = 0.7). The reaction mixture was concentrated and purification by flash column chromatography afforded the desired product 107 as a white solid (385 mg, 54%); mp 68-72 °C dec.

νmax/cm⁻¹ (neat) 3432 (OH, NH), 3290 (CH), 3098 (CH), 2955 (CH), 1755, 1708, 1655 (C=O), 1637 (C=O), 1567, 1473 (CH), 1402, 1365, 1259, 1218, 1198, 1153, 1100 (C-O), 1060, 1042, 1014, 955, 902, 852, 782.
1H NMR (600 MHz, DMSO-d6): δ = 1.79 (d, J = 1.1 Hz, 3 H, CH3T), 2.04 (ddd, J = 3.3 Hz, J1;2a = 6.3 Hz, J2a,2b = 13.5 Hz, 1 H, H2’a), 2.13 (ddd, J = 6.5 Hz, J1;2b = 7.9 Hz, J2a,2b = 13.5 Hz, 1 H, H2’b), 3.24-3.30 (m, 3 H, CH2), 3.40 (dd, J = 6.5 Hz, J1’,2’b = 7.9 Hz, J1’,2’a = 6.3 Hz, J2’a,2’b = 13.5 Hz, 1 H, H4’), 4.13-4.16 (m, 1 H, H3’), 5.30 (d, J = 4.0 Hz, 1 H, OH), 6.14 (dd, J1’,2’a = 6.3 Hz, J1’,2’b = 7.9 Hz, 1 H, H1’), 7.46 (app. d, J = 1.1 Hz, 1 H, H6), 8.28 (app. t, J = 5.8 Hz, CONH), 11.29 (bs, 1 H, NHT) ppm.

13C NMR (150 MHz, DMSO-d6): δ = 12.0 (CH3T), 38.4 (C2’), 41.0 (C5’), 42.2 (α-CH2), 51.8 (OCH3), 71.1 (C3’), 83.7 (C1’), 84.8 (C4’), 109.8 (C5), 136.2 (C6), 150.5 (C2), 163.7 (C4), 165.5 (CONH), 168.4 (COONa) ppm.

HRMS (APCI): m/z calc. 342.1296 [M + H]+, found: 342.1299

**N-(5’-Deoxythymidin-5’-yl) amido sodium malonate (108)**

KOH (164 mg, 2.92 mmol) was dissolved in MeOH (6 mL). Ester 107 (100 mg, 293 µmol) was added and the reaction was stirred for 2 h. After this time, TLC analysis (CH2Cl2-MeOH, 4:1) showed the complete consumption of starting material (Rf = 0.8) and the formation of a product (Rf = 0.0). The reaction mixture was eluted through Diaion resin WT01S(H) (H form) followed by elution through Diaion resin WT01S(H) (Na form) and concentrated to afford the desired product 108 as a white foam (100 mg, 98%).

νmax/cm⁻¹ (neat) 3281 (OH, NH), 2932 (CH), 1648 (C=O), 1595 (C=O), 1471 (CH), 1373 (C=O), 1316, 1270, 1087 (CH), 1049, 731.

1H NMR (400 MHz, D2O): δ = 1.89 (d, J = 1.1 Hz, 3 H, CH3T), 2.31-2.43 (m, 2 H, H2’a, H2’b), 3.21 (s, 2 H, α-CH2), 3.50-3.59 (m, 2 H, H5’a, H5’b), 4.02-4.06 (m, 1 H, H4’), 4.43 (app. dt, J = 4.3 Hz, J = 6.3 Hz, 1 H, H3’), 6.28 (app. t, J = 6.9 Hz, 1 H, H1’), 7.45 (app. d, J = 1.1 Hz, 1 H, H6) ppm.

13C NMR (100 MHz, D2O): δ = 12.0 (CH3T), 37.8 (C2’), 40.6 (C5’), 45.0 (α-CH2), 71.2 (C3’), 85.0 (C1’), 84.1 (C4’), 111.7 (C5), 137.0 (C6), 154.6 (C2), 170.3 (C4), 171.6 (CONH), 174.9 (COONa) ppm.
HRMS (ESI\(^+\)): m/z calc. 372.0778 [M + Na]\(^+\), found: 372.0791

**N-(5'-Deoxythymidin-5'-yl) amido \(N\)-hydroxymalonamide (109)**

![Structure of 109](image)

Hydroxylamine hydrochloride (407 mg, 5.86 mmol) was suspended in MeOH (6 mL) and KOH (411 mg, 7.33 mmol) was added. The suspension was warmed to 40 °C to aid dissolution and the precipitate was removed by filtration. Ester 107 (100 mg, 293 \(\mu\)mol) was dissolved in the filtrate and the reaction was stirred at r.t. for 4 h. After this time, TLC analysis showed the complete consumption of starting material (CH\(_2\)Cl\(_2\)-MeOH, 4:1, \(R_t = 0.8\)) and the formation of a product (H\(_2\)O-\(-i\)-PrOH-EtOAc, 1:5:4, \(R_t = 0.6\)). The pH was adjusted to pH 7 using 1 M aq. HCl to quench the reaction. Purification by flash column chromatography (H\(_2\)O-\(-i\)-PrOH-EtOAc, 1:10:9) afforded the desired product 109 as a white foam (83 mg, 83%).

\(\nu_{\text{max}}/\text{cm}^{-1}\) (neat) 3205 (OH, NH), 3068 (CH), 2934 (CH), 2826 (CH), 1638 (C=O), 1551, 1475 (CH), 1414, 1368, 1270, 1088 (C=O), 1053.

\(^1\)H NMR (600 MHz, DMSO-\(d_6\)): \(\delta = 1.80\) (d, \(J = 1.0\) Hz, 3 H, CH\(_3\)), \(2.02\) (ddd, \(J = 3.1\) Hz, \(J_{1':2'a} = 6.2\) Hz, \(J_{2'a,2'b} = 13.5\) Hz, 1 H, H2'a), \(2.15\) (ddd, \(J = 6.6\) Hz, \(J_{1':2'b} = 7.9\) Hz, \(J_{2'a,2'b} = 13.5\) Hz, 1 H, H2'b), \(2.93\) (d, \(J_{\alpha a,\alpha b} = 14.2\) Hz, 1 H, \(\alpha\)-CH\(_a\)), \(2.97\) (d, \(J_{\alpha a,\alpha b} = 14.2\) Hz, 1 H, \(\beta\)-CH\(_b\)), \(3.29-3.34\) (m, 2 H, H5'a, H5'b), \(3.72-3.74\) (m, 1 H, \(\beta\)-CH\(_a\)), \(4.14-4.17\) (m, 1 H, H3'), \(5.33\) (d, \(J = 4.3\) Hz, 1 H, 3'-OH), \(6.14\) (dd, \(J_{1':2'a} = 6.2\) Hz, \(J_{1':2'b} = 7.9\) Hz, 1 H, H1'), \(7.54\) (app. d, \(J = 4.0\) Hz, 1 H, H6), \(8.24\) (app. t, \(J = 5.9\) Hz, CONHC), \(8.91\) (s, 1 H, NOH), \(10.60\) (bs, 1 H, NHO), \(11.28\) (bs, 1 H, NH\(_T\)) ppm.

\(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)): \(\delta = 12.1\) (CH\(_3\)), \(38.4\) (C2'), \(40.7\) (\(\alpha\)-CH\(_2\)), \(40.9\) (C5'), \(71.0\) (C3'), \(83.5\) (C1'), \(84.8\) (C4'), \(109.8\) (C5), \(136.3\) (C6), \(150.5\) (C2), \(163.8\) (C4), \(163.9\) (COONa), \(166.7\) (CONH) ppm.

HRMS (APCI\(^+\)): m/z calc. 343.1248 [M + H]\(^+\), found: 343.1251
6.1.3.2 Synthesis of 3'-modified nucleosides

Attempted synthesis of 3'-malonyl thymidine 169

\[ \text{Thymidine (1) (10.0 g, 41.3 mmol) was dissolved in pyridine under argon.} \]
\[ 4,4'\text{-Dimethoxytrityl chloride (14.9 g, 44.0 mmol) was added in four portions over 1 h} \]
\[ \text{and the reaction mixture was stirred at r.t. for 20 h. After this time, TLC analysis} \]
\[ \text{(EtOAc) showed the consumption of starting material (R_f = 0.1) and the formation of} \]
\[ \text{the product (R_f = 0.5). The mixture was diluted with sat. aq. NaHCO}_3 \text{ (250 mL),} \]
\[ \text{CH}_2\text{Cl}_2 \text{ (500 mL) and H}_2\text{O (500 mL). The layers were separated and the aq. layer} \]
\[ \text{was extracted with CH}_2\text{Cl}_2 \text{ (2 x 250 mL). The combined organic extracts were} \]
\[ \text{washed with H}_2\text{O (2 x 500 mL) and brine (500 mL), dried over MgSO}_4 \text{, filtered and} \]
\[ \text{concentrated. The residue was dissolved in EtOAc (50 mL) and poured into} \]
petroleum ether (1 L). The precipitate was collected by vacuum filtration and recrystallized from CH₂Cl₂ to afford the desired product 170 as a white crystalline solid (17.0 g, 76%); mp (CH₂Cl₂) 138-140 °C (lit. 294-122-124 °C).

νmax/cm⁻¹ (neat) 3346 (OH, NH), 2970 (CH), 2837 (CH), 1693 (C=O), 1669 (C=O), 1607, 1508, 1469 (CH), 1250, 1175, 1109, 1094 (C-O), 1033, 962, 826, 735, 700.

¹H NMR (400 MHz, DMSO-d₆): δ = 1.45 (ap. s, 3 H, CH₃), 2.15 (ddd, J = 3.7 Hz, J = 6.3 Hz, J = 13.4 Hz, 1 H, H2'a), 2.21-2.28 (m, 1 H, H2'b), 3.17 (dd, J₄',₅ₐ = 2.9 Hz, J₅ₐ,₅ₐ = 10.4 Hz, 1 H, H5'a), 3.21 (dd, J₄',₅₅ = 4.5 Hz, J₅ₐ,₅ₐ = 10.4 Hz, 1 H, H5'b), 3.73 (s, 6 H, 2 x OCH₃), 3.87-3.90 (m, 1 H, H4'), 4.31-4.33 (m, 1 H, H3'), 5.32 (d, J₃',OH = 2.8 Hz, 1 H, OH), 6.20 (app. t, J = 6.8 Hz, 1 H, H1'), 6.88-6.90 (4 H, m, H₂ar.DMTr), 7.21-7.32 (7 H, m, H₂ar.DMTr), 7.38-7.40 (2 H, m, H₂ar.DMTr), 7.50 (app. d, J = 0.9 Hz, 1 H, H6), 11.33 (s, 1 H, NH) ppm.

¹³C NMR (100 MHz, DMSO-d₆): δ = 11.7 (CH₃T), 40.0 (C₂'), 55.1 (OCH₃), 63.8 (C₅'), 70.6 (C₃'), 83.8 (C₁'), 85.5 (C₄'), 85.9 (qC, DMTr), 109.6 (C₅), 113.3 (DMTr), 126.8 (DMTr), 127.7 (DMTr), 127.9 (DMTr), 129.8 (DMTr), 135.3 (qC, DMTr), 135.5 (qC, DMTr), 137.5 (C₆), 144.7 (qC, DMTr), 150.4 (C₂), 158.16 (qC, DMTr), 158.18 (qC, DMTr), 163.7 (C₄) ppm.

HRMS (ESI⁺): m/z calc. 567.2102 [M + Na]⁺, found: 567.2093

The spectroscopic data are in agreement with those reported in the literature.³⁰⁶

1-[5'-O-(4,4'-Dimethoxytrityl)-2'-deoxy-β-D-furanosyl]thymine (171)

Alcohol 171 was prepared according to a modified published procedure.²⁹⁵

Protected thymidine 170 (5.00 g, 9.18 mmol) was dissolved in dry THF (40 mL) under argon, Et₃N (3.2 mL, 22.95 mmol) was added and the solution was cooled to 0 °C. Methylsulfonate chloride (1.1 mL, 13.77 mmol) was added dropwise and the resulting suspension was gradually warmed to r.t. and stirred for 1 h. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material (Rf = 0.5) and the formation of the intermediate (Rf = 0.6). EtOH (20 mL) and aq. NaOH (50 mL, 10 M) were added to the reaction mixture and it was heated to 60 °C for 17 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 19:1) showed the complete
consumption of the intermediate ($R_f = 0.6$) and the formation of the product ($R_f = 0.1$). The reaction mixture was cooled to r.t. and the organic solvents were removed under reduced pressure. The mixture was neutralised with aq. HCl and extracted with CH$_2$Cl$_2$ (3 x 150 mL). The combined organic extracts were washed with H$_2$O (200 mL) and brine (200 mL), dried over MgSO$_4$, filtered and concentrated to afford the desired product 171 as a yellow foam (4.48 g, 90%).

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3396 (OH, NH), 3171 (OH, NH), 3004 (CH), 2954 (CH), 2932 (CH), 2836, 1682 (C=O), 1607 (C=O), 1508, 1464 (CH), 1445, 1271, 1247, 1175, 1065 (C-O), 1032, 903, 828, 755, 701.

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.64$ (s, 3 H, CH$_3$T), 1.86 (dd, $J_{1',2'a} = 2.0$ Hz, $J_{2'a,2'b} = 14.6$ Hz, 1 H, H2'a), 2.52-2.57 (m, 1 H, H2'b), 3.19 (dd, $J_{4',5'a} = 2.8$ Hz, $J_{5'a,5'b} = 10.3$ Hz, 1 H, H5'a), 3.38 (dd, $J_{4',5'b} = 8.1$ Hz, $J_{5'a,5'b} = 10.3$ Hz, 1 H, H5'b), 3.73 (s, 6 H, 2 x OCH$_3$), 4.08 (app. dt, $J = 2.8$ Hz, $J = 8.1$ Hz, 1 H, H4'), 4.18-4.21 (m, 1 H, H3'), 5.19 (d, $J_{3',OH} = 3.3$ Hz, 1 H, OH), 6.11 (dd, $J_{1',2'a} = 2.0$ Hz, $J_{1',2'b} = 8.1$ Hz, 1 H, H1'), 6.86-6.89 (m, 4 H, H$^{\text{ar,DMTr}}$), 7.20-7.24 (m, 1 H, H$^{\text{ar,DMTr}}$), 7.27-7.31 (m, 6 H, H$^{\text{ar,DMTr}}$), 7.42-7.43 (m, 2 H, H$^{\text{ar,DMTr}}$), 7.60 (s, 1 H, H6), 11.26 (s, 1 H, NH) ppm.

$^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta = 12.4$ (CH$_3$T), 40.8 (C2'), 55.0 (OCH$_3$), 62.8 (C5'), 69.0 (C3'), 83.3 (C4'), 84.2 (C1'), 85.5 (qC, DMTr), 108.3 (C5), 113.1 (DMTr), 126.6 (DMTr), 127.75 (DMTr), 127.78 (DMTr), 129.7 (DMTr), 129.8 (DMTr), 135.5 (qC, DMTr), 135.7 (qC, DMTr), 136.8 (C6), 144.9 (qC, DMTr), 150.5 (C2), 158.0 (qC, DMTr), 163.8 (C4) ppm.

HRMS (APCI$^+$): $m/z$ calc. 543.2137 [M - H]$^+$, found: 543.2141

The spectroscopic data are in agreement with those reported in the literature.\textsuperscript{295}
Mesylated thymidine 172 was prepared according to a published procedure. Inverted thymidine 171 (300 mg, 0.55 mmol) was dissolved in dry pyridine (5 mL) under argon and the solution was cooled to 0 °C. Methylsulfonate chloride (130 µL, 1.68 mmol) was added dropwise and the reaction mixture was gradually warmed to r.t. and stirred for 18 h. After this time, TLC analysis (CH2Cl2-MeOH, 19:1) showed the complete consumption of starting material (Rf = 0.1) and the formation of the product (Rf = 0.6). The reaction mixture was cooled to 0 °C and H2O (0.4 mL) was added. After 5 min, the reaction mixture was poured into ice-H2O (60 mL) with vigorous stirring. The resulting precipitate was collected by vacuum filtration and washed with H2O (10 mL) to afford the desired product 172 as a white solid (309 mg, 90%); mp 88-92 °C dec (lit. 85.4-86.7 °C).

νmax/cm⁻¹ (neat) 3181 (OH, NH), 3058 (CH), 2955 (CH), 2930 (CH), 2856 (CH), 1689 (C=O), 1608, 1508, 1464 (CH), 1446, 1250, 1175, 1097 (C-O), 1033, 831, 779, 702. 1H NMR (400 MHz, acetone-d6): δ = 1.73 (d, 4J = 1.1 Hz, 3 H, CH3T), 2.45 (ddd, J2a,3' = 1.0 Hz, J1'a,2'a = 3.2 Hz, J2a,2b = 15.7 Hz, 1 H, H2'a), 2.97 (ddd, J2b,3' = 5.5 Hz, J1'a,2'b = 8.0 Hz, J2a,2b = 15.7 Hz, 1 H, H2'b), 3.02 (s, 3 H, SO2CH3), 3.39 (dd, J4',5'a = 4.9 Hz, J5'a,5'b = 10.1 Hz, 1 H, H5'a), 3.61 (dd, J4',5'b = 6.6 Hz, J5'a,5'b = 10.1 Hz, 1 H, H5'b), 3.79 (s, 6 H, 2 x OCH3), 4.43-4.46 (m, 1 H, H4'), 5.41-5.44 (m, 1 H, H3'), 6.29 (dd, J1'a,2'a = 3.2 Hz, J1'a,2'b = 8.0 Hz, 1 H, H1'), 6.89-6.91 (m, 4 H, Harn,DMTr), 7.22-7.26 (m, 1 H, Harn,DMTr), 7.30-7.34 (m, 2 H, Harn,DMTr), 7.36-7.41 (m, 5 H, H6, 4 x Harn,DMTr), 7.51-7.53 (m, 2 H, Harn,DMTr), 9.96 (bs, 1 H, NH) ppm.

13C NMR (100 MHz, acetone-d6): δ = 12.6 (CH3T), 38.4 (SO2CH3), 40.0 (C2'), 55.5 (OCH3), 62.5 (C5'), 80.4 (C3'), 82.1 (C4'), 84.6 (C1'), 87.5 (qC, DMTr), 110.8 (C5), 113.96 (DMTr), 113.99 (DMTr), 127.7 (DMTr), 128.7 (DMTr), 129.0 (DMTr), 131.01 (DMTr), 131.03 (DMTr), 136.2 (C6), 136.5 (qC, DMTr), 136.6 (qC, DMTr), 145.9 (qC, DMTr), 151.3 (C2), 159.7 (qC, DMTr), 164.2 (C4) ppm.

HRMS (APCI-): m/z calc. 621.1912 [M - H]+, found: 621.1928

The spectroscopic data are in agreement with those reported in the literature.
2,3’-Anhydro-5’-O-(4,4’-dimethoxytrityl)thymidine (174)

Anhydrosugar 174 was prepared according to a published procedure.\textsuperscript{298} DMTr-protected thymidine 170 (3.00 g, 5.51 mmol) was dissolved in dry pyridine (14 mL) under argon. Methylsulfonate chloride (0.55 mL, 7.11 mmol) was added dropwise and the reaction mixture was stirred at r.t. for 18 h. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material (R\textsubscript{f} = 0.5) and the formation of the intermediate (R\textsubscript{f} = 0.6). The reaction was quenched by the addition of H\textsubscript{2}O (1.5 mL) and the mixture was concentrated. The residue was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (50 mL) and washed with H\textsubscript{2}O (30 mL) followed by brine (30 mL). The organic extracts were dried over MgSO\textsubscript{4}, filtered and concentrated to a foam and dissolved in dry MeCN (30 mL) under argon. Potassium carbonate (3.00 g, 21.71 mmol) was added and the suspension was stirred for 120 h. After this time, TLC analysis (CH\textsubscript{2}Cl\textsubscript{2}-MeOH, 19:1) showed the consumption of the intermediate (R\textsubscript{f} = 0.6) and the formation of the product (R\textsubscript{f} = 0.2). Purification by flash column chromatography (CH\textsubscript{2}Cl\textsubscript{2}-MeOH, 19:1) afforded the desired product 174 as a white foam (1.84 g, 63%).

\[
\begin{align*}
\text{H} & \text{NMR (400 MHz, acetone-}\text{d}_6): \delta = 1.82 \text{ (s, 3 H, CH}_3T\text{)}, 2.54-2.59 \text{ (m, 1 H, H2’a)}, \\
& 2.68 \text{ (app. d, } J = 13.0 \text{ Hz, 1 H, H2’b)}, 3.22 \text{ (dd, } J_{4’,5’a} = 5.8 \text{ Hz, } J_{5’a,5’b} = 9.7 \text{ Hz, 1 H, H5’a)}, \\
& 3.30 \text{ (dd, } J_{4’,5’b} = 7.7 \text{ Hz, } J_{5’a,5’b} = 9.7 \text{ Hz, 1 H, H5’b)}, 3.77 \text{ (s, 6 H, 2 x OCH}_3\text{)}, \\
& 4.45-4.49 \text{ (m, 1 H, H4’)}, 5.33 \text{ (bs, 1 H, H3’)}, 5.83 \text{ (app. d, } J = 4.0 \text{ Hz, 1 H, H1’)}, 6.86 \text{ (app. t, } J = 8.1 \text{ Hz, 4 H, H}^{\text{ar,DMTr}}\text{)}, 7.17-7.21 \text{ (m, 1 H, H}^{\text{ar,DMTr}}\text{)}, 7.28 \text{ (app. t, } J = 7.6 \text{ Hz, 2 H, H}^{\text{ar,DMTr}}\text{)}, 7.32-7.34 \text{ (m, 4 H, H}^{\text{ar,DMTr}}\text{)}, 7.41 \text{ (s, 1 H, H6)}, 7.46-7.48 \text{ (m, 2 H, H}^{\text{ar,DMTr}}\text{)} \text{ ppm.}
\end{align*}
\]

\[
\begin{align*}
\text{C} & \text{ NMR (100 MHz, acetone-}\text{d}_6): \delta = 13.5 \text{ (CH}_3T\text{)}, 34.1 \text{ (C2’)}, 55.5 \text{ (OCH}_3\text{)}, 63.2 \text{ (C5’)}, 78.0 \text{ (C3’)}, 84.8 \text{ (C4’)}, 87.2 \text{ (qC, DMTr)}, 88.4 \text{ (C1’)}, 113.92 \text{ (DMTr)}, 113.95 \text{ (DMTr)}, 117.9 \text{ (C5)}, 127.5 \text{ (DMTr)}, 128.6 \text{ (DMTr)}, 128.9 \text{ (DMTr)}, 130.88 \text{ (DMTr)}, \\
& 13.5 \text{ (CH}_3T\text{)}, 34.1 \text{ (C2’)}, 55.5 \text{ (OCH}_3\text{)}, 63.2 \text{ (C5’)}, 78.0 \text{ (C3’)}, 84.8 \text{ (C4’)}, 87.2 \text{ (qC, DMTr)}, 88.4 \text{ (C1’)}, 113.92 \text{ (DMTr)}, 113.95 \text{ (DMTr)}, 117.9 \text{ (C5)}, 127.5 \text{ (DMTr)}, 128.6 \text{ (DMTr)}, 128.9 \text{ (DMTr)}, 130.88 \text{ (DMTr)}, \\
& ...}
\]

\[\text{ν}_\text{max}/\text{cm}^{-1} \text{ (neat) 3063 (CH), 2929 (CH), 2836 (CH), 1659 (C=O), 1631 (C=O), 1608 (C=O), 1529, 1508, 1466 (CH), 1301, 1269, 1247, 1221, 1175, 1157, 1134, 1074 (C=O), 1030, 1012, 993, 878, 827, 783, 754, 728, 701.}\]
130.92 (DMTr), 136.4 (qC, DMTr), 136.5 (qC, DMTr), 136.8 (C6), 146.0 (qC, DMTr), 154.4 (C2), 159.6 (qC, DMTr), 171.6 (C4) ppm.

HRMS (APCI+) \( m/z \) calc. 527.2177 \([M + H]^+\), found: 527.2175.

The spectroscopic data are in agreement with those reported in the literature.\(^\text{298}\)

\textit{C-(5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl) benzyl methyl malonate (169) ( Attempted)\)

\begin{center}
\includegraphics[width=0.2\textwidth]{image.png}
\end{center}

\textbf{Method A:}

NaH (60% dispersion in mineral oil, 39 mg, 964 µmol) was suspended in dry DMF (1.0 mL) and benzyl methyl malonate (136) (175 µL, 1964 µmol) was added dropwise. Mesylate 172 (100 mg, 161 µmol) was dissolved in dry DMF (0.5 mL) under argon and added dropwise to the mixture of NaH and benzyl methyl malonate. The reaction mixture was heated to 100 °C for 120 h. After this time, TLC analysis (petroleum ether-EtOAc, 2:1) showed the consumption of starting material (\(R_f = 0.2\)) and the formation of a product (\(R_f = 0.6\)). The reaction mixture was cooled to r.t. and \(H_2O\) (10 mL) was added. The mixture was extracted with EtOAc (3 x 20 mL) and the combined organic extracts were washed with brine (20 mL), dried over \(MgSO_4\), filtered and concentrated. Purification by flash column chromatography (EtOAc- petroleum ether, 1:3→EtOAc) did not afford the desired product 169 but afforded byproduct 173.

\textbf{Method B:}

Anhydrosugar 174 (50 mg, 95 µmol) was dissolved in dry DMF (0.5 mL) under argon. NaH (60% dispersion in mineral oil, 23 mg, 570 µmol) was suspended in dry DMF (0.5 mL) and benzyl methyl malonate (103 µL, 570 µmol) was added dropwise. The anhydrosugar solution was added dropwise to the reaction and the reaction was heated to 140 °C for 72 h. TLC analysis (\(CH_2Cl_2\)-MeOH, 19:1) showed the consumption of starting material 174 (\(R_f = 0.2\)) and the formation of a product (\(R_f = 0.9\)). The reaction mixture was diluted with toluene (5 mL) and concentrated to a brown oil. The residue was taken up in \(CH_2Cl_2\) (30 mL), washed with \(H_2O\) (30 mL)
and brine (30 mL). The aq. layers were extracted with CH₂Cl₂ (2 x 30 mL), dried over MgSO₄, filtered and concentrated to an oil. The desired product 169 was not obtained.

3'-Deoxy-2',3'-didehydro-5'-O-(4,4'-dimethoxytrityl)-N²-methylthymidine (173)

Compound 173 was obtained from the attempted synthesis of compound 169 (Method A) as a white solid (40 mg, 46%).

ν_max/cm⁻¹ (CH₂Cl₂) 3057 (CH), 2955 (CH), 2929 (CH), 2871 (CH), 2839 (CH), 1702 (C=O), 1667 (C=O), 1638 (C=O), 1608 (C=C), 1508, 1467 (CH), 1446, 1294, 1248, 1176, 1089 (C-O), 1033, 991, 829, 766, 733, 700.

¹H NMR (600 MHz, acetone-d₆): δ = 1.33 (d, 4J = 0.9 Hz, 3 H, CH₃), 3.27 (s, 3 H, N-CH₃), 3.33 (dd, J₄',5ₐ = 2.7 Hz, J₅ₐ,₅ₖ = 10.4 Hz, 1 H, H₅'ₐ), 3.39 (dd, J₄',₅ₖ = 4.8 Hz, J₅ₐ,₅ₖ = 10.4 Hz, 1 H, H₅'b), 3.78 (2 x s, 6 H, 2 x OCH₃), 5.02-5.04 (m, 1 H, H₄'), 6.02-6.03 (m, 1 H, H₂'), 6.55 (app. dt, J = 1.6 Hz, J = 6.0 Hz, 1 H, H₃'), 6.86-6.88 (m, 4 H, H_ar,DMTr), 7.06 (app. dt, J = 1.6 Hz, J = 3.5 Hz, 1 H, H₁'), 7.23-7.25 (m, 1 H, H_ar,DMTr), 7.29-7.31 (m, 2 H, H_ar,DMTr), 7.33-7.34 (m, 4 H, H_ar,DMTr), 7.43 (app. d, 4J = 0.9 Hz, 1 H, H₆), 7.47-7.48 (m, 2 H, H_ar,DMTr) ppm.

¹³C NMR (150 MHz, acetone-d₆): δ = 12.5 (CH₃), 27.8 (N-CH₃), 55.5 (OCH₃), 66.1 (C₅'), 86.5 (C₄'), 87.0 (qC, DMTr), 91.2 (C₁'), 110.0 (C₅), 113.89 (DMTr), 113.90 (DMTr), 127.1 (C₂'), 127.7 (DMTr), 128.6 (DMTr), 129.1 (DMTr), 131.02 (DMTr), 131.05 (DMTr), 135.0 (C₆), 135.4 (C₃'), 136.4 (qC, DMTr), 136.5 (qC, DMTr), 145.8 (qC, DMTr), 152.2 (C₂), 159.70 (qC, DMTr), 159.73 (qC, DMTr), 163.9 (C₄) ppm.

HRMS (APCI⁺): m/z calc. 563.2153 [M + Na]⁺, found: 563.2151
Attempted activation of anhydrothymidine 174 with benzyl triflate

\[
\text{BnOH + TfO} \rightarrow \text{[BnOTf]} + \text{DMTrO}
\]

\[
\text{174}
\]

\[
\text{136}
\]

\[
\text{175}
\]

\[
\text{C-(N}^2\text{-Benzyl-5'}-\text{O-(4,4'-Dimethoxytrityl)thymidin-3'}\text{-yl) benzyl methyl malonate (175) (Attempted)}
\]

Trifluoromethanesulfonic anhydride (33 µL, 197 µmol) was added to dry THF (1 mL) at -78 °C under argon. Benzyl alcohol (21 µL, 202 µmol) and 2,6-di-tert-butyl-4-methylpyridine (41 mg, 200 µmol) were dissolved in dry THF (1 mL) under argon, added to the anhydride solution and stirred at -78 °C for 15 min. Anhydrosugar 174 (100 mg, 190 µmol) and 2,6-di-tert-butyl-4-methylpyridine (10 mg, 49 µmol) were dissolved in dry THF (1 mL) under argon and added dropwise to the reaction. The reaction was warmed to -20 °C and stirred for 20 min. NaH (60% dispersion in mineral oil, 15 mg, 375 µmol) was suspended in dry THF (1 mL) and benzyl methyl malonate (69 µL, 386 µmol) was added. The malonate solution was added dropwise to the reaction and it was warmed to 10 °C over the course of 2 h and then stirred for a further 2 h. After this time, the reaction was quenched by the addition of H₂O (0.5 mL) and concentrated. TLC analysis (EtOAc-petroleum ether, 9:1) showed the complete consumption of starting material (R_t = 0.2) and the formation of products (R_t = 0.7, R_t = 0). The residue was diluted with EtOAc (25 mL) and washed with H₂O (10 mL) and brine (10 mL), dried over MgSO₄, filtered and concentrated. Purification by flash column chromatography (EtOAc-petroleum ether, 2:1 → EtOAc-MeOH, 9:1) did not afford the desired product 175.
Attempted activation of anhydrothymidine 174 with different activating agents

a) 

\[
\begin{align*}
\text{BnOH} + \text{Tf}_2\text{O} \rightarrow \text{[BnOTf]} + \\
\text{THF} \quad -65 \, ^\circ\text{C}, 15 \, \text{min}
\end{align*}
\]

b) 

\[
\begin{align*}
\text{Me}_3\text{SiOTf} \rightarrow \text{[Me}_3\text{SiOTf]} + \\
\text{THF} \quad 0 \, ^\circ\text{C}, 1 \, \text{h}
\end{align*}
\]

c) 

\[
\begin{align*}
\text{MeOTf} \rightarrow \text{[MeOTf]} + \\
\text{THF} \quad -20 \, ^\circ\text{C}, 10 \, \text{min}
\end{align*}
\]

3’-Azido-N\textsuperscript{3}-benzyl-3’-deoxy-5’-O-(4,4’-dimethoxytrityl)thymidine (176)

(Attempted)

Trifluoromethanesulfonic anhydride (20 µL, 119 µmol) was added to dry THF (1 mL) at -65 °C under argon. Benzyl alcohol (13 µL, 125 µmol) and 2,6-di-tert-butyl-4-methylpyridine (26 mg, 127 µmol) were dissolved in dry THF (1 mL) under argon, added to the anhydride solution and stirred at -65 °C for 15 min. Anhydrosugar 174 (61 mg, 116 µmol) and 2,6-di-tert-butyl-4-methylpyridine (8 mg, 39 µmol) were dissolved in dry THF (1 mL) under argon and added dropwise to the reaction. The reaction was warmed to -20 °C over the course of 30 min and stirred for a further 1 h. NaN\textsubscript{3} (12 mg, 185 µmol) was added to the reaction and it was warmed to 10 °C
over the course of 30 min and then stirred for a further 90 min. The reaction was then warmed to r.t. and stirred for a further 36 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed the consumption of starting material ($R_f = 0.5$) and the formation of products ($R_f = 0.3$, $R_f = 0.5$). The reaction was quenched by the addition of H$_2$O (0.5 mL) and concentrated. The residue was diluted with EtOAc (20 mL) and washed with brine (10 mL), dried over MgSO$_4$, filtered and concentrated. Purification by flash column chromatography (CH$_2$Cl$_2$→CH$_2$Cl$_2$-MeOH, 9:1) did not afford the desired product 176.

3'-Azido-3'-deoxy-5'-O(4,4'-dimethoxytrityl)thymidine (177) (Attempted)

Anhydrosugar 174 (60 mg, 114 µmol) and 2,6-di-tert-butyl-4-methylpyridine (6 mg, 29 µmol) were dissolved in dry THF (2 mL) under argon and cooled to 0 °C. Trimethylsilyl triflate (21 µL, 116 µmol) was added dropwise and the reaction was stirred at 0 °C for 1 h. NaN$_3$ (12 mg, 185 µmol) was added, the solution was slowly warmed to r.t. and stirred for 72 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed no consumption of starting material ($R_f = 0.5$) and no formation of a product. The reaction was quenched by the addition of H$_2$O (1 mL). The desired product 177 was not obtained.

C-(5'-O-(4,4'-Dimethoxytrityl)-N$^\alpha$-methylthymidin-3'-yl) benzyl methyl malonate 178 (Attempted)

Anhydrosugar 174 (60 mg, 114 µmol) and 2,6-di-tert-butyl-4-methylpyridine (6 mg, 29 µmol) were dissolved in dry THF (2 mL) under argon and cooled to 10 °C. Methyl triflate (13 µL, 115 µmol) was added dropwise and the reaction was stirred for 10 min. NaH (60% dispersion in mineral oil, 6.7 mg, 168 µmol) was suspended in
dry THF (0.5 mL) and benzyl methyl malonate (136) (41 µL, 226 µmol) was added dropwise. The malonate solution was added dropwise to the reaction and it was stirred for 2.5 h. After this time, TLC analysis (EtOAc-MeOH, 19:1) showed the complete consumption of starting material (Rf = 0.2) and the formation of a product (Rf = 0.7). The reaction mixture was diluted with EtOAc (20 mL) and washed with brine (2 x 10 mL), dried over MgSO₄, filtered and concentrated to an oil. Purification by flash column chromatography (EtOAc-petroleum ether, 1:1→2:1) did not afford the desired product 178 but byproduct 179.

5'-O-(4,4'-Dimethoxytrityl)-N²-methyl thymidine (179)

Compound 179 was obtained from the attempted synthesis of compound 178 as a white solid (20 mg, 31%).

νmax/cm⁻¹ (neat) 3429 (OH), 3058 (CH), 2952 (CH), 2933 (CH), 2837, 1694 (C=O), 1625 (C=O), 1609 (C=O), 1508, 1475, 1445, 1296 (CH), 1247, 1175, 1157, 1105 (C-O), 1072 (C-O), 1031, 828, 766, 754, 734, 701.

¹H NMR (400 MHz, acetone-d₆): δ = 1.75 (d, ⁴J = 1.1 Hz, 3 H, CH₃T), 2.02-2.07 (m, 1 H, H2'a), 2.68 (dd, J = 5.2 Hz, J₁',₂b = 8.2 Hz, J = 13.4 Hz, 1 H, H2'b), 3.22 (s, 3 H, N-CH₃), 3.39 (dd, J = 3.6 Hz, J₅'a,₅'b = 10.2 Hz, 1 H, H5'a), 3.61 (dd, J = 7.5 Hz, J₅'a = 10.2 Hz, 1 H, H5'b), 3.78 (s, 6 H, 2 x OCH₃), 4.19-4.22 (m, 1 H, H4'), 4.40-4.45 (m, 1 H, H5'), 6.23 (dd, J = 2.3 Hz, J₁',₂b = 8.2 Hz, 1 H, H1'), 6.86-6.89 (m, 4 H, H₁',₂b,DMTr), 7.20-7.24 (m, 1 H, H₁',DMTr), 7.28-7.32 (m, 2 H, H₂',DMTr), 7.37-7.42 (m, 4 H, H₁',₂b,DMTr), 7.52-7.54 (m, 2 H, H₂',DMTr), 7.73 (app. d, ⁴J = 1.1 Hz, 1 H, H6) ppm.

¹³C NMR (100 MHz, acetone-d₆): δ = 13.5 (CH₃T), 27.6 (N-CH₃), 42.2 (C₂'), 55.5 (OCH₃), 63.7 (C₅'), 70.8 (C₃'), 84.7 (C₄'), 86.7 (C₁'), 87.0 (qC, DMTr), 108.7 (C5), 113.8 (DMTr), 113.9 (DMTr), 127.5 (DMTr), 128.5 (DMTr), 129.0 (DMTr), 130.98 (DMTr), 131.01 (DMTr), 136.1 (C6), 136.8 (qC, DMTr), 137.0 (qC, DMTr), 146.2 (qC, DMTr), 151.9 (C2), 159.6 (qC, DMTr), 164.0 (C4) ppm.

HRMS (APCI⁺): m/z calc. 559.2439 [M + H]⁺, found: 559.2435
Chapter 6 – Experimental procedures

Synthesis of 3’-malonate ester 115 and carboxylate salt 116 from AZT (181)

\[
\text{N} \quad 181 \quad H_2, Pd/C, \text{MeOH} \quad \text{r.t., 2 h} \quad 99\% \\
\text{N} \quad 180 \quad \text{MeO} \quad \text{EDC, DMAP, DMF} \quad 0^\circ \text{C to r.t., 23 h} \quad 34\%
\]

\[
\text{O} \quad \text{O} \quad 115 \quad 1.00 \text{ g, 3.74 mmol} \quad \text{was dissolved in MeOH (35 mL) and added to a dried} \\
\text{flask containing Pd/C (10%, 150 mg) and H}_2 \text{ was bubbled through the suspension while stirring at r.t. for 2 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed} \\
\text{the complete consumption of starting material, (R}_f \text{ = 0.8) and the formation of the} \\
\text{product (R}_f \text{ = 0.1). The reaction mixture was filtered through celite and the filtrate was} \\
\text{concentrated to afford the desired product 180 as a white foam (893 mg, 99%).}
\]

\[
\text{ν}_{\text{max}}/\text{cm}^{-1} \text{ (neat) 3363 (OH, NH), 3338 (OH, NH), 3290 (OH, NH), 2955 (CH), 2902} \\
\text{(CH), 2760 (CH), 1706 (C=O), 1663 (C=O), 1607 (C=O), 1477 (CH), 1441, 1421,} \\
1370, 1360, 1245, 1207, 1092 (C-O), 1048, 1029, 955, 916, 869, 762.
\]

\[
^1\text{H NMR (400 MHz, DMSO-}d_6\text{): δ = 1.76 (d, } ^4\text{J} = 1.1 \text{ Hz, 3 H, CH}_3, 1.98} \\
\text{(dt, J}_{1',2'a} = 6.7 \text{ Hz, J}_{2'a,3'} = 6.7 \text{ Hz, J}_{2'a,2'b} = 13.1 \text{ Hz, 1 H, H2'a}, 2.08 \text{ (ddd, J}_{1',2'b} = 5.2 \text{ Hz,} \\
J_{2'b,3'} = 7.1 \text{ Hz, J}_{2'a,2'b} = 13.1 \text{ Hz, 1 H, H2'b}), 3.37-3.42 \text{ (m, 1 H, H3'), 3.50-3.53} \\
\text{(m, 1 H, H4'), 3.56} \text{ (dd, J}_{4',5'a} = 3.8 \text{ Hz, J}_{5'a,5'b} = 11.8 \text{ Hz, 1 H, H5'a), 3.65} \text{ (dd,} \\
J_{4',5'b} = 3.1 \text{ Hz, J}_{5'a,5'b} = 11.8 \text{ Hz, 1 H, H5'b), 4.98} \text{ (bs, 1 H, OH), 6.08} \text{ (dd,} \\
J_{1',2'b} = 5.2 \text{ Hz, J}_{1',2'a} = 6.7 \text{ Hz, 1 H, H1'), 7.75} \text{ (app. d,} ^4\text{J} = 1.1 \text{ Hz, 1 H, H6) ppm.}
\]

3’-Amino-3’-deoxythymidine (180)

Amine 180 was prepared according to a published procedure.\textsuperscript{300} AZT (181) (1.00 g, 3.74 mmol) was dissolved in MeOH (35 mL) and added to a dried flask containing Pd/C (10%, 150 mg) and H\textsubscript{2} was bubbled through the suspension while stirring at r.t. for 2 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed the complete consumption of starting material, (R\textsubscript{f} = 0.8) and the formation of the product (R\textsubscript{f} = 0.1). The reaction mixture was filtered through celite and the filtrate was concentrated to afford the desired product 180 as a white foam (893 mg, 99%).
\( ^{13} \text{C NMR (100 MHz, DMSO-} d_6 \text{):} \delta = 12.3 \text{ (CH}_3^+ \text{T), 40.7 (C2'), 50.8 (C3'), 60.8 (C5'), 83.5 (C1'), 87.6 (C4'), 108.9 (C5), 136.3 (C6), 150.4 (C2), 163.8 (C4) ppm.} \)

HRMS (ESI\(^{+}\)): \( m/z \) calc. 264.0955 [M + Na]\(^+\), found: 264.0956

The spectroscopic data are in agreement with those reported in the literature.\(^{308}\)

\( N-(3'-\text{Deoxythymidin}-3'-\text{yl}) \text{ amido methyl malonamide (115)} \)

EDC (318 mg, 1.66 mmol) was suspended in dry DMF (8 mL) under argon and cooled to 0 °C. Monomethyl potassium malonate (285 mg, 1.66 mmol) was added and the suspension was stirred at 0 °C for 1 h. After this time, aminothymidine 180 (400 mg, 1.66 mmol) was added followed by 4-dimethylaminopyridine (20 mg, 0.16 mmol) the reaction was slowly warmed to r.t. and stirred for 26 h. After this time, TLC analysis (CH\(_2\)Cl\(_2\)-MeOH, 4:1) showed the consumption of amine starting material 180 (\( R_f = 0.1 \)) and the formation of the product (\( R_f = 0.5 \)). The reaction mixture was concentrated to an oil and purification by flash column chromatography afforded the desired product 115 as a colourless solid (190 mg, 34%); \( \text{mp} \) 153-154 °C.

\( \nu_{\max }/\text{cm}^{-1} \) (neat) 3316 (OH, NH), 3247 (OH, NH), 3059 (CH), 2933 (CH), 1702 (C=O), 1655 (C=O), 1643 (C=O), 1551, 1476 (CH), 1438, 1272, 1101 (C-O), 1065 (C-O), 969, 768.

\( ^1 \text{H NMR (400 MHz, DMSO-} d_6 \text{):} \delta = 1.78 \text{ (d,} J = 1.0 \text{ Hz, 3 H, CH}_3^+ \text{T), 2.05-2.11 (m, 1 H, H2'a), 2.19-2.27 (m, 1 H, H2'b), 3.25 (s, 2 H, COCH}_2\text{CO), 3.52-3.57 (m, 1 H, H5'a), 3.61-3.66 (m, 4 H, H5'b, COCH}_2\text{CO), 3.75-3.78 (m, 1 H, H4'), 4.28-4.34 (m, 1 H, H3'), 5.09 (app. t,} J = 5.2 \text{ Hz, 1 H, OH), 6.19 (app. t,} J = 6.7 \text{ Hz, 1 H, H1'}, 7.76 (app. d,} J = 1.0 \text{ Hz, 1 H, H6), 8.57 (d,} J = 7.2 \text{ Hz, 1 H, CONH), 11.29 (s, 1 H, NH}^+ \text{T) ppm.} \)

\( ^{13} \text{C NMR (100 MHz, DMSO-} d_6 \text{):} \delta = 12.3 \text{ (CH}_3^+ \text{T), 36.8 (C2'), 42.2 (COCH}_2\text{CO), 49.5 (C3'), 51.9 (OCH}_3\text{), 61.4 (C5'), 83.5 (C1'), 85.1 (C4'), 109.5 (C5), 136.1 (C6), 150.5 (C2), 163.8 (C4), 165.1 (CONH), 168.2 (COCH}_2\text{CO) ppm.} \)

HRMS (ESI\(^{+}\)): \( m/z \) calc. 364.1115 [M + Na]\(^+\), found: 364.1121
Chapter 6 – Experimental procedures

*N-(3’-Deoxythymidin-3’yI) amido sodium malonamide (116)*

KOH (25 mg, 446 µmol) was dissolved in MeOH (1 mL) and cooled to 0 °C. Ester 115 (50 mg, 147 µmol) was added, the solution was slowly warmed to r.t. and stirred for 5.5 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 4:1) showed the consumption of starting material (Rᵣ = 0.8) and the formation of a product (Rᵣ = 0.0). The reaction mixture was eluted through Diaion resin WT01S(H) (H form), then eluted through Diaion resin WT01S(H) (Na form) and concentrated to a white solid. After purification by flash column chromatography (CH₂Cl₂-MeOH, 4:1→MeOH) the residue was taken up in MeOH and eluted through Diaion resin WT01S(H) (Na form). The desired product 116 was obtained as a white foam (42 mg, 82%); mp 202-205 °C dec.

ν_max/cm⁻¹ (neat) 3431 (OH, NH), 3263 (OH, NH), 3071 (CH), 2954 (CH), 2926 (CH), 1642 (C=O), 1593 (C=O), 1473 (CH), 1441, 1379 (C=O), 1318, 1302, 1276, 1233, 1102 (C-O), 1073, 978, 927, 886, 785.

¹H NMR (600 MHz, DMSO-d₆): δ = 1.75 (d, J = 0.7 Hz, 3 H, CH₃), 2.05-2.09 (m, 1 H, H2’a), 2.19 (ddd, J₁;₂b = 6.5 Hz, J = 8.0 Hz, J = 13.3 Hz, 1 H, H2'b), 2.70 (s, 2 H, COCH₂CO), 3.53 (dd, J = 4.2 Hz, J₅’a,₅’b = 11.9 Hz, 1 H, H5’a), 3.62 (dd, J = 2.9 Hz, J₅’a,₅’b = 11.9 Hz, 1 H, H5’b), 3.70-3.73 (m, 1 H, H4’), 4.27-4.31 (m, 1 H, H3’), 5.06 (bs, 1 H, 5’-OH), 6.15 (app. t, J = 6.5 Hz, 1 H, H1’), 7.63 (app. s, 1 H, H6), 9.95-9.97 (m, 1 H, CONH) ppm.

¹³C NMR (150 MHz, DMSO-d₆): δ = 12.7 (CH₃), 37.4 (C2’), 44.8 (COCH₂CO), 48.5 (C3’), 61.4 (C5’), 83.5 (C1’), 85.0 (C4’), 109.3 (C5), 135.6 (C6), 152.4 (C2), 166.4 (C4), 170.0 (CONH), 170.4 (CO₂Na) ppm.

HRMS (ESI⁺): m/z calc. 350.0959 [M + H]⁺, found: 350.0967
Synthesis of 3’-hydroxamic acid malonate 117

EDC (83 mg, 433 µmol) was suspended in dry DMF under argon and cooled to 0 °C. Carboxylate salt 160 (100 mg, 433 µmol) was added and the reaction was stirred at 0 °C for 1 h. Amine 180 (105 mg, 435 µmol) was added, followed by 4-dimethylaminopyridine (6 mg, 49 µmol), and the reaction was slowly warmed to r.t. and stirred for 49 h. After this time, TLC analysis (EtOAc-MeOH, 9:1) showed the consumption of malonate 160 (Rf = 0.9) and the formation of the product (Rf = 0.6). The reaction mixture was concentrated and coevaporated with toluene. Purification by flash column chromatography (EtOAc-MeOH, 19:1→9:1, then CH2Cl2-MeOH, 14:1, 9:1) afforded the desired product 182 as a colourless film (25 mg, 13%).

νmax/cm\(^{-1}\) (CH2Cl2) 3279 (OH, NH), 3064 (OH, NH), 2967 (CH), 2926 (CH), 2854 (CH), 1656 (C=O), 1552, 1470 (CH), 1272, 1102 (C-O), 1059, 751, 699.
Chapter 6 – Experimental procedures

$\text{H NMR (400 MHz, acetone-}\text{d}_6): \delta = 1.82$ (d, $J = 1.2$ Hz, 3 H, CH$_3^T$), 2.32-2.44 (m, 2 H, H2'a, H2'b), 3.13 (s, 2 H, COCH$_2$CO), 3.77 (ddd, $J = 3.4$ Hz, $J_{5'a,\text{OH}} = 5.5$ Hz, $J_{5'a,5'b} = 12.1$ Hz, 1 H, H5'a), 3.84 (ddd, $J = 2.4$ Hz, $J_{5'b,\text{OH}} = 5.5$ Hz, $J_{5'a,5'b} = 12.1$ Hz, 1 H, H5'b), 3.90-3.93 (m, 1 H, H4'), 4.27 (t, $J_{5'a,\text{OH}} = 5.5$ Hz, $J_{5'b,\text{OH}} = 5.5$ Hz, 1 H, OH), 4.54 (ddd, $J_{3',\text{NH}} = 6.2$ Hz, $J = 7.5$ Hz, $J = 13.5$ Hz, 1 H, H3'), 4.89 (s, 2 H, O-CH$_2$-Ph), 6.25 (app. t, $J = 6.3$ Hz, 1 H, H1'), 7.32-7.39 (m, 3 H, Ph), 7.43-7.45 (m, 2 H, Ph), 7.85 (app. d, $J = 1.2$ Hz, 1 H, H6), 7.97 (d, $J_{3',\text{NH}} = 6.2$ Hz, 1 H, CONHC), 9.94 (bs, 1 H, NH$_T$), 10.34 (bs, 1 H, CONHO) ppm.

$\text{C NMR (150 MHz, acetone-}\text{d}_6): \delta = 12.6$ (CH$_3^T$), 38.2 (C2'), 41.7 (COCH$_2$CO), 50.4 (C3'), 62.6 (C5'), 78.4 (O-CH$_2$-Ph), 85.0 (C1'), 86.4 (C4'), 110.7 (C5), 129.2 (Ph), 129.9 (Ph), 136.9 (C6), 137.0 (qC, Ph), 151.4 (C2), 164.3 (C4), 165.2 (CONHO), 167.7 (CONHC) ppm.

HRMS (ESI$^+$): $m/z$ calc. 455.1537 [M + Na]$^+$, found: 455.1539

$N$-(3'-Deoxythymidin-3'-yl) amido $N$-hydroxymalonamide (117)

Method A:
Hydroxylamine hydrochloride (407 mg, 5.86 mmol) was suspended in MeOH (6 mL) and KOH (411 mg, 7.33 mmol) was added. The suspension was warmed to 40 °C to aid dissolution and the precipitate was removed by filtration. Ester 115 (100 mg, 293 µmol) was dissolved in the filtrate and the reaction was stirred at r.t. for 2.5 h, during which a white precipitate formed. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 4:1) showed the complete consumption of starting material, ($R_t = 0.7$) and the formation of the product ($R_t = 0.1$). The reaction mixture was cooled to 0 °C and neutralised to pH 7 using aq. HCl (1 M). The white precipitate was collected by vacuum filtration to afford the desired product 117 as a white amorphous solid (70 mg, 70%); mp 166-170 °C dec.

Method B:
Protected hydroxamic acid 182 (32 mg, 53 µmol) was dissolved in MeOH (2 mL) and added to a dried flask containing Pd/C (10%, 3.5 mg) and H$_2$ was bubbled
through the suspension while stirring at r.t. for 32 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 4:1) showed the complete consumption of starting material, (Rᵢ = 0.7) and the formation of the product (Rᵢ = 0.1). The reaction mixture was filtered through celite and the filtrate was concentrated to a brown film. Purification by flash column chromatography (CH₂Cl₂-MeOH, 4:1) afforded the desired product 117 alongside organic contaminants as a white solid.

ν_max/cm⁻¹ (neat) 3253 (OH, NH), 3065 (C–H), 2955 (CH), 2826 (CH), 1643 (C=O), 1599 (C=O), 1552, 1431, 1274, 1101, 1072, 971, 883, 762.

¹H NMR (600 MHz, DMSO-d₆): δ = 1.77 (s, 3 H, CH₃), 2.08-2.12 (m, 1 H, H2’a), 2.18-2.23 (m, 1 H, H2’b), 2.83 (s, 2 H, COCH₂CO), 3.54 (dd, J = 3.9 Hz, J₅₉₆ = 12.0 Hz, 1 H, H5’a), 3.62 (dd, J = 2.4 Hz, J₅₉₆ = 12.0 Hz, 1 H, H5’b), 3.75-3.77 (m, 1 H, H4’), 4.28-4.32 (m, 1 H, H3’), 6.18 (app. t, J = 6.6 Hz, 1 H, H1’), 7.74 (s, 1 H, H6), 8.78 (bs, 1 H, NHO-C) ppm.

¹³C NMR (150 MHz, DMSO-d₆): δ = 12.3 (CH₃), 37.0 (C2’), 40.8 (COCH₂CO), 49.1 (C3’), 61.4 (C5’), 83.5 (C1’), 85.0 (C4’), 109.4 (C5), 136.1 (C6), 150.8 (C2), 163.1 (CONHOH), 164.3 (C4), 167.6 (CONHC) ppm.

HRMS (ESI⁺): m/z calc. 341.1103 [M - H], found: 341.1109

6.1.3.3 Synthesis of modified dinucleosides

Synthesis of protected 3’-amino thymidine 183

3’-Azido-3’-deoxy-5’-O-(4,4’-dimethoxytrityl)thymidine (184)

Protected AZT 184 was prepared according to a published procedure.³⁰¹ AZT (181) (5.00 g, 18.71 mmol) was suspended in CH₂Cl₂ (60 mL) under argon and DIPEA (9.8 mL, 56.13 mmol) was added. 4,4’-Dimethoxytrityl chloride (6.97 g, 204
20.58 mmol) was added the resulting solution was stirred for 30 min. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 19:1) showed the complete consumption of starting material ($R_f = 0.3$) and the formation of the product ($R_f = 0.6$). The reaction mixture was diluted with CH$_2$Cl$_2$ (90 mL), washed with H$_2$O (80 mL) and brine (80 mL), dried over MgSO$_4$, filtered and concentrated. Purification by flash column chromatography (CH$_2$Cl$_2$→CH$_2$Cl$_2$-MeOH, 19:1) afforded the desired product 184 as a yellow foam (10.01 g, 94%).

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3183 (NH), 3058 (CH), 2930 (CH), 2837 (CH), 2100 (N=N=N), 1686 (C=O), 1662 (C=O), 1607 (C=O), 1508, 1464 (CH), 1446, 1300, 1247, 1175, 1072 (C=O), 1031, 827, 790, 754, 727, 701.

$^1$H NMR (400 MHz, acetone-d$_6$): $\delta = 1.53$ (s, 3 H, CH$_3$T), 2.47 (app. dt, $J = 6.4$ Hz, $J = 13.8$ Hz, 1 H, H2'a), 2.59-2.66 (m, 1 H, H2'b), 3.41 (dd, $J_{4',5'a} = 3.6$ Hz, $J_{5'a,5'b} = 10.6$ Hz, 1 H, H5'a), 3.46 (dd, $J_{4',5'b} = 3.0$ Hz, $J_{5'a,5'b} = 10.6$ Hz, 1 H, H5'b), 3.79 (s, 6 H, 2 x OCH$_3$), 4.00-4.03 (m, 1 H, H4'), 4.46-4.68 (m, 1 H, H3'), 6.25 (app. t, $J = 6.4$ Hz, 1 H, H1'), 6.89-6.91 (m, 4 H, H$_{\text{ar,DMTr}}$), 7.23-7.27 (m, 1 H, H$_{\text{ar,DMTr}}$), 7.31-7.38 (m, 6 H, H$_{\text{ar,DMTr}}$), 7.49-7.51 (m, 2 H, H$_{\text{ar,DMTr}}$), 7.61 (s, 1 H, H6), 10.03 (bs, 1 H, NH) ppm.

$^{13}$C NMR (100 MHz, acetone-d$_6$): $\delta = 12.3$ (CH$_3$T), 37.8 (C2'), 55.5 (OCH$_3$), 61.7 (C3'), 64.1 (C5'), 83.9 (C4'), 85.0 (C1'), 87.6 (qC, DMTr), 111.1 (C5), 114.0 (DMTr), 127.8 (DMTr), 128.7 (DMTr), 129.0 (DMTr), 131.0 (DMTr), 136.4 (C6), 136.5 (qC, DMTr), 145.8 (qC, DMTr), 151.2 (C2), 159.77 (qC, DMTr), 159.78 (qC, DMTr), 164.2 (C4) ppm.

HRMS (APCI-): $m/z$ calc. 568.2202 [M - H]$^-$, found: 568.2215.

The spectroscopic data are in agreement with the literature. 301

3'-Amino-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)thymidine (183)

Amine 183 was prepared according to a published procedure. 301

DMT-protected AZT 184 (3.00 g, 5.27 mmol) was suspended in $i$-PrOH (100 mL) and NaBH$_4$ (1.00 g, 26.43 mmol) was added. The suspension was heated to reflux and stirred for 17 h. After this time, TLC analysis (CH$_2$Cl$_2$-MeOH, 9:1) showed the
complete consumption of starting material ($R_f = 0.7$) and the formation of the product ($R_f = 0.4$). The reaction mixture was diluted with CH$_2$Cl$_2$ (200 mL), washed with H$_2$O (200 mL) and brine (200 mL), dried over MgSO$_4$, filtered and concentrated to afford the desired product **183** as a white foam (2.73 g, 95%) which was used without further purification.

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3164 (NH), 3057 (CH), 2930 (CH), 2836, 1682 (C=O), 1607 (C=O), 1508, 1463 (CH), 1445, 1299, 1247, 1175, 1031 (C-O), 826, 771, 756, 727, 701.

$^1$H NMR (400 MHz, acetone-$d_6$): $\delta = 1.55$ (s, 3 H, CH$_3$), 2.29 (app. dt, $J = 6.7$ Hz, $J = 13.2$ Hz, 1 H, H2’a), 2.42 (ddd, $J_{1';2'b} = 5.5$ Hz, $J_{2'b,3'} = 7.5$ Hz, $J_{2'a,2'b} = 13.2$ Hz, 1 H, 2'-b), 3.24 (dd, $J_{4';5'a} = 3.7$ Hz, $J_{5'a,5'b} = 10.6$ Hz, 1 H, H5’a), 3.43 (dd, $J_{4',5'b} = 2.9$ Hz, $J_{5'a,5'b} = 10.6$ Hz, 1 H, H5’b), 3.79 (s, 6 H, 2 x OCH$_3$), 4.03-4.08 (m, 1 H, H4’), 4.40-4.45 (m, 1 H, H3’), 6.31 (dd, $J_{1';2'b} = 5.5$ Hz, $J_{1';2'b} = 6.7$ Hz, 1 H, H1’), 6.89-6.91 (m, 4 H, Har.DMTr), 7.22-7.26 (m, 1 H, Har.DMTr), 7.31-7.39 (m, 6 H, Har.DMTr), 7.49-7.51 (m, 2 H, Har.DMTr), 7.74 (s, 1 H, H6), 10.02 (bs, 1 H, NH) ppm.

$^{13}$C NMR (151 MHz, acetone-$d_6$): $\delta = 12.4$ (CH$_3$), 39.8 (C2’), 55.5 (OCH$_3$), 60.0 (C3’), 64.0 (C5’), 85.7 (C1’), 85.9 (C4’), 87.3 (qC, DMTr), 110.6 (C5), 113.97 (DMTr), 114.03 (DMTr), 127.7 (DMTr), 128.7 (DMTr), 129.0 (DMTr), 130.97 (DMTr), 130.99 (DMTr), 131.01 (DMTr), 136.6 (C6), 136.7 (qC, DMTr), 146.0 (qC, DMTr), 151.2 (C2), 159.7 (qC, DMTr), 164.3 (C4) ppm.

HRMS (ESI): $m/z$ calc. 542.2297 [M - H]$^-$, found: 542.2305 

The spectroscopic data are in agreement with the literature.\textsuperscript{309}
Synthesis of dinucleoside target 124 from modified nucleosides 139 and 183

Carboxylic acid 139 (375 mg, 0.82 mmol) was dissolved in dry DMF (15 mL) under argon and cooled to 0 °C. HOAt (168 mg, 1.23 mmol) and EDC (236 mg, 1.23 mmol) were added, followed by the addition of amine 183 (469 mg, 0.86 mmol). The reaction mixture was warmed to r.t and stirred at r.t. for 18 h. After this time, TLC analysis (CH₂Cl₂-MeOH, 9:1) showed the consumption of starting materials (Rᵣ = 0.2, Rᵣ = 0.5) and the formation of the product (Rᵣ = 0.8). The reaction was diluted with EtOAc (100 mL) and washed with H₂O (50 mL), sat. aq. NaHCO₃ (50 mL) and brine (50 mL). The combined organic extracts were dried over MgSO₄, filtered and concentrated. Purification by flash column chromatography (EtOAc-
petroleum ether, 9:1) afforded the desired product 18 as a white solid (614 mg, 76%).

Compound 185 was isolated as 4:3 mixture of interconverting diastereoisomers.

$\nu_{\text{max}}/\text{cm}^{-1}$ (neat) 2950 (CH), 2930 (CH), 2855 (CH), 1685 (C=O), 1608 (C=O), 1508, 1465 (CH), 1249, 1175, 1032 (C=O), 831, 777.

Major diastereoisomer:

$^1$H NMR (600 MHz, acetone-$d_6$): $\delta = 0.13$-$0.14$ (2 x s, 6 H, CH$_3$TBDMS), 0.92 (s, 9 H, t-BuTBDMS), 1.43 (s, 3 H, 5'-CH$_3$T), 1.84 (s, 3 H, 3'-CH$_3$T), 2.09-2.20 (m, 2 H, 3'-H2'a, 3'-H5'a), 2.30-2.41 (m, 4 H, 5'-H2'a, 5'-H2'b, 3'-H2'b, 3'-H5'b), 3.37-3.45 (m, 2 H, 5'-H5'a, 5'-H5'b), 3.50-3.54 (m, 1 H, 1'H), 3'-H6'), 3.56 (s, 3 H, CO$_2$CH$_3$), 3.79 (s, 6 H, 2 x OCH$_3$), 3.84-3.91 (m, 1 H, 3'-H4'), 4.04-4.05 (m, 1 H, 5'-H4'), 4.33-4.36 (m, 1 H, 3'-C3'), 4.74-4.82 (m, 1 H, 5'-H3'), 6.18 (app. t, $J = 7.0$ Hz, 1 H, 3'-H1'), 6.27 (app. t, $J = 6.4$ Hz, 1 H, 5'-H1'), 6.88-6.91 (m, 4 H, H$_{\text{ar}}$), 7.23-7.26 (m, 1 H, H$_{\text{ar}}$), 7.31-7.39 (m, 6 H, H$_{\text{ar}}$), 7.45 (s, 1 H, 3'-H6), 7.49-7.52 (m, 2 H, H$_{\text{ar}}$), 7.61 (s, 1 H, 5'-H6), 7.86 (d, $J_{3',NH} = 7.8$ Hz, 1 H, CONH), 9.95-9.98 (m, 2 H, 5'-NH$_T$, 3'-NH$_T$) ppm.

Minor diastereoisomer:

$^1$H NMR (600 MHz, acetone-$d_6$): $\delta = 0.09$ (s, 3 H, CH$_3$TBDMS), 0.11 (s, 3 H, CH$_3$TBDMS), 0.88 (s, 9 H, t-BuTBDMS), 1.44 (s, 3 H, 5'-CH$_3$T), 1.82 (s, 3 H, 3'-CH$_3$T), 2.09-2.20 (m, 2 H, 3'-H2'a, 3'-H5'a), 2.30-2.41 (m, 3 H, 5'-H2'a, 3'-H2'b, 3'-H5'b), 2.48-2.53 (m, 1 H, 5'-H2'b), 3.37-3.45 (m, 2 H, 5'-H5'a, 5'-H5'b), 3.50-3.54 (m, 1 H, 1'H), 3'-H6'), 3.66 (s, 3 H, CO$_2$CH$_3$), 3.74-3.76 (m, 1 H, 3'-H4'), 3.79 (s, 6 H, 2 x OCH$_3$), 4.06-4.08 (m, 1 H, 5'-H4'), 4.33-4.36 (m, 1 H, 3'-H3'), 4.74-4.82 (m, 1 H, 5'-H3'), 6.22 (app. t, $J = 6.9$ Hz, 1 H, 3'-H1'), 6.32 (app. t, $J = 6.8$ Hz, 1 H, 5'-H1'), 6.88-6.91 (m, 4 H, H$_{\text{ar}}$), 7.23-7.26 (m, 1 H, H$_{\text{ar}}$), 7.31-7.39 (m, 6 H, H$_{\text{ar}}$), 7.45 (s, 1 H, 3'-H6), 7.49-7.52 (m, 2 H, H$_{\text{ar}}$), 7.61 (s, 1 H, 5'-H6), 7.86 (d, $J_{3',NH} = 7.8$ Hz, 1 H, CONH), 9.95-9.98 (m, 2 H, 5'-NH$_T$, 3'-NH$_T$) ppm.
Chapter 6 – Experimental procedures

7.23-7.26 (m, 1 H, H^ar), 7.31-7.39 (m, 6 H, H^ar), 7.42 (s, 1 H, 3'-H6), 7.49-7.52 (m, 2 H, H^ar), 7.65 (s, 1 H, 5'-H6), 7.91 (d, J_{3',NH} = 7.5 Hz, 1 H, CONH), 9.95-9.98 (m, 2 H, 5'-NH^T, 3'-NH^T) ppm.

$^{13}$C NMR (151 MHz, acetone-d$_6$): δ = -4.58 (CH$_3$TBDMS), -4.51 (CH$_3$TBDMS), 12.13 (5'-CH$_3$T), 12.46 (3'-CH$_3$T), 18.53 (qC, t-BuTBDMS), 26.17 (t-BuTBDMS), 33.3 (3'-C5'), 38.32 (5'-C2'), 40.1 (3'-C2'), 50.0 (3'-C6'), 51.3 (5'-C3'), 52.6 (CO$_2$CH$_3$), 55.55 (OCH$_3$DMT), 64.5 (5'-C5'), 76.3 (3'-C3'), 84.6 (5'-C4'), 84.9 (5'-C1'), 85.0 (3'-C4'), 85.5 (3'-C1'), 87.42 (qC, DMTr), 111.08 (3'-C5, 5'-C5), 114.01 (CH, DMTr), 114.02 (CH, DMTr), 127.73 (CH, DMTr), 128.74 (CH, DMTr), 129.1 (CH, DMTr), 131.06 (CH, DMTr), 131.07 (CH, DMTr), 136.3 (5'-C6), 136.6 (qC, DMTr), 136.8 (qC, DMTr), 136.9 (3'-C6), 145.91 (qC, DMTr), 151.3 (3'-C2, 5'-C2), 159.72 (qC, DMTr), 159.74 (qC, DMTr), 164.18 (3'-C4), 164.20 (5'-C4), 168.4 (CONH), 171.2 (CO$_2$) ppm.

HRMS (APCI$^+$): m/z calc. 1004.4084 [M + Na]$^+$, found: 1004.4068

$N$-(3'-Deoxy-5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl)-C-(5'-deoxythymidin-5'-yl) amido methyl malonate (186)

Protected dimer 185 (580 mg, 0.59 mmol) was dissolved in THF (8 mL). TBAF (316 mg, 1.06 mmol) was added and the solution was stirred at r.t. for 24 h. After this time, TLC analysis (EtOAc-MeOH, 9:1) showed the complete consumption of the starting material ($R_f = 0.7$) and product formation as a mixture of diastereomers ($R_f = 0.3$ and $R_f = 0.4$). The suspension was concentrated and purification by flash column chromatography (EtOAc-MeOH, 19:1→9:1) afforded the desired product 186 as a white solid (472 mg, 92%).

Compound 186 was isolated as a 3:1 mixture of interconverting diastereoisomers.

$\nu_{\max}$/cm$^{-1}$ (neat) 3310 (OH, NH), 3073 (CH), 2950 (CH), 2930 (CH), 2839 (CH), 1655 (C=O), 1607 (C=O), 1508, 1466 (CH), 1248, 1175, 1031 (C=O), 828.
Major diastereoisomer:

$^1$H NMR (600 MHz, acetone-$d_6$): $\delta = 1.43$ (d, $4J = 1.0$ Hz, 3 H, 5'-CH$_3$), 1.86 (d, $4J = 1.0$ Hz, 3 H, 3'-CH$_3$), 2.10-2.23 (m, 2 H, 3'H$_2$a, 3'-H5'a), 2.27-2.38 (m, 4 H, 5'-H2'a, 5'-H2'b, 3'-H2'b, 3'-H5'b), 3.37-3.46 (m, 2 H, 5'-H5'a, 5'-H5'b), 3.52-3.56 (m, 4 H, 3'-H6', CO$_2$CH$_3$), 3.78 (s, 6 H, 2 x OCH$_3$), 3.90 (app. dt, $J = 3.6$ Hz, $J = 10.2$ Hz, 1 H, 3'-H4'), 4.05-4.07 (m, 1 H, 5'-H4'), 4.25-4.28 (m, 1 H, 3'-H3'), 4.57 (d, $J_{3',OH} = 3.3$ Hz, 1 H, OH), 4.75-4.80 (m, 1 H, 5'-H3'), 6.21 (dd, $J = 6.2$ Hz, $J = 7.6$ Hz, 1 H, 3'-H1'), 6.27 (app. t, $J = 6.3$ Hz, 1 H, 5'-H1'), 6.88-6.91 (m, 4 H, H$^a$), 7.22-7.25 (m, 1 H, H$^a$), 7.30-7.38 (m, 6 H, H$^a$), 7.46 (app. d, $4J = 1.0$ Hz, 1 H, 3'-H6), 7.49-7.52 (m, 2 H, H$^a$), 7.63 (app. d, $4J = 1.0$ Hz, 1 H, 5'-H6), 7.90 (d, $J_{3',NH} = 7.9$ Hz, 1 H, CONH), 10.09-10.13 (m, 2 H, 5'-NH$^T$, 3'-NH$^T$) ppm.

$^{13}$C NMR (151 MHz, acetone-$d_6$): $\delta = 12.2$ (5'-CH$_3$), 12.54 (3'-CH$_3$), 33.6 (3'-C5'), 38.4 (5'-C2'), 39.5 (3'-C2'), 50.5 (5'-C3'), 50.7 (3'-C6'), 52.5 (CO$_2$CH$_3$), 55.53 (OCH$_3$DMTr), 64.0 (5'-C5'), 75.3 (3'-C3'), 84.4 (5'-C4'), 84.93 (5'-C1'), 86.0 (3'-C4'), 86.2 (3'-C1'), 87.36 (qC, DMTr), 111.0 (3'-C5), 111.2 (5'-C5), 113.98 (CH, DMTr), 114.00 (CH, DMTr), 114.02 (CH, DMTr), 127.68 (CH, DMTr), 128.71 (CH, DMTr), 129.1 (CH, DMTr), 131.07 (CH, DMTr), 131.09 (CH, DMTr), 136.3 (5'-C6), 136.5 (qC, DMTr), 136.63 (qC, DMTr), 137.6 (3'-C6), 145.92 (qC, DMTr), 151.40 (5'-C2), 151.41 (3'-C2), 159.66 (qC, DMTr), 159.70 (qC, DMTr), 164.4 (5'-C4), 164.5 (3'-C4), 169.5 (CONH), 170.7 (CO$_2$) ppm.

Minor diastereoisomer:

$^1$H NMR (600 MHz, acetone-$d_6$): $\delta = 1.44$ (d, $4J = 1.0$ Hz, 3 H, 5'-CH$_3$), 1.83 (d, $4J = 1.0$ Hz, 3 H, 3'-CH$_3$), 2.10-2.23 (m, 2 H, 3'-H2'a, 3'-H5'a), 2.27-2.33 (m, 2 H, 3'-H2'b, 3'-H5'b), 2.39-2.42 (m, 1 H, 5'-H2'a), 2.49-2.54 (m, 1 H, 5'-H2'b), 3.37-3.46 (m, 2 H, 5'-H5'a, 5'-H5'b), 3.52-3.56 (m, 1 H, 3'-H6'), 3.65 (s, 3 H, CO$_2$CH$_3$), 3.76-3.78 (m, 7 H, 3'-H4', 2 x OCH$_3$), 4.08-4.10 (m, 1 H, 5'-H4'), 4.25-4.28 (m, 1 H, 3'-H3'), 4.51 (d, $J_{3',OH} = 4.2$ Hz, 1 H, OH), 4.75-4.80 (m, 1 H, 5'-H3'), 6.24 (app. t, $J = 6.9$ Hz, 1 H, 3'-H1'), 6.33 (app. t, $J = 6.6$ Hz, 1 H, 5'-H1'), 6.88-6.91 (m, 4 H, H$^a$), 7.22-7.25 (m, 1 H, H$^a$), 7.30-7.38 (m, 6 H, H$^a$), 7.42 (app. d, $4J = 1.0$ Hz, 1 H, 3'-H6), 7.49-7.52 (m, 2 H, H$^a$), 7.66 (app. d, $4J = 1.0$ Hz, 1 H, 5'-H6), 7.98 (d, $J_{3',NH} = 7.5$ Hz, 1 H, CONH), 10.09-10.13 (m, 2 H, 5'-NH$^T$, 3'-NH$^T$) ppm.

$^{13}$C NMR (151 MHz, acetone-$d_6$): $\delta = 12.1$ (5'-CH$_3$), 12.47 (3'-CH$_3$), 33.6 (3'-C5'), 38.3 (5'-C2'), 39.7 (3'-C2'), 50.0 (3'-C6'), 51.2 (5'-C3'), 52.6 (CO$_2$CH$_3$), 55.55 (OCH$_3$DMTr), 64.4 (5'-C5'), 75.1 (3'-C3'), 84.6 (5'-C4'), 84.87 (3'-C4'), 84.94 (5'-C1'), 210
85.3 (3'-C1'), 87.40 (qC, DMTr), 111.1 (5'-C5, 3'-C5), 113.6 (CH, DMTr), 113.98 (CH, DMTr), 114.00 (CH, DMTr), 114.02 (CH, DMTr), 127.72 (CH, DMTr), 128.73 (CH, DMTr), 129.1 (CH, DMTr), 131.1 (CH, DMTr), 136.44 (5'-C6), 136.55 (qC, DMTr), 136.66 (qC, DMTr), 136.9 (3'-C6), 145.93 (qC, DMTr), 151.37 (3'-C2), 151.40 (5'-C2), 159.68 (qC, DMTr), 159.71 (qC, DMTr), 164.4 (5'-C4, 3'-C4), 168.7 (CONH), 171.2 (CO₂) ppm.

HRMS (APCI): m/z calc. 866.3254 [M - H]⁻, found: 866.3262

N-(3'-Deoxythymidin-3'-yl)-C-(5'-deoxythymidin-5'-yl) amido methyl malonate (124)

Protected dimer 186 (100 mg, 115 µmol) was dissolved in CH₂Cl₂ (980 µL). Triethylsilane (184 µL, 1.15 mmol) and trifluoroacetic acid (20 µL, 261 µmol) were added and the solution was stirred at r.t. The solution turned bright red, followed by the formation of a white precipitate and loss of the red colour after 10 min. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed the complete consumption of starting material (Rᵥ = 0.6) and the formation of the product (Rᵥ = 0.4). The supernatant was removed and the precipitated product was washed with toluene (2 x 1 mL). The washings and supernatant were combined and concentrated, and the resulting white solid was washed with CH₂Cl₂ (3 x 5 mL) to remove impurities. The precipitates were combined to afford the desired product 124 as a white amorphous solid (60 mg, 92%).

Compound 124 was isolated as a 3:1 mixture of interconverting diastereomers.

ν_max/cm⁻¹ (neat) 3398 (OH, NH), 3261 (OH, NH), 3174 (OH, NH), 3050 (CH), 2955 (CH), 2819 (CH), 1742 (C=O), 1677 (C=O), 1655 (C=O), 1638 (C=O), 1547, 1473 (CH), 1369, 1273, 1203, 1097 (C-O), 1047 (C-O), 988.

211
Major diastereoisomer:

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 1.77\) (bs, 3 H, 5'-CH\(_3\)), 1.79 (bs, 3 H, 3'-CH\(_3\)), 1.88-1.96 (m, 1 H, 3'-H5'a), 1.99-2.23 (m, 5 H, 5'-H2'a, 5'-H2'b, 3'-H2'a, 3'-H2'b, 3'-H5'b), 3.42-3.45 (m, 1 H, 3'-H6'), 3.49-3.53 (m, 1 H, 5'-H5'a), 3.59-3.60 (m, 1 H, 5'-H5'b), 3.63 (s, 3 H, CO\(_2\)CH\(_3\)), 3.66-3.71 (m, 1 H, 3'-H4'), 3.73-3.76 (m, 1 H, 5'-H4'), 4.04-4.09 (m, 1 H, 3'-H3'), 4.23-4.29 (m, 1 H, 5'-H3'), 5.29 (bs, 2 H, 5'-OH, 3'-OH), 6.10 (app. t, \(J = 7.0\) Hz, 1 H, 3'-H1'), 6.19 (app. t, \(J = 6.7\) Hz, 1 H, 5'-H1'), 7.38 (app. d, \(4J = 0.9\) Hz, 1 H, 3'-H6), 7.73 (app. d, \(4J = 0.9\) Hz, 1 H, 5'-H6), 8.63 (d, \(J_{\text{3',NH}} = 7.4\) Hz, 1 H, CONH), 11.25-11.27 (2 x s, 2 H, 5'-NH\(_T\), 3'-NH\(_T\)) ppm.

\(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta = 12.1\) (3'-CH\(_3\)), 12.3 (5'-CH\(_3\)), 32.37 (3'-C5'), 36.5 (5'-C2'), 38.2 (3'-C2'), 49.0 (3'-C6'), 49.5 (5'-C3'), 52.1 (CO\(_2\)CH\(_3\)), 61.3 (5'-C5'), 73.3 (3'-C3'), 83.49 (5'-C1'), 83.69 (3'-C1'), 84.1 (3'-C4'), 84.9 (5'-C4'), 109.5 (5'-C5), 109.7 (3'-C5), 136.1 (5'-C6), 136.3 (3'-C6), 150.4 (5'-C2, 3'-C2), 163.7 (5'-C4, 3'-C4), 168.0 (CONH), 169.9 (CO\(_2\)) ppm.

Minor diastereoisomer:

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 1.78\) (d, \(4J = 1.1\) Hz, 3 H, 5'-CH\(_3\)), 1.80 (d, \(4J = 1.1\) Hz, 3 H, 3'-CH\(_3\)), 1.97-2.25 (m, 6 H, 5'-H2'a, 5'-H2'b, 3'-H2'a, 3'-H2'b, 3'-H5'a, 3'-H5'b), 3.41-3.45 (m, 1 H, 3'-H6'), 3.52-3.58 (m, 2 H, 5'-H5'a, 3'-H4'), 3.61-3.66 (m, 4 H, 5'-H5'b, CO\(_2\)CH\(_3\)), 3.76-3.78 (m, 1 H, 5'-H4'), 4.04-4.09 (m, 1 H, 3'-H3'), 4.30-4.35 (m, 1 H, 5'-H3'), 5.29 (bs, 2 H, 5'-OH, 3'-OH), 6.15 (app. t, \(J = 7.1\) Hz, 1 H, 3'-H1'), 6.20 (app. t, \(J = 6.7\) Hz, 1 H, 5'-H1'), 7.42 (app. d, \(4J = 1.1\) Hz, 1 H, 3'-H6), 7.76 (app. d, \(4J = 1.1\) Hz, 1 H, 5'-H6), 8.71 (d, \(J_{\text{3',NH}} = 7.3\) Hz, 1 H, CONH), 11.29-11.30 (2 x s, 2 H, 5'-NH\(_T\), 3'-NH\(_T\)) ppm.

\(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta = 12.1\) (3'-CH\(_3\)), 12.3 (5'-CH\(_3\)), 32.42 (3'-C5'), 36.7 (5'-C2'), 38.0 (3'-C2'), 48.5 (3'-C6'), 49.7 (5'-C3'), 52.1 (CO\(_2\)CH\(_3\)), 61.5 (5'-C5'), 73.3 (3'-C3'), 83.47 (3'-C4'), 83.53 (5'-C1'), 83.7 (3'-C1'), 85.0 (5'-C4'), 109.5 (5'-C5), 109.8 (3'-C5), 136.1 (5'-C6), 136.2 (3'-C6), 150.4 (5'-C2), 150.5 (3'-C2), 163.7 (5'-C4, 3'-C4), 167.5 (CONH), 170.3 (CO\(_2\)) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 588.1912 [M + Na]\(^+\), found: 588.1922
Synthesis of carboxylate dinucleoside 125

\[
\text{N-(3'-Deoxymethidin-3'-yl)-C-(5'-deoxymethidin-5'-yl) amido sodium malonate (125)}
\]

KOH (18 mg, 320 µmol) was dissolved in MeOH (1 mL) and cooled to 0 °C. Ester 124 (30 mg, 53 µmol) was added, the solution was slowly warmed to r.t. and stirred for 7 h. After this time, TLC analysis (H₂O-i-PrOH-EtOAc, 1:5:4) showed the consumption of starting material (R_f = 0.8) and the formation of a product (R_f = 0.3). The reaction mixture was concentrated and eluted through Diaion resin WT01S(H) (H form), then eluted through Diaion resin WT01S(H) (Na form) and concentrated to afford the desired product 125 as a white solid (27 mg, 89%).

The product was isolated as a 1:1 mixture of interconverting diastereomers.

\[\nu_{\text{max}}/\text{cm}^{-1} \text{ (neat) } 3259 \text{ (OH, NH), 3063 (CH), 2925 (CH), 2820 (CH), 1655 (C=O), 1587 (C=O), 1474 (CH), 1366, 1270, 1090 (C-O), 1050 (C-O), 962, 766.}\]

\[\delta \text{ (CD₃OD): } 1.89-1.91 \text{ (m, } 12 \text{ H, } 4 \times \text{CH₃}) \text{, 2.05-2.41 (m, } 12 \text{ H, } 3'-\text{H2'a, 3'-H2'b, 3'-H5'a, 3'-H5'b, 5'-H2'a, 5'-H5'b), 3.23 (dd, } J = 5.5 \text{ Hz, } J = 8.0 \text{ Hz, } 1 \text{ H, } 3'-\text{H6'}), 3.31-3.35 \text{ (m, } 1 \text{ H, } 3'-\text{H6'}), 3.73-3.91 \text{ (m, } 8 \text{ H, } 3'-\text{H4'}, 5'-\text{H4'}, 5'-\text{H5'a, 5'-H5'b), 4.14-4.19 (m, } 2 \text{ H, } 3'-\text{H3'}), 4.46 \text{ (app. q, } J = 7.2 \text{ Hz, } 1 \text{ H, } 5'-\text{H3'}), 4.51 \text{ (app. q, } J = 7.2 \text{ Hz, } 1 \text{ H, } 5'-\text{H3'}), 6.15-6.25 \text{ (m, } 4 \text{ H, } 3'-\text{H1'}, 5'-\text{H1'}), 7.48-7.50 \text{ (2 x s, } 2 \text{ H, H6), 7.88-7.90 (2 x s, } 2 \text{ H, H6) ppm.}\]
\(^{13}\)C NMR (151 MHz, CD\(_3\)OD): \(\delta = 12.45\) (CH\(_3\)), 12.51 (CH\(_3\)), 12.53 (CH\(_3\)), 35.4 (3'-C5'), 35.5 (3'-C5'), 38.5 (5'-C2'), 38.8 (5'-C2'), 39.9 (3'-C2'), 40.1 (3'-C2'), 49.7 (5'-C3'), 50.0 (5'-C3'), 53.6 (3'-C6'), 55.1 (3'-C6'), 62.0 (5'-C5'), 62.3 (5'-C5'), 75.4 (3'-C3'), 75.3 (3'-C3'), 85.9 (3'-C1', 5'-C1'), 86.0 (3'-C1', 5'-C1'), 86.15 (3'-C4'), 86.19 (5'-C4'), 86.6 (5'-C4'), 87.5 (3'-C4'), 111.3 (C5), 111.4 (C5), 111.7 (C5), 112.0 (C5), 137.8 (C6), 138.1 (C6), 138.2 (C6), 152.39 (C2), 152.43 (C2), 152.45 (C2), 152.54 (C2), 166.5 (C4), 166.6 (C4), 166.68 (C4), 166.70 (C4), 174.3 (CONH), 174.8 (CONH), 176.8 (CO\(_2\)), 177.1 (CO\(_2\)) ppm.

HRMS (ESI\(^+\)): \(m/z\) calc. 596.1575 [M + Na]\(^+\), found: 596.1594

Synthesis of hydroxamic acid dinucleoside 126

\(N\)-(3'-Deoxy-5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl)-C-(5'-deoxythymidin-5'-yl) amido \(N\)-(tert-butyldimethylsilyloxy) malonamide (187)
Protected ester 186 (20 mg, 23 µmol) was suspended in MeOH (0.5 mL) and aq. NaOH (1 M, 23 µL, 23 µmol) was added. The reaction was stirred at r.t. for 18 h and after this time, TLC analysis (EtOAc-MeOH, 4:1) showed the complete consumption of starting material (R_f = 0.6) and the formation of a product (R_f = 0.0). The suspension was concentrated and the crude product (14 mg) was suspended in dry DMF (0.5 mL) under argon and cooled to 0 °C. EDC (4.7 mg, 25 µmol) and HOAt (3.4 mg, 25 µmol) were added and the reaction was stirred at 0 °C for 10 min. O-(tert-Butyldimethylsilyl)hydroxylamine (2.5 mg, 17 µmol) was added, the reaction was warmed to r.t. and stirred for 45 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed no change. The reaction was concentrated and the desired product 187 was not obtained.

\[ N-(3'-Deoxythymidin-3'-yl)-C-(5'-deoxythymidin-5'-yl)\text{ amido } N\text{-hydroxy malonamide} (126) \]

Hydroxylamine hydrochloride (123 mg, 1.77 mmol) was suspended in MeOH (2 mL) and KOH (124 mg, 2.21 mmol) was added. The suspension was warmed to 40 °C to aid dissolution and the precipitate was removed by filtration. Ester 124 (50 mg, 88 µmol) was dissolved in the filtrate and the reaction was stirred at r.t. for 48 h, during which a white precipitate formed. After this time, TLC analysis (H_2O-\text{-}i\text{-PrOH-EtOAc, 1:5:4}) showed the consumption of starting material (R_f = 0.8) and the formation of the product (R_f = 0.4). The reaction mixture was neutralised to pH 7 using aq. HCl (1 M) and concentrated. Purification by flash column chromatography (H_2O-\text{-}i\text{-PrOH-EtOAc, 1:5:4}) afforded the desired product 126 as a white amorphous solid (37 mg, 74%).

Compound 126 was isolated as a 5:4 mixture of interconverting diastereomers.

\[ \nu_{\text{max}}/\text{cm}^{-1} \text{ (neat) 3409 (OH, NH), 3162 (OH, NH), 2994 (CH), 2923 (CH), 1664 (C=O), 1543, 1474 (CH), 1364, 1271, 1089 (C-O), 1049 (C-O), 1019, 855, 765.} \]
Major diastereomer:

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.77-1.78$ (m, 3 H, 5'-CH$_3$T), 1.81 (d, $^4J = 0.9$ Hz, 3 H, 3'-CH$_3$T), 1.85-1.94 (m, 1 H, 5'-H5'a), 1.99-2.04 (m, 1 H, 3'-H2'a), 2.08-2.22 (m, 4 H, 5'-H'2a, 5'-H2'b, 3'-H2'b, 3'-H5'b), 3.18 (dd, $J = 6.0$ Hz, $J = 8.5$ Hz, 1 H, 3'-H6'), 3.50-3.54 (m, 1 H, 5'-H5'a), 3.55-3.58 (m, 1 H, 3'-H4'), 3.59-3.64 (m, 1 H, 5'-H5'b), 3.76-3.80 (m, 1 H, 5'-H4'), 4.02-4.05 (m, 1 H, 3'-H3'), 4.26-4.33 (m, 1 H, 5'-H3'), 5.08-5.11 (m, 1 H, 5'-OH), 5.32 (d, $J = 4.4$ Hz, 1 H, 3'-OH), 6.08-6.11 (m, 1 H, 3'-H1'), 6.17-6.19 (m, 1 H, 5'-H1'), 7.44 (bs, 1 H, 5'-H6), 7.75 (app. d, $^4J = 1.0$ Hz, 1 H, 3'-H6), 8.37 (d, $J_{\text{5'-NH}}$ = 7.3 Hz, 1 H, CONH), 8.96 (bs, 1 H, CONHO), 10.59 (bs, 1 H, NH$_T$ or NHOH), 11.27 (bs, 1 H, NH$_T$ or NHOH) ppm.

$^{13}$C NMR (151 MHz, DMSO-d$_6$): $\delta = 12.08$ (3'-CH$_3$T), 12.3 (5'-CH$_3$T), 32.4 (3'-C5'), 36.9 (5'-C2'), 38.4 (53-C2'), 47.2 (3'-C6'), 49.3 (5'-C3'), 61.2 (5'-C5'), 73.2 (3'-C3'), 83.5 (5'-C1'), 83.6 (3'-C1'), 83.9 (5'-C4'), 84.8 (5'-C4'), 109.4 (5'-C5), 109.77 (3'-C5), 136.19 (3'-C6), 136.21 (5'-C6), 150.39 (3'-C2), 150.44 (5'-C2), 163.7 (5'-C4, 3'-C4), 165.6 (CONHOH), 168.7 (CONHC) ppm.

Minor diastereomer:

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta = 1.77-1.78$ (m, 3 H, 5'-CH$_3$T), 1.81 (d, $^4J = 0.9$ Hz, 3 H, 3'-CH$_3$T), 1.85-1.94 (m, 1 H, 3'-H5'a), 1.99-2.04 (m, 1 H, 3'-H2'a), 2.08-2.22 (m, 4 H, 5'-H'2a, 5'-H2'b, 3'-H2'b, 3'-H5'b), 3.21 (dd, $J = 5.6$ Hz, $J = 8.6$ Hz, 1 H, 3'-H6'), 3.50-3.54 (m, 1 H, 5'-H5'a), 3.55-3.58 (m, 1 H, 3'-H4'), 3.59-3.64 (m, 1 H, 5'-H5'b), 3.76-3.80 (m, 1 H, 5'-H4'), 4.02-4.05 (m, 1 H, 3'-H3'), 4.26-4.33 (m, 1 H, 5'-H3'), 5.08-5.11 (m, 1 H, 5'-OH), 5.32 (d, $J = 4.4$ Hz, 1 H, 3'-OH), 6.08-6.11 (m, 1 H, 3'-H1'), 6.17-6.19 (m, 1 H, 5'-H1'), 7.44 (bs, 1 H, 5'-H6), 7.77 (app. d, $^4J = 1.0$ Hz, 1 H, 3'-H6), 8.45 (d, $J_{\text{5'-NH}}$ = 7.3 Hz, 1 H, CONH), 8.91 (bs, 1 H, CONHO), 10.59 (bs, 1 H, NH$_T$ or NHOH), 11.27 (bs, 1 H, NH$_T$ or NHOH) ppm.

$^{13}$C NMR (151 MHz, DMSO-d$_6$): $\delta = 12.12$ (3'-CH$_3$T), 12.3 (5'-CH$_3$T), 32.5 (3'-C5'), 36.7 (5'-C2'), 38.3 (53-C2'), 46.9 (3'-C6'), 49.4 (5'-C3'), 61.3 (5'-C5'), 73.2 (3'-C3'), 83.5 (5'-C1'), 83.7 (3'-C1'), 83.9 (5'-C4'), 85.1 (5'-C4'), 109.4 (5'-C5), 109.80 (3'-C5), 136.16 (5'-C6), 136.24 (3'-C6), 150.41 (3'-C2), 150.44 (5'-C2), 163.7 (5'-C4, 3'-C4), 166.0 (CONHOH), 168.3 (CONHC) ppm.

HRMS (ESI): $m/z$ calc. 565.1900 [M - H], found: 565.1906
Synthesis of 3’-oxyamine 188 from alcohol 171

Phthalimide 189 was prepared according to a published procedure.\(^{245}\)

Inverted thymidine 171 (3.00 g, 5.51 mmol) was dissolved in dry THF (50 mL) under argon and PPh\(_3\) (1.88 g, 7.16 mmol) and \(N\)-hydroxypthalimide (1.17 g, 7.16 mmol) were added. The solution was cooled to 0 °C and DIAD (1.4 mL, 7.13 mL) was added dropwise. The solution was slowly warmed to r.t. and stirred for 5 h. After this time, TLC analysis (EtOAc) showed the complete consumption of starting material (\(R_f = 0.5\)) and the formation of the product (\(R_f = 0.7\)). The reaction mixture was concentrated. Repeated flash column chromatography (EtOAc-petroleum ether, 1:1→2:1 followed by CH\(_2\)Cl\(_2\)-MeOH, 49:1→19:1) afforded the desired product 189 as a yellow foam (2.24 g, 59%).

\(\nu_{\text{max}}/\text{cm}^{-1}\) (neat) 3179 (NH), 3060 (CH), 2932 (CH), 2836 (CH), 1733 (C=O), 1685 (C=O), 1607, 1508, 1466 (CH), 1370, 1248, 1176, 1112 (C-O), 1072 (C-O), 1031, 982, 877, 828, 790, 755, 727, 699.

\(^1\)H NMR (400 MHz, acetone-d\(_6\)): \(\delta = 1.46\) (d, \(^4J = 1.0\) Hz, 3 H, CH\(_3\)T), 2.58 (ddd, \(J = 6.3\) Hz, \(J_{1';2'a} = 8.8\) Hz, \(J = 14.9\) Hz, 1 H, 2'-a), 2.76-2.82 (m, 1 H, H2'b), 3.43-3.50 (m, 2 H, H5'a, H5'b), 3.78 (s, 6 H, 2 x OCH\(_3\)), 4.48-4.50 (m, 1 H, H4'), 5.27-5.29 (m, 1 H, H3'), 6.55 (dd, \(J_{1';2'b} = 6.4\) Hz, \(J_{1';2'a} = 8.8\) Hz, 1 H, H1'), 6.84-6.87 (m, 4 H, H\text{ar,DMTr}), 7.20-7.24 (m, 1 H, H\text{ar,DMTr}), 7.27-7.34 (m, 6 H, H\text{ar,DMTr}), 7.43-7.45 (m, 2 H, H\text{ar,DMTr}), 7.59 (app. d, \(^4J = 1.0\) Hz, 1 H, H6), 7.87-7.92 (m, 4 H, H\text{ar,Phth}), 10.00 (bs, 1 H, NH) ppm.

\(^{13}\)C NMR (100 MHz, acetone-d\(_6\)): \(\delta = 12.1\) (CH\(_3\)T), 37.3 (C2'), 55.5 (OCH\(_3\)), 64.7 (C5'), 83.0 (C4'), 85.2 (C1'), 87.7 (qC, DMTr), 89.2 (C3'), 111.3 (C5), 113.9 (DMTr), 217
3'-O-Amino-5'-O-(4,4'-dimethoxytrityl)thymidine (188)

Oxyamine 188 was prepared according to a published procedure.\(^{251}\) Phthalimide 189 (200 mg, 290 \(\mu\)mol) was suspended in MeOH (2 mL) and hydrazine hydrate solution (53 \(\mu\)L, 80\%, 867 \(\mu\)mol) was added. The reaction mixture initially became clear, followed by the formation of a precipitate. After 7 h, TLC analysis (EtOAc-petroleum ether, 2:1) showed the complete consumption of starting material (\(R_f = 0.4\)) and the formation of the product (\(R_f = 0.5\)). The reaction mixture was diluted with Et\(_2\)O (20 mL), washed with sat. aq. NaHCO\(_3\) (20 mL) and the aq. layer was extracted with Et\(_2\)O (3 x 20 mL). The combined organic layers were dried over MgSO\(_4\), filtered and concentrated. Purification by flash column chromatography (EtOAc-petroleum ether, 1:1) afforded the desired product 188 as a white foam (129 mg, 80\%).

\[\text{Oxyamine 188} \]

\[\text{Phthalimide 189} \]

\(\nu_{\text{max}}/\text{cm}^{-1}\) (neat) 3182 (NH), 3060 (CH), 2926 (CH), 2837 (CH), 1686 (C=O), 1607 (C=O), 1508, 1464 (CH), 1446, 1248, 1175, 1108 (C-O), 1032, 827, 791, 755, 701.

\(^1\)H NMR (400 MHz, acetone-\(d_6\)): \(\delta = 1.43\) (d, \(^4J = 1.1\) Hz, 3 H, CH\(_3\)), 2.42-2.54 (m, 2 H, H2‘a, 2’b), 3.41 (dd, \(J = 3.5\) Hz, \(J_{5'a,5'b} = 10.3\) Hz, 1 H, H5’a), 3.45 (dd, \(J = 3.1\) Hz, \(J_{5'a,5'b} = 10.3\) Hz, 1 H, H5’b), 3.79 (s, 6 H, 2 x OCH\(_3\)), 4.21-4.23 (m, 1 H, H4’), 4.90-4.93 (m, 1 H, H3’), 6.35 (dd, \(J = 6.0\) Hz, \(J = 8.4\) Hz, 1 H, H1’), 6.90-6.93 (m, 4 H, H\(_{\text{ar,DMTr}}\)), 7.23-7.28 (m, 1 H, H\(_{\text{ar,DMTr}}\)), 7.32-7.39 (m, 6 H, H\(_{\text{ar,DMTr}}\)), 7.49-7.52 (m, 2 H, H\(_{\text{ar,DMTr}}\)), 7.66 (app. d, \(^4J = 1.1\) Hz, 1 H, H6), 9.96 (bs, 1 H, NH) ppm.

\(^{13}\)C NMR (100 MHz, acetone-\(d_6\)): \(\delta = 12.1\) (CH\(_3\)), 38.1 (C2’), 55.5 (OCH\(_3\)), 65.2 (C5’), 83.4 (C3’), 84.1 (C4’), 85.3 (C1’), 87.6 (qC, DMTr), 111.1 (C5), 114.0 (DMTr), 127.8 (DMTr), 128.8 (DMTr), 129.1 (DMTr), 131.0 (DMTr), 131.1 (DMTr), 136.4
(C6), 136.5 (qC, DMTr), 136.6 (qC, DMTr), 145.9 (qC, DMTr), 151.3 (C2), 159.78 (qC, DMTr), 159.80 (qC, DMTr), 164.2 (C4) ppm.

HRMS: not found

**Attempted amide coupling of modified nucleosides 139 and 188**

\[
\text{C-(3'}-\text{O-(tert-Butyldimethylsilyl)-5'}-\text{deoxythymidin-5'}-\text{yl)-N(5'}-\text{O-(4,4'}-\text{dimethoxytrityl)-3'}-\text{amidothymidin-3'}-\text{yl) methyl malonate (190) (Attempted)}
\]

Carboxylic acid **139** (122 mg, 251 µmol) was dissolved in dry DMF (4 mL) under argon and cooled to 0 °C. EDC (62 mg, 323 µmol), HOAt (44 mg, 323 µmol) and DIPEA (56 µL, 321 µmol) were added and the solution was stirred at 0 °C for 1 h. Oxyamine **188** (90 mg, 161 µmol) was added and the solution was slowly warmed to r.t. and stirred for 3.5 d. After this time, TLC analysis (EtOAc-petroleum ether, 2:1) showed the complete consumption of malonate **139** \((R_f = 0.1)\) and the formation of a product \((R_f = 0.6)\). The reaction mixture was diluted with EtOAc (20 mL), washed with \(\text{H}_2\text{O}\) (20 mL) and the aq. layer was extracted with EtOAc (20 mL). The combined organic layers were washed with brine (20 mL), dried over MgSO\(_4\), filtered and concentrated to a brown foam. Purification by flash column chromatography (EtOAc-petroleum ether, 1:1) did not afford the desired product **190**. Starting material **40** was recovered (61 mg, 68%) and byproduct **191** was obtained.
2-C-(3'-O-(tert-Butyldimethylsilyl)-5'-deoxythymidin-5'-yl) methyl acrylate (191)

Compound 191 was obtained from the attempted synthesis of compound 190 as a colourless glassy solid (57 mg, 54%).

$\nu_{\text{max}}$/cm$^{-1}$ (neat) 3180 (NH), 3047 (CH), 2953 (CH), 2930 (CH), 2893 (CH), 2857 (CH), 1687 (C=O), 1471 (CH), 1439, 1362, 1275, 1252, 1196, 1131, 1100 (C-O), 1059, 1056, 1005, 938, 832, 776.

$^1$H NMR (400 MHz, acetone-d$_6$): $\delta = 0.11$ (2 x s, 6 H, CH$_3$TBDMS), 0.91 (s, 9 H, t-Bu$_{\text{TBDMS}}$), 1.84 (d, $^4J = 1.0$ Hz, 3 H, CH$_3$T), 2.20 (ddd, $J_{2'a,3'} = 3.2$ Hz, $J_{1',2'a} = 6.3$ Hz, $J_{2'a,2'b} = 13.5$ Hz, 1 H, H2'a), 2.35 (ddd, $J_{2'b,3'} = 6.2$ Hz, $J_{1',2'a} = 7.6$ Hz, $J_{2'a,2'b} = 13.5$ Hz, 1 H, H2'b), 2.68 (dd, $J_{4',5'a} = 8.1$ Hz, $J_{5'a,5'b} = 14.3$ Hz, 1 H, H5'a), 2.75 (dd, $J_{4',5'b} = 5.8$ Hz, $J_{5'a,5'b} = 14.3$ Hz, 1 H, H5'b), 3.73 (s, 3 H, CO$_2$CH$_3$), 4.04 (ddd, $J_{3',4'} = 3.2$ Hz, $J_{4',5'b} = 5.8$ Hz, $J_{4',5'a} = 8.1$ Hz, 1 H, H4''), 4.37 (app. dt, $J = 3.2$ Hz, $J = 6.2$ Hz, 1 H, H3'), 5.82 (d, $J = 1.1$ Hz, 1 H, C=CH$_a$Hb), 6.23 (d, $J = 1.1$ Hz, 1 H, C=CH$_a$Hb), 6.25 (dd, $J_{1',2'a} = 6.3$ Hz, $J_{1',2'a} = 7.6$ Hz, 1 H, H1'), 7.47 (app. d, $^4J = 1.0$ Hz, 1 H, H6), 10.00 (bs, 1 H, NH) ppm.

$^{13}$C NMR (100 MHz, acetone-d$_6$): $\delta = -4.7$ (CH$_3$TBDMS), -4.5 (CH$_3$TBDMS), 12.5 (CH$_3$T), 18.5 (qC, t-Bu$_{\text{TBDMS}}$), 26.1 (t-Bu$_{\text{TBDMS}}$), 36.8 (C5'), 40.1 (C2'), 52.2 (CO$_2$CH$_3$), 75.8 (C3'), 85.4 (C1'), 86.0 (C4'), 110.9 (C5), 127.9 (C=CH$_2$), 136.7 (C6), 137.9 (C=CH$_2$), 151.3 (C2), 164.3 (C4), 167.6 (CO$_2$CH$_3$) ppm.

HRMS (APCI$^+$): m/z calc. 425.2102 [M + H]$^+$, found: 425.2106
6.2 Synthesis of modified oligonucleotides

Oligonucleotides of the sequence 5'-XTAG CAG TCA GTC AGT CAT CGY-3', where X = modified nucleoside or thymidine and Y = OH, Cy3 or Biotin-TEG, were synthesised using solid-phase oligonucleotide synthesis. Standard DNA phosphoramidites, solid supports and reagents were purchased from Link Technologies and Applied Biosystems. Automated solid phase synthesis of oligonucleotides was performed on an Applied Biosystems 394 synthesiser. Synthesis was performed on 1.0 μmol scale involving cycles of acid-catalysed detritylation, coupling, capping, and iodine oxidation. All 2-cyanoethyl phosphoramidite monomers were dissolved in dry MeCN to a concentration of 0.1 M immediately before use. Standard DNA phosphoramidites were coupled for 60 seconds while extended coupling time of 10 min was used for modified phosphoramidites. Coupling efficiencies and overall synthesis yields, excluding coupling of the modified nucleotides, were determined by the inbuilt automated trityl cation conductivity monitoring facility and were ≥98.0% in all cases. The oligonucleotides, with the exception of 5'-amino-5’-deoxyoligonucleotides, were then cleaved from the solid support and protecting groups from the nucleobase and backbone were removed by heating in conc. aq. NH₄OH in a sealed tube for 5 h at 55 °C. 5'-Amino-5’-deoxyoligonucleotides were deprotected by washing with Et₂NH in MeCN (20%) followed by 0.4 M NaOH in MeOH-H₂O 4:1 for 10 min at r.t. and 2.5 h at 55 °C and desalted using NAP-5 columns (GE Healthcare). The oligonucleotides were purified by reversed-phase HPLC on a Gilson system using a Luna 10 μ C8 100Å pore Phenomenex column (10 x 250 mm) with a gradient of MeCN in triethylammonium bicarbonate (0% to 50% over 12.5 min, flow rate 4 mL/min), (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.6, buffer B: 0.1 M triethylammonium bicarbonate, pH 7.6, with 50% MeCN). Elution was monitored by UV absorption at 290 nm or 298 nm. Electrospray mass spectrometry of oligonucleotides was performed in H₂O using a Waters ESI HRMS. Data were processed using MaxEnt. Oligonucleotide concentrations were determined by measuring absorbance in H₂O at 260 nm.
### Experimental procedures

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>5'-Modification X</th>
<th>3'-Modification Y</th>
<th>Mass (calc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81a</td>
<td>thymidine</td>
<td>OH</td>
<td>6420 (6421)</td>
</tr>
<tr>
<td>81b</td>
<td>thymidine</td>
<td>Cy3</td>
<td>6927 (6928)</td>
</tr>
<tr>
<td>81c</td>
<td>thymidine</td>
<td>Biotin-TEG</td>
<td>6987 (6991)</td>
</tr>
<tr>
<td>90a</td>
<td>5'-oxyamide thymidine</td>
<td>OH</td>
<td>6477 (6478)</td>
</tr>
<tr>
<td>90b</td>
<td>5'-oxyamide thymidine</td>
<td>Cy3</td>
<td>6984 (6985)</td>
</tr>
<tr>
<td>90c</td>
<td>5'-oxyamide thymidine</td>
<td>Biotin-TEG</td>
<td>7045 (7048)</td>
</tr>
<tr>
<td>93a</td>
<td>5'-amino-5'-deoxythymidine</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>93b</td>
<td>5'-amino-5'-deoxythymidine</td>
<td>Cy3</td>
<td>-</td>
</tr>
<tr>
<td>93c</td>
<td>5'-amino-5'-deoxythymidine</td>
<td>Biotin-TEG</td>
<td>-</td>
</tr>
</tbody>
</table>

**Synthesis of 5'-phosphorylated oligonucleotide 82**

Oligonucleotide 81b (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 82.

**Squaramide-containing oligonucleotides 94a-c and 100a-b**

5'-amino oligonucleotide 93a-c (400-700 nmol) was dissolved in 1.0 M sodium borate buffer (1.0 mL, pH 8.5). Aq. dimethyl squarate solution (2 mL, 300 eq.) or aq. diethyl squarate solution (2 mL, 300 eq.) was added and the solution was left at r.t. for 3 h. After this time, unreacted squarate ester and salts were removed by NAP-10 column (GE Healthcare) according to manufacturer instructions to give 5'-squaryl monoamide oligonucleotides 94a-c and 100a-b.

**Squaramide-containing oligonucleotides 92a-b and 95a-b**

A solution of 5'-squaryl monoamide oligonucleotide 94a-b in H₂O (1.5 mM, 20 µL) was dissolved in a solution of Et₂NH in H₂O (125 mM, 80 µL) or MeNH₂ in H₂O (125 mM, 80 µL) and the solution was left at r.t. for 4 h. After this time, unreacted amine was removed by NAP-5 column according to manufacturer instructions to give 5'-squaryl diamide oligonucleotides 92a-b and 95a-b.
Squaramide-containing oligonucleotides 101a-b

A solution of 5'-squaryl monoamide oligonucleotide 100a-b in H$_2$O (50 µM, 280 µL) was mixed with a solution of ethanolamine in H$_2$O (150 mM, 20 µL) and the solution was left at r.t. for 19 h. After this time, unreacted amine was removed by NAP-5 column according to manufacturer instructions to give 5'-squaryl diamide oligonucleotides 101a-b.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>5'-Modification X</th>
<th>3'-Modification Y</th>
<th>Mass (calc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94a</td>
<td>5'-squaryl monoamide thymidine (MeO)</td>
<td>OH</td>
<td>6530 (6530)</td>
</tr>
<tr>
<td>94b</td>
<td>5'-squaryl monoamide thymidine (MeO)</td>
<td>Cy3</td>
<td>7036 (7037)</td>
</tr>
<tr>
<td>94c</td>
<td>5'-squaryl monoamide thymidine (MeO)</td>
<td>Biotin-TEG</td>
<td>7099 (7100)</td>
</tr>
<tr>
<td>101a</td>
<td>5'-squaryl monoamide thymidine (EtO)</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>101b</td>
<td>5'-squaryl monoamide thymidine (EtO)</td>
<td>Cy3</td>
<td>-</td>
</tr>
<tr>
<td>92a</td>
<td>5'-squaryl diamide thymidine (Et$_2$NR)</td>
<td>OH</td>
<td>6571 (6571)</td>
</tr>
<tr>
<td>92b</td>
<td>5'-squaryl diamide thymidine (Et$_2$NR)</td>
<td>Cy3</td>
<td>7077 (7078)</td>
</tr>
<tr>
<td>95a</td>
<td>5'-squaryl diamide thymidine (MeNHR)</td>
<td>OH</td>
<td>6528 (6529)</td>
</tr>
<tr>
<td>95b</td>
<td>5'-squaryl diamide thymidine (MeNHR)</td>
<td>Cy3</td>
<td>7036 (7036)</td>
</tr>
<tr>
<td>101a</td>
<td>5'-squaryl diamide thymidine (ethanolamine)</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>101b</td>
<td>5'-squaryl diamide thymidine (ethanolamine)</td>
<td>Cy3</td>
<td>-</td>
</tr>
</tbody>
</table>
6.3 Biological experimental procedures

6.3.1 Gel-based assays

SNM1A (698-1040) was stored as a 1.0 µM solution in reaction buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.05% Triton-X, 0.1 mg/mL BSA, 5% glycerol, 0.5 mM DTT).

All incubations were carried out with gentle shaking (80 rpm).

Inhibition assay of modified nucleosides
Modified nucleosides were added to the assay in 40% DMSO solution (10 mM or as specified). Modified nucleosides (10 nmol or as specified) were mixed with SNM1A (698-1040) (25 fmol) in reaction buffer containing 4% DMSO (10 µL) on ice and incubated at 37 °C for 5 min. Phosphorylated oligonucleotide 82 (0.8 pmol) was added and the reaction was incubated at 37 °C for 60 min. The reaction was stopped by the addition of stop solution (2 µL, 95% formamide, 10 mM EDTA) followed by heating to 95 °C for 3 min. The products of the reaction were analysed using gel electrophoresis; 3 µL of each reaction was used.

Stability assay of modified oligonucleotides
Oligonucleotides 82, 90b, 92b, 94b, 95b and 101b (0.8 pmol or as specified) were mixed with SNM1A (698-1040) (25 fmol or as specified) in reaction buffer (10 µL) on ice and incubated at 37 °C for 60 min or as specified. The reaction was stopped by the addition of stop solution (2 µL, 95% formamide, 10 mM EDTA) followed by heating to 95 °C for 3 min. The products of the reaction were analysed using gel electrophoresis; 3 µL of each reaction was used.

Inhibition assay of modified oligonucleotides
Oligonucleotides 81a, 90a, 92a, 94a, 95a and 101a (0.8 pmol) and phosphorylated oligonucleotide 82 (0.8 pmol) were mixed with SNM1A (698-1040) (25 fmol) in reaction buffer (10 µL) on ice and incubated at 37 °C for 60 min. The reaction was stopped by the addition of stop solution (2 µL, 95% formamide, 10 mM EDTA) followed by heating to 95 °C for 3 min. The products of the reaction were analysed using gel electrophoresis; 3 µL of each reaction was used.
**Gel electrophoresis**

Oligonucleotides were separated on a 15% acrylamide 6.5 M urea gel (2.9 g urea, 2.7 mL 40% acrylamide-bisacrylamide 25:1, 1.4 mL 5X TBE (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA pH 8.0), 0.6 mL H$_2$O) in 1X TBE at 150 V for 60-90 min alongside bromophenol blue and xylene cyanol as markers for 8 nt and 28 nt, respectively, and imaged using Typhoon FLA 9500 (Method Cy3).

**6.3.2 Membrane permeability assay**

Permeability studies were carried out using the lipid–PAMPA method described by Merck Millipore.$^{259}$ A 96-well MultiScreen Filter Plate (Merck), with underdrain removed, was used as the donor plate, and a 96-well MultiScreen Transport Receiver Plate (Merck) as the acceptor plate. Carbamazepine and furosemide were used to confirm the integrity of the membrane. Solutions of hydroxamic acid 63 (500 µM), carbamazepine (500 µM) and furosemide (500 µM) in PBS pH 7.4 containing 5% DMSO were prepared. PBS pH 7.4 containing 5% DMSO (300 µL) was added to each well of the acceptor plate. A solution of lecithin in dodecane (5 µL, 1% w/v) was added to the filter within each donor well to form an artificial membrane. The drug solutions (150 µL) were immediately added to each well of the donor plate in quadruplicate. The donor plate was then placed into the acceptor plate and incubated at room temperature for 16 h. After the incubation, a sample of each donor well solution (100 µL) and of each acceptor well solution (250 µL) were transferred into a UV-star 96-well plate (Greiner Bio-one) and solutions of the equilibrium concentration were prepared by combining drug solution (150 µL) and PBS containing 5% DMSO (300 µL) and samples of the equilibrium solution (250 µL) were also measured. Absorbance was measured from 250-500 nm using a SpectraMax Plus 384 plate reader (Molecular Devices). Concentrations were determined using a calibration curve. The effective permeability $P_e$ was calculated using the following equation:

$$P_e = -\ln(1 - r) \left( \frac{V_D V_A}{(V_D + V_A) A t_i} \right)$$

where

$$r = \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}$$

$V_D = $ volume of donor well, 0.15 cm$^3$  
$V_A = $ volume of acceptor well, 0.30 cm$^3$  
$A = $ area of the filter, 0.3 cm$^2$  
$t_i = $ incubation time, 57 600 s

$P_e$ (Hydroxamic acid 63) = $3.01727 \times 10^{-8}$

$\log P_e$ (Hydroxamic acid 63) = -7.5
7 References


162. Dyrkheeva, N. S.; Khodyreva, S. N.; Sukhanova, M. V.; Safronov, I. V.; Dezhurov, S. V.; Lavrik, O. I., 3′–5′ exonuclease activity of human apurinic/apyrimidinic endonuclease 1 towards DNAs containing dNMP and their


164. Sukhanova, M. V.; D’Herin, C.; Auffret van der Kemp, P.; Koval, V. V.; Boiteux, S.; Lavrik, O. I., Ddc1 checkpoint protein and DNA polymerase ε interact with nick-containing DNA repair intermediate in cell free extracts of Saccharomyces cerevisiae. *DNA Repair 2011*, 10 (8), 815-825.


208. Miller, M. J.; Anderson, K. S.; Braccolino, D. S.; Cleary, D. G.; Gruys, K. J.; Han, C. Y.; Lin, K.-C.; Pansegrau, P. D.; Ream, J. E.; Douglas Sammons, R.; Sikorski, J. A., EPSP synthase inhibitor design II. The importance of the 3-phosphate group for ligand binding at the shikimate-3-phosphate site & the


234. Tornøe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem.* **2002**, *67* (9), 3057-3064.


238. El-Sagheer, A. H.; Sanzone, A. P.; Gao, R.; Tavassoli, A.; Brown, T., Biocompatible artificial DNA linker that is read through by DNA polymerases and is


266. Dürr, E.-M.; McGouran, J. F., Probing the Binding Requirements of Modified Nucleosides with the DNA Nuclease SNM1A. *Molecules* 2021, 26 (2), 320.


