Design and Synthesis of Triazole-Based Inhibitors of the DNA Repair Enzyme Alkyladenine Glycosylase (AAG)

by

Balqees Al Yahyaei

A Thesis Submitted for the Degree of Doctor of Philosophy

Department of Chemistry
Faculty of Engineering and Physical Sciences
University of Surrey
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Declaration

The work contained in this thesis was carried out in the Department of Chemistry, University of Surrey between October 2013 and September 2017. All the work is my own unless otherwise indicated. It has not previously been submitted for a degree at this or any other university.

Student’s name: Balqees Al Yahyaei
Date: September 2017
Abstract

The base excision DNA repair (BER) enzyme alkyladenine glycosylase (AAG) can drive DNA damage-induced cell death in specific cell types in mice and can induce frameshift mutagenesis and microsatellite instability in yeast and in human cells. It was hypothesised that humans with overactive AAG, or who encounter higher levels of alkylating agents in the form of pollution, diet or chemotherapy, or suffer an ischaemic reperfusion event such as a stroke, may incur increased tissue damage through this mechanism. An inhibitor of AAG is required to further study this mechanism and form a potential lead for future drug discovery.

In previous work, to discover an inhibitor, a published X-ray co-crystal structure of AAG was used in a virtual screen of two million compounds for potential binding activity. Of the top 49 virtual hits, one real hit triazole-thione-based inhibitor (UNIS00021) with an IC$_{50}$ of ~60 µM was identified in a biochemical assay. In this thesis, efforts to design and synthesise analogues of UNIS00021 with improved potency against AAG are described.

Successful divergent syntheses were developed which provided access to: 1. analogues varying at the alkyl group of the amide (six different amides); 2. analogues with a free amine in place of the amide and with variation of the length of the alkyl linkage group (five different amines); and 3. analogues bearing a C$_{5}$-methyl group instead of thiol/thione at the core (one cyclohexylamide triazole). Work was also begun on the synthesis of analogues varying the N$_{4}$-CH$_{2}$-aryl group but was not completed due to time constraints.

Two main types of microplate biochemical assay were investigated for assessment of the candidate inhibitors’ potencies against AAG using: 1. a surface-bound fluorescein-conjugated substrate DNA-oligomer; and 2. a free substrate oligomer and LCMS. Despite much experimentation, these assays continued to show inconsistent and irreproducible inhibition curves so it was not possible to make conclusions about the candidate inhibitors’ potencies.
Acknowledgements

First and foremost all thanks go to ALLAH ALMIGHTY, worthy of all prayers, the most beneficent the most merciful, without ALLAH's help none of this could have happened.

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# Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>$^{13}$C NMR</td>
<td>carbon-13 Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>$^{1}$H NMR</td>
<td>proton Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>3MeA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>5'-dRP</td>
<td>5'-deoxyribose-phosphate</td>
</tr>
<tr>
<td>7-deaza-Hx</td>
<td>7-deazahypoxanthine</td>
</tr>
<tr>
<td>7MeG</td>
<td>7-methylguanine</td>
</tr>
<tr>
<td>AAG</td>
<td>alkyladenine glycosylase</td>
</tr>
<tr>
<td>AIC</td>
<td>5-amino-4-imidazolecarboxamide</td>
</tr>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>AP</td>
<td>apurinic/apyrimidinic</td>
</tr>
<tr>
<td>APE1</td>
<td>apurinic/apyrimidinic endonuclease 1</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>c</td>
<td>concentration (g/mL)</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethyl sulfate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>dt</td>
<td>doublet of triplets</td>
</tr>
<tr>
<td>edGuo</td>
<td>1,N2-etheno-2'-deoxyguanosine</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FTIR</td>
<td>fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography - mass spectrometry</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond coherence</td>
</tr>
<tr>
<td>HNE</td>
<td>trans-4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HRP</td>
<td>(goat anti-fluorescin) horseradish peroxidases</td>
</tr>
<tr>
<td>Hx</td>
<td>hypoxanthine</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography - mass spectrometry</td>
</tr>
<tr>
<td>LP-BER</td>
<td>long-patch base excision repair</td>
</tr>
<tr>
<td>LPO</td>
<td>lipid peroxidation</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge ratio</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MeOSO$_2$Et-Lex</td>
<td>MeOSO$_2$(CH$_2$)$_2$-lexitropsin</td>
</tr>
<tr>
<td>MGMT</td>
<td>O$_6$-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methanesulfonate</td>
</tr>
<tr>
<td>MNU</td>
<td>methyl nitrosourea</td>
</tr>
<tr>
<td>MTIC</td>
<td>5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide</td>
</tr>
<tr>
<td>mtSSB</td>
<td>mitochondrial single-stranded binding protein</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>NNN</td>
<td>N'-nitrosonornicotine</td>
</tr>
<tr>
<td>O$_6$MeG</td>
<td>O$_6$-methylguanine</td>
</tr>
<tr>
<td>Pol β</td>
<td>polymerase β</td>
</tr>
<tr>
<td>pyr:T</td>
<td>pyrrolidine opposite thymine duplex oligomer</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>RONS</td>
<td>reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>RP</td>
<td>retinitis pigmentosa</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
</tbody>
</table>
s  singlet
SAM  S-adenosylmethionine
SP-BER  short-patch base excision repair
SSB  single stranded DNA break
t  triplet
TMB  3,3',5,5'-tetramethylbenzidine
TLC  thin layer chromatography
WT  wild type
δ  chemical shift in ppm
εA  ethenoadenine
List of UNIS compounds and their structures

Compounds thought to inform structure-activity relationships for inhibition of AAG and to be tested in the bioassays were labelled with a code beginning **UNIS000** and are shown in the following figure:
1. Introduction

1.1 General Introduction and Project Aim

Alkyladenine glycosylase (AAG) is one of the monofunctional glycosylase enzymes responsible for initiating the base excision DNA repair (BER) pathway. AAG protects against alkylative and oxidative DNA damage which can interfere with DNA replication and transcription and would otherwise lead to mutagenesis and cytotoxicity.\(^1\) AAG initiates BER by flipping substrate damaged nucleotides out of the DNA double-helix (through bond rotations about the phosphodiester-deoxyribose group) and into its active site where it facilitates hydrolytic cleavage of the $N$-glycosidic bond to release the damaged nucleobase.\(^2\) Downstream enzymes replace the resulting abasic site with the correct nucleotide. The BER pathway and AAG’s structure and mechanism will be described in detail in Sections 1.2.2 and 1.3, respectively.

However, AAG has been shown to drive DNA damage-induced cell death in certain tissue types in mice, for example leading to alkylation-induced retinal degeneration,\(^3\) and cell/tissue damage in the cerebellum, spleen, thymus, liver, brain and kidney.\(^4,5\) AAG has also been implicated in tissue damage during experimentally induced ischaemia/reperfusion in mice, a procedure that can be used to simulate stroke.\(^5\) Finally, AAG overexpression has been associated with increased risk for lung cancer in humans.\(^6\) These detrimental activities of AAG will be discussed in detail in Section 1.3.2.

Given the above, small molecule inhibitors of AAG would be beneficial for use as tool molecules for the investigation of DNA damage-induced cell death mediated by AAG and its role in disease. Also, such inhibitors could be used as drug
discovery leads for 'chemoprotectives' for patients undergoing alkylation-based chemotherapy or as a rapid treatment for stroke.

Known inhibitors include DNA oligomers containing either an ethenocytidine nucleotide or an abasic pyrrolidine nucleotide inhibit AAG *in vitro* with a 50% inhibitory concentration (IC$_{50}$) of 39 nM and a K$_d$ of 21 ± 3 pM, respectively.$^7, ^8$ However, these inhibitors are 13 nucleotides in length and too polar to work in cell and animal models or be suitable as drug leads *in vivo*. During the research reported in this dissertation, the polyhydroxy natural product flavonoid morin was also identified as an inhibitor with IC$_{50}$ of 2.6 uM.$^9$

The aim of this project was to design and synthesise small molecule inhibitors of AAG which are potent (IC$_{50}$ < 100 nM), selective for AAG, cell permeable and not rapidly metabolised. Inhibitor discovery was based on the hit triazole-thione **UNIS00021** (Figure 1.1) which was discovered using a high throughput virtual screen of ~2 million lead-like (250 ≤ MW < 350, logP < 3.5) compounds.$^{10}$ Biochemical assay showed it to have an IC$_{50}$ of ~68 µM against AAG. Inhibitors with improved potency and properties were therefore sought through the synthesis and testing of analogues which vary at the triazolothione core, the benzyl group and the amide, as will be discussed in detail in Section 1.6.

![Figure 1.1: UNIS00021](image-url)
1.2 DNA Damage and Repair

Genomic DNA is constantly attacked by endogenous and exogenous DNA damaging agents and most damage occurs in the form of lesions to the DNA bases. Due to environmental factors and metabolic processes inside the cell, the DNA damage is estimated to occur at a rate of 1 000 to 1 000 000 molecular lesions per cell per day. Such damage can generate errors in the transcription of DNA and, following that, mistranslation of proteins that are necessary for signalling and cellular function. In addition, if damaged DNA is not repaired prior to cellular replication, genomic mutations can be carried over to the following generations of cells. That may lead to cell death or, in the case of a number of mutations in tumor suppressor genes and oncogenes, cancer.

1.2.1 Causes of DNA damage

DNA damage can result from exposure to reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation (LPO) products and other reactive alkylating agents.

ROS are usually formed from metabolic and other biochemical reactions in all living cells. If ROS production exceeds the body’s natural antioxidant defence mechanisms, oxidative stress occurs which leads to the damage of DNA, lipids, and proteins. ROS include hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), and hydroxyl radicals (OH$^-$) which damage DNA directly by oxidising the bases or breaking the DNA backbone. Endogenous RNS include nitric oxide and peroxynitrite.

An indirect cause of DNA damage by ROS involves peroxidation of lipids to give LPO products which lead to the generation of etheno DNA adducts – nucleobases which possess extra five membered rings. Malondialdehyde (MDA) 1 and trans-4-hydroxy-2-nonenal (HNE) 2 are examples of LPO reactive aldehyde products that
generate etheno adducts such as 1,N\textsuperscript{2}-etheno-2'-deoxyguanosine (1,N\textsuperscript{2}-\textepsilon dGuo), ethenoadenine (\textepsilon A) and ethenocytidine (\textepsilon C) (Figure 1.2).\textsuperscript{16}

![Figure 1.2: Structures of two of LPO-derived reactive aldehydes and their induced etheno adducts.\textsuperscript{17}](image)

The mechanism by which HNE can ethenylate DNA bases is shown for the example of \textepsilon A in Scheme 1.1.\textsuperscript{16}

![Scheme 1.1: Proposed mechanism for the formation of \textepsilon A by HNE.\textsuperscript{17}](image)

Other alkylating agents react with exocyclic oxygen (\textit{O}) and exo- and endocyclic ring nitrogen (\textit{N}) atoms of DNA bases.\textsuperscript{18} S-Adenosylmethionine (AdoMet or SAM) 4 is an example of weak endogenous methylating agent whose reactivity stems
from the positive charge on the sulfur atom making the methyl group electrophilic. It has been shown to induce mutations in DNA mainly through generating 3-methyladenine (3MeA) and 7-methylguanine (7MeG) (Figure 1.3).\textsuperscript{19}

![Methyladenine and Methylguanine](image)

\textit{Figure 1.3:} S-Adenosyl-L-Methionine with two of its mutated base products

Exogenous alkylating agents are found in low concentrations in air, food, and water and can be from natural food plant metabolism, drugs or pollution. One example are \textit{N}-nitroso compounds formed in tobacco smoke. Among these compounds the most effective are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) 5, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) 6, and \textit{N}'-nitrosonornicotine (NNN) 7 and it has been reported that they give rise to the DNA alkylations by generating $O^6$-methylguanine ($O^6$-MeG), 7-meG, and $O^4$-methylthymine ($O^4$-MeT) shown in Scheme 1.2.\textsuperscript{20}
DNA damage is purposefully induced during many types of cancer chemotherapy. The drugs involved are mostly chloroethylating agents (e.g. lomustine, carmustin, and fotemustine) or methylating agents (e.g. temozolomide and streptozotocin). For example, temozolomide (TMZ) is an alkylating agent prodrug which delivers a methyl group to purine bases of DNA. It has been reported that temozolomide first binds noncovalently in the major groove of DNA. It then undergoes ring-opening and decarboxylation to give the bioactive agent 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC) (Scheme 1.3). Fragmentation of this liberates 5-amino-4-imidazolecarboxamide (AIC) and methyldiazonium cation. DNA methylation by the reactive methyldiazonium intermediate is facilitated by the basic microenvironment of guanine-rich sequences.
Different factors contribute to the damage profile induced by a given alkylating agent. One is the number of reactive sites in the alkylating agent. Those with one reactive group are called monofunctional alkylating agents, (e.g. Dacarbazine 12, Temozolomide 8, and Procarbazine 13) while those with two are bifunctional alkylating agents (e.g. Altretamine 14, Mitomycin C 15, and Thiotepa 16), as shown in Figure 1.4, and can cross-link DNA.

In addition, the type of chemical reactivity of the alkylating agents affects its damage profile – whether the mechanism proceeds with more S_N1 or S_N2 character influences the alkylation of DNA bases. S_N1-like alkylating agents, which tend to react via cationic intermediates such as methyldiazonium ions, can target both ring nitrogen atoms and exocyclic oxygen atoms in DNA bases due to their hard electrophilic nature and preference for reaction with atoms bearing more electron density. S_N2 alkylating agents preferentially target softer ring nitrogen atoms. Many of the anticancer drugs are monofunctional S_N1-like methylating agents such us, dacarbazine 12, and procarbazine 13.
1.2.2 Base Excision Repair (BER)

DNA base damage includes deaminated cytosine, deaminated adenine, 5-methylcytosine and oxidation and alkylation products of the four bases. However, most of these lesioned base units do not go on to harm the cell since they are repaired by endogenous pathways such as the base excision repair (BER) pathway. The BER pathway can be reconstructed in vitro with a glycosylase, AP endonuclease, polymerase β, and a ligase. The steps involved are shown in Scheme 1.4 and described below.\textsuperscript{11}
The process starts with recognition and hydrolysis of the damaged base from the deoxyribose-phosphate backbone by initiator enzymes known as DNA glycosylases, such as alkyladenine DNA glycosylase (AAG). The initiator enzymes can be divided into two mechanistic subclasses: the monofunctional glycosylases and the bifunctional glycosylases.\textsuperscript{12}

Monofunctional DNA glycosylases only catalyse the hydrolysis of the $N$-glycosidic bonds of damaged residues, and do not cleave the phosphodiester bonds.\textsuperscript{22} This is achieved by flipping the damaged base out of the double helix (through bond rotations about the deoxyribose-phosphate backbone) and intercalating an aromatic amino acid into the resulting space left in the DNA. With the damaged

Scheme 1.4: The base excision repair (BER) pathway of the monofunctional DNA glycosylases
base now undergoing several intermolecular interactions in its active site, its leaving group ability is increased and the enzyme catalyses hydrolysis of its N-glycosidic bond using an activated water molecule as a nucleophile which attacks the anomeric carbon atom.\textsuperscript{24,25} This leaves an apurinic/apyrimidinic (AP) site which must be repaired by downstream enzymes. The first of these is apurinic/apyrimidinic endonuclease 1 (APE1) which hydrolyses the phosphate P-O bond at 5' to the AP site generating a single stranded DNA break (SSB) which will leave a 3’-OH group and a 5’-deoxyribose-phosphate moiety (5’-dRP) at the termini next to the nucleotide gap. The abasic 5’-dRP is then removed by the next enzyme in the pathway, polymerase β (Pol β), to leave a complete nucleotide gap on which Pol β acts to replicate the correct replacement nucleotide (matching the opposite DNA strand) onto the 3’-OH terminus. After this step, a break in the polymer still remains between the 3’-OH of the new nucleotide and the 5’-phosphate terminus. This is joined to make the required phosphodiester group by the enzyme DNA ligase.

Alternatively, bifunctional DNA glycosylases do not use an active site water molecule to hydrolyse the damaged base but an N-terminal proline or lysine side chain amine moiety which results in an imine (Schiff base) between the anomeric carbon and the enzyme (\textbf{Scheme 1.5}).\textsuperscript{26} These enzymes then also possess AP lyase activity which eliminates the 3’-phosphate group from the damaged nucleotide to leave an α,β-unsaturated imine which undergoes hydrolysis to the α,β-unsaturated aldehyde. Downstream enzyme APE1 recognises these as substrates and hydrolyses the phosphate P-O bond at 5' to the AP site to leave a
nucleotide gap. The remaining steps are the same as for monofunctional DNA glycosylases.

Scheme 1.5: The base excision repair (BER) pathway initiated by bifunctional DNA glycosylases.²⁶

BER can then proceed through either short-patch (SP-BER) or long-patch (LP-BER) processes. In SP-BER a single nucleotide is introduced by polymerase β and then the DNA nick is sealed by the enzyme ligase IIIα. In the LP-BER polymerase α/ε introduces two to eight nucleotides past the abasic site. Then the DNA nick is sealed by DNA ligase I after the removal of the DNA overhang by FEN1 endonuclease.¹²
1.3 Alkyladenine Glycosylase (AAG)

1.3.1 AAG Structure and Mechanism

The project described in this thesis is concerned with the monofunctional DNA BER glycosylase alkyladenine DNA glycosylase (AAG\textsuperscript{27}), also known as methylpurine-DNA glycosylase (MPG), and alkyl N-purine glycosylase (ANPG).\textsuperscript{28} It is able to recognise and excise several different alkylated bases including 3-methyladenine (3MeA) and 7-methylguanine (7MeG) which can be caused by alkylating agents such as methyl methanesulfonate (MMS) (Figure 1.5). It also recognises damaged bases caused directly or indirectly (through LPO) by reactive oxygen and nitrogen species (RONS) such as 1,N\textsuperscript{6}-ethenoadenine (εA), deaminated adenine (hypoxanthine, Hx), and deaminated guanine (xanthine).\textsuperscript{29,30,31}

Several crystal structures of AAG in complex with DNA oligomers containing lesions have been published.\textsuperscript{1} Figure 1.6 shows that (PDB: 1EWN) of E125Q mutant AAG bound to a DNA oligomer containing εA, one of AAG’s substrates.\textsuperscript{32} The mutation is for the glutamate residue which normally holds and deprotonates the active site nucleophilic water molecule and so glycosylase activity is removed.
Interestingly, in the same study, it was also found that purified active AAG protein is inhibited by MgCl$_2$, which was contained in the crystallisation buffer. Thus, a crystal structure of wild-type AAG in complex with an εA-containing DNA oligomer could be solved. The reason for inhibition by MgCl$_2$ is unknown and no electron density for a Mg$^{2+}$ ion was observed in the crystal structure.

Figure 1.6: Crystal structure of AAG with DNA containing ethenoadenine lesion (PDB: 1EWN) and ligand interactions$^{33}$ [drawn using PyMOL]

Based on this and other X-ray crystal structures, a mechanism by which AAG catalyses the hydrolysis of the base from the nucleotide and differentiates between normal adenine and guanine and the damaged bases has been proposed.$^8$ AAG flips the damaged base, in this case εA, to the outside of the DNA through rotations about the phosphodiester backbone. The εA base fills a pocket in the active site where it π-stacks on top of Tyr-127 and makes an edge-to-face π-stacking interaction with Tyr-159 above. It also stacks against His-136 and makes a hydrogen bond between N$^6$ of εA and His-136. Meanwhile, Tyr-162
intercalates into the DNA duplex between the bases that were flanking εA to fill the empty site in the DNA. Lau. et al. generated a mutant AAG where Tyr-162 was replaced with alanine (Y162A mutant) and showed it to have minimal glycosylase activity and to only weakly bind to εA-DNA and pyr-DNA (an inhibitory oligomer containing an abasic pyrrolidine nucleotide mimetic, discussed later) \textit{in vitro}, showing the importance of this intercalation mechanism. Furthermore, cells expressing the Y162A mutant were observed to show higher sensitivity to MMS than the wild-type.\footnote{32}

AAG cleaves the \textit{N}-glycosylic bond between the εA base and the deoxyribose sugar through acid/base catalysis and a nucleophilic water molecule.\footnote{34} This water molecule is held in place by hydrogen bonds with residues Val-262 and Arg-182 and is deprotonated by Glu-125 to facilitate attack of the anomeric position and glycosyl bond cleavage (Figure 1.7).\footnote{32} The crystal structure of the εA complex (PDB: 1EWN) also showed that N\textsuperscript{7} of εA was in contact with another water molecule and the same is observed if hypoxanthine (Hx) is modelled onto the same crystal structure as the damaged base.\footnote{8} This water molecule is thought to protonate the base and the protonated form is then stabilized by a hydrogen bond between N\textsuperscript{7}H\textsuperscript{+} of εA and the backbone carbonyl oxygen atom of Ala-134.
The lesioned bases that are recognised by AAG do not appear to have common structural features. Some of them, such as 3MeA and 7MeG, are positively charged which could be selectively recognised by a tight-binding interaction with an aromatic side chain of the AAG active site, and they are already good leaving groups that weaken the glycosylic bond. However, other lesioned bases are neutral such as Hx and εA and they are still recognised and cleaved by AAG. Lau. et. al. proposed some reasons for AAG’s specificity towards its substrate damaged bases based on the shape and electrostatic properties of the binding pocket of the enzyme. Comparing εA with adenine in the AAG active site, a hydrogen bond is donated from the backbone amide of His-136 to the etheno N6 nitrogen of εA in the AAG crystal structure (Figure 1.8) and it has a large aromatic surface that is snugly accommodated into the active site. Yet, in adenine (A) the 6-amino group cannot accept a hydrogen bond so easily due to delocalisation of the lone pair. Hypoxanthine, another of AAG’s substrates, is able to make the same hydrogen bond using its O-atom. In the case of guanine (G), which could also make the hydrogen bond, there is a steric clash between its 2-amino group and the Asn-169 side chain which disfavors its binding. Although the alkylated base, and AAG

Figure 1.7: Mechanism in the AAG active site with εA [reproduced from reference 33].
substrate, 7-methylguanine also has a 2-amino group, it is positively charged which increases its interaction with Tyr-127 and His-136 by cation-π interaction and that would be enough to outweigh the steric clash with Asn-169.\textsuperscript{32}

Figure 1.8: AAG catalytic specificity towards damage bases based on the shape and electrostatic properties of the binding pocket of the enzyme.\textsuperscript{2}

1.3.2 Reasons to inhibit AAG

1.3.2.1 Alkylation-Induced Retinal Degeneration

Growing research suggests that increased levels of certain DNA repair enzymes can result in an imbalance of intermediates between the enzymatic steps in the BER pathway and that can negatively affect cellular growth.\textsuperscript{6,35,36} It was found that the accumulation of BER intermediates such as the AP sites and single strand breaks (SSB) is cytotoxic.\textsuperscript{37,38} These two repair intermediates can block transcription and replication which can lead to cell death.\textsuperscript{37}

In the retina there are two types of neuronal photoreceptor cells which are responsible for perception of light and colour; they are called rods and cones. It has been found that retinal degeneration via photoreceptor cell death leads to blindness and is a hallmark of a group of diseases together referred to as retinitis pigmentosa (RP).\textsuperscript{39} Oxidative stress and oxidation-dependent DNA damage are thought to play roles in RP-associated photoreceptor apoptosis. It has been shown that exposure to excessive light stimulates the shedding of rod outer segments
which are subsequently phagocytosed by retinal pigment epithelium (RPE) cells in a process that generates an excess of reactive oxygen and nitrogen species (RONS).\textsuperscript{40,41} In addition, studies have shown that treatment of rodents with the \textit{Sn1} DNA alkylating agent methyl nitrosourea (MNU) causes retinal degeneration. MNU reacts with both oxygen and nitrogen atoms in DNA to form $O^6$-methylguanine ($O^6$MeG), 7MeG, and 3MeA.\textsuperscript{42}

In 2009 Meira \textit{et al.} showed that retina photoreceptor cell degeneration was also observed in mice treated with the \textit{Sn2} alkylating agent, methyl methanesulfonate (MMS).\textsuperscript{3} They showed that there was a dramatic reduction in the number of photoreceptors in the outer nuclear layer of the retina of wild-type (\textit{Aag}\textsuperscript{+/+}) mice treated with MMS. On the other hand, mice with the \textit{Aag} gene knocked out (\textit{Aag}\textsuperscript{-/-}) treated with MMS did not show a reduction of photoreceptors and a heterozygous group (\textit{Aag}\textsuperscript{+/-}) suffered intermediate levels of retinal degeneration. Therefore, it was concluded that alkylation-induced retinal cell death is proportional to the gene copy number of \textit{Aag}. It is now hypothesised that the action of AAG on alkylated bases leads to an accumulation of abasic sites and SSB with which the downstream enzymes cannot keep up and which eventually drive photoreceptors to death. The single strand breaks resulting from action of downstream enzyme APE1 are known to activate poly(ADP-ribose) polymerase 1 (Parp1) whose action can lead to the depletion of ATP and NAD$^+$ and resultant cell death.\textsuperscript{4}

Based on these studies, it is hypothesised that individuals with overactive AAG or those undergoing alkylation-based chemotherapy may be susceptible to retinal degeneration and an inhibitor of AAG could slow the production of abasic sites and single strand breaks thus reducing retinal degeneration.

\textbf{1.3.2.2 Ischaemic Stroke}

Ischemia/reperfusion (I/R)-induced tissue inflammation results from blockage of the blood supply to a tissue followed by release of the blockage and reperfusion of
the tissue with blood. It leads to a wide range of human diseases such as, myocardial infarction, hepatic and renal failure and stroke. I/R causes the production of large amounts of reactive oxygen and nitrogen species (RONS) that can lead to direct and indirect DNA damage which is a substrate for DNA repair by BER.\textsuperscript{5}

It was found that the absence of Uracil and Ogg1 DNA glycosylases lead to an increase in brain damage induced by I/R which indicate that BER initiated by these enzymes protects against I/R-induced tissue damage.\textsuperscript{43,44} In contrast, Ebrahimbhani \textit{et. al.} found that BER initiated by AAG increased the I/R-induced tissue damage in brain, liver and kidney in mice. For instance, it was found that in wild type (WT) mice, a protocol where a 90-min liver ischemia was followed by 24 h reperfusion resulted in hepatic injury that was much more severe than that found in \textit{Aag}^{-/-} liver.\textsuperscript{5} The authors correlate this increased toxicity with the accumulation of BER abasic sites in WT liver DNA. In \textit{Aag}^{-/-} liver DNA, the number of abasic sites induced was found to be reduced by half.\textsuperscript{45,46} The group also found that there was a high level of Parp1 activation combined with greater depletion of NAD\textsuperscript{+} and ATP which can lead to cell death, as described previously. Yet, in \textit{Aag}^{-/-} livers, which were treated similarly, there was a decrease in the activation of Parp1 which indicate that Aag-initiated BER promotes BER intermediate generation, Parp-1 activation, tissue injury and inflammation in liver following I/R.\textsuperscript{5}

1.3.2.3 Alkylation-Induced Degeneration of Specific Tissue Types

It was found that bone marrow (BM) cells are deficient in recognising and repairing DNA alkylation damage which makes them sensitive to alkylating agent cytotoxicity.\textsuperscript{47} However, a study by Roth and Samson showed unexpected alkylation resistance of \textit{Aag}^{-/-} BM cells compared to WT BM cells when using alkylating agents such as MMS and TMZ and \textit{ex vivo} BM survival assays.\textsuperscript{48}
Another study by Calvo et al. found that AAG also drives alkylation-induced cell damage in the rapidly proliferating tissues of the spleen and thymus as well as cells in the cerebellum. Alkylation-induced toxicity was apparent in wild-type mice but completely suppressed in Aag<sup>−</sup> mice. 4

Coquerelle et. al. showed that Chinese hamster ovary (CHO) cells overexpressing AAG are more sensitive to alkylating agents MMS and Dimethyl Sulfate (DMS) than normal ovary cells expressing low AAG activity. 49 In addition, these cells showed more double-strand breaks and greater inhibition of DNA replication compared to normal cells. 49

Repair of mitochondrial DNA (mtDNA) by AAG has also been investigated. Fishel. et al. engineered mitochondrial targeting AAG and showed that its overexpression increases cell death of breast cancer cells when treated with MMS 50. van Loon and Samson showed that wild type AAG naturally localises to mitochondria as a result of association with mitochondrial single stanced binding protein (mtSSB). 50-52 Fishel. et al. hypothesised that the overexpression of AAG in mitochondria leads to an imbalance in the BER pathway resulting in an accumulation of AP sites, or single-strand and double-strand breaks causing cell death. 50

1.3.2.4 Other toxicity correlating to AAG overactivity

A study related to AAG activity in lung cancer patients by Crosbie et al. showed it is not related to sex or smoking status but patients with high levels of AAG had a three fold increased probability of lung cancer compared to patients with lower Aag levels. 6 The association between AAG overexpression and increased breast cancer cell sensitivity to alkylation was also reported. 53 It is postulated that this is due to the imbalance in the BER pathway creating many abasic sites, overloading the proteins involved in the downstream steps and causing cytotoxic single and double strand break formation. 53
1.3.3 Possible Side Effects of AAG Inhibition

Inhibiting the AAG enzyme could result in detrimental side effects. For instance, Engelward et al. showed that AAG in mouse protects embryonic stem (ES) cells against MeOSO$_2$(CH$_2$)$_2$-lexitropsin (MeOSO$_2$Et-Lex), alkylating agent used for cancer chemotherapy, which is an S$_2$N$_2$ alkylating agent that forms almost exclusively 3MeA lesions.$^{54,55}$ They found that Aag null cells are more sensitive than WT and heterozygous cells to this methylating agent due to their inability to repair 3MeA which leads to cell death by blocking DNA replication.$^{55}$ Also, they found the same result using two other cancer chemotherapy agents: mitomycin C and chloroethylnitrosourea.$^{55}$ The same group showed that Aag$^{-/-}$ mouse ES cells are more sensitive than WT cells to chromosome damage.$^{56}$ However, they also found that Aag null ES cells divided normally although these cells show no detectable repair of 3MeA in vitro. They relate this result to one of three possible reasons: to a low level of formation of 3MeA causing no detectable change in growth rate; the replication process can bypass endogenous levels of 3MeA lesions; or other possible pathways that remove the 3MeA produced endogenously.$^{55}$ Meira et al. reported that AAG deficiency in mouse increases inflammation-associated colon tumorigenesis.$^{57}$ They found that Aag$^{-/-}$ mouse colon was more sensitive compared to Aag$^{+/+}$ mouse after treating them with carcinogen agents azoxymethane (AOM) and dextran sulfate sodium (DSS). They showed that 8-oxoG, εA, and εC induced by inflammation increased dramatically in the colonic epithelium of Aag$^{-/-}$ mice in response to DSS.$^{57}$

From all these reported results, we conclude that inhibiting AAG might protect several tissue types from cell death in the case of ischemic reperfusion or alkylative chemotherapy. However, such an inhibitor could be detrimental to developing embryos and could have tumorigenic effects in those suffering from inflammation of the colon.
1.4 Published Inhibitors of AAG

1.4.1 Pyrrolidine-Based Inhibitor

A double stranded 25 nucleotide DNA oligomer containing an abasic pyrrolidine nucleotide 17 has been reported to be a very potent inhibitor (Figure 1.9) of *Escherichia coli* 3-methyladenine DNA glycosylase II (AlkA).\(^7\)

![Figure 1.9](image)

*Figure 1.9*: The pyrrolidine nucleotide-containing DNA oligomer inhibitor (left, 17) and the proposed transition state for enzyme mediated glycosidic bond cleavage which it mimics.

In their study the authors proposed that the protonated pyrrolidine moiety mimics the positive charge developed in the transition state of the glycosidic bond cleavage reaction in DNA glycosylase active sites.\(^{28}\) Their experimental results showed that all DNA glycosylases, except uracil DNA glycosylase, interact with oligomer 17, including AAG which had a dissociation constant (for a pyrrolidine opposite thymine duplex oligomer, pyr:T) of 23 ± 4 pM.\(^{28}\) This high potency of pyrrolidine containing DNA was also proven by measuring the release of methylated bases from [\(^{3}\)H] dimethyl sulfate-treated calf thymus DNA by the DNA glycosylase enzymes in its presence. The pyrrolidine-duplex showed almost complete inhibition compared to a normal A-T duplex.

In 1998 Lau. *et al.* reported the crystal structure (PDB: 1F6O) of the AAG enzyme in complex with pyr:T oligomer 17.\(^{58}\) This showed the pyrrolidine rotated outwards from the duplex and into the AAG active site while AAG’s Tyr-162 intercalates into the empty space left in the DNA to stabilize the DNA distortion. A hydrogen
bonding network is found between the nucleophilic water molecule in the AAG active site, pyrrolidine, Glu-125, Arg-182, and the main chain carbonyl of Val-262. Comparing this with the crystal structure of AAG complexed with the εA-containing oligomer, described in Section 1.3.1, it is seen that the conformation of the protein is the same, including the pocket for binding the base despite the absence of a base in the pyrrolidine-oligomer. However, there is a slight rotation of the pyrrolidine ring away from the corresponding position of the εA-deoxyribose ring in its crystal structure and this is thought to be to allow hydrogen bonding between N\(^4\) of the pyrrolidine and the nucleophilic water molecule. However, when these crystal structures are compared with one of AAG in complex with another inhibitory oligomer containing the smaller base ethenocytidine (εC, see below) then the pyrrolidine group is in the same position as that deoxyribose ring (Figure 1.10) indicating the possibility that it is the larger εA which pushes the deoxyribose back.

**Figure 1.10:** Superposition of the active sites of the E125Q/εA-DNA (blue), E125Q/εC-DNA (pink), and E125Q/pyr-DNA (green) X-ray crystal structures.
1.4.2 3,N4-Ethenocytosine (εC)-containing DNA oligomer

Although AAG is able to excise a variety of lesions, there are still many which it fails to remove including 3-methyluracil, 3-ethyluracil, 3-methylcytosine, 3-methylthymine, and εC. Interestingly, it has been found that an εC-containing oligomer forms a stable, abortive complex with AAG and inhibits AAG activity in human cells. Such abortive complexes sequester AAG and can lead to the accumulation of more DNA damage. Lingaraju et al. compared the binding affinity of AAG to the εA:T duplex oligomer using 25 and 13-mers to that to the εC:G 13-mer. It was found that AAG binds to the εC:G 13-mer duplex ($K_d=21 \pm 3 \text{ nM}$) with ~2-fold higher affinity than to the εA:T 13-mer duplex ($K_d=46 \pm 6 \text{ nM}$) and that binding strength increased with increasing duplex length. Also, it was found that both the truncated AAG used for crystallography and full length AAG are inhibited by the εC:G 13-mer, according to a gel-based assay. Lingaraju et al. measured the IC$_{50}$ of the εC:G 13-mer duplex using competition DNA glycosylase assay and it was calculated to be 39 nM which was ~4-fold stronger than the εA:T 13-mer duplex (IC$_{50}=163 \text{ nM}$).

The crystal structure solved by the group showed that the εC base lesion is stabilised in the same AAG active site pocket that binds the εA by hydrogen bonds and van der Waals interactions. The way that AAG recognises the εC is similar to the way it does so for εA and that is achieved by a hydrogen bond donated from the main chain amide of His-136 to the N$^9$ of εC or N$^{12}$ of εA (Figure 1.11). However, there is an additional hydrogen bond between the carboxamide nitrogen of the side chain of Asn-169 and the O$^2$ of εC which is thought to increase the affinity of AAG for εC. That was proved by replacing the normal Asn-169 with leucine or alanine in N169L and N169A mutant forms of AAG and showing a ~2-4 fold reduced affinity for the εC:G 25-mer compared to the wild type AAG. The reason εC does not act as a substrate of AAG could be due to the failure of AAG to activate the it as a leaving group by protonation due to the presence of a carbon
atom ($C^5$) in $\varepsilon C$ where a basic nitrogen atom ($N^7$) is present in $\varepsilon A$ (Figure 1.11). This was also proven in their experiment comparing the substrate hypoxanthine (Hx) with non-substrate 7-deaza-Hx where the corresponding basic N-atom has been changed into a carbon atom: AAG failed to cleave the 7-deaza-Hx.\(^8\)

![Figure 1.11: Comparison of binding and excision of $\varepsilon A$ (left), in the AAG active site, with binding and non-excision of $\varepsilon C$ (right) and the structure of 7-deaza-Hx and Hx (box).](image)

Based on this, Chu et al. at the University of Surrey synthesised the single ethenocytidine nucleoside along with three phosphate derivatives to test their activity as small molecule inhibitors of AAG when not part of a larger oligomer. However, there was no inhibition up to 1 mM concentration which indicates the importance of the extended DNA strands in the inhibitory oligomer for action as an inhibitor.\(^10\)
1.4.3 Morin

In 2015, while the research described in this thesis was taking place, the naturally occurring polyphenol morin was reported by the Dixon group to be an inhibitor of AAG with an IC$_{50}$ of 2.6 µM.$^{62}$ In their report, a gel-based assay with εA-containing duplex oligonucleotides as substrate was used against a library of small molecules and morin was identified as a hit. They also calculated the IC$_{50}$ of morin in live cells and cell extracts to be ~100 and 300 µM, respectively. An important counter-assay in this work showed that morin does not bind to DNA thus eliminating that as a possible mechanism of inhibition.

The authors’ molecular docking of morin into the AAG crystal structure (PDB: 1EWN) revealed that the amino residues which interact with morin are His-136, Ala-134, and Val-262. They also showed that there is an interaction between the 5'-OH in the A ring of morin and Ala-134 and His-136 while the 2'-OH in the B ring of morin interacts with Val-262.

This docking was repeated for this dissertation using MOE (Figure 1.12) and compared with Dixon’s. The new result showed that Ala-134 and Val-262 were not close enough to the 5'-OH on the A ring or the 2'-OH on the B ring, respectively, to make strong hydrogen bonds. Interestingly the docking showed a hydrogen bond between the nucleophilic water molecule in the Aag active site and the carbonyl group in the C ring which might contribute to inhibition by morin.
Virtual Screen for Novel Inhibitors of AAG

In the absence of a small molecule lead in 2012, a virtual screen was set up using the published crystal structure of AAG bound to the inhibitory εC-containing oligomer (PDB: 3UBY). The digital structures of two million compounds were downloaded from the ZINC ‘lead-like, commercially available’ library which has been filtered to only include compounds with molecular weights of 250-350 Da, clogP ≤3.5 and rotatable bond count ≤ 7. The screen was carried out using the molecular modelling software MOE which generated the lowest energy conformation of each compound, docked it into the AAG structure and predicted a binding energy against which the compounds were ranked. The top 3000 molecules were re-screened using a more thorough docking mode and these were again ranked according to binding energy and visually inspected for binding to the nucleophilic water molecule and synthetic tractability. Forty-nine of these were purchased and tested for actual inhibition of AAG in a fluorescence-based biochemical assay. Of these only one showed real inhibitory activity of AAG;
UNIS00021 whose IC$_{50}$ was 60 µM (Figure 1.13 and Figure 1.14). In addition, it was shown to be a reversible inhibitor using a jump dilution bioassay$^{10}$ which involved incubation with AAG for 15 or 30 minutes followed by 100-fold dilution and monitoring of AAG activity by fluorescence over 60 min.$^{10}$

![UNIS00021](image)

**Figure 1.13:** UNIS00021

![AAG Inhibition by UNIS00021](image)

**Figure 1.14:** UNIS00021 IC$_{50}$ curve, % inhibition values calculated from apparent AAG activity obtained by interpolation of absorbance values into the AAG standard curve.

UNIS00021 has a central triazole-thione, which can act as a hydrogen bond donor and acceptor, and also an amide bond connected to a cyclohexyl group. Amide bonds play an important role in medicinal chemistry - about 25% of known drugs contain an amide group$^{64}$ and that is because amides are neutral, stable, have both hydrogen-bond acceptor and hydrogen-bond donor sites and are relatively easy to synthesise.

Figure 1.15 and Figure 1.16 show the predicted docking of UNIS00021 in crystal structure 3UBY, with εC-containing DNA oligomer removed, using MOE. Figure 1.15 is overlayed with εA-containing oligomer-AAG crystal structure 1EWN for
comparison. It is thought that the hydrogen bond between the amide N-H and AAG’s nucleophilic water molecule is of utmost importance because this water molecule is held in the active site by three hydrogen bonds with Val-262, Arg-182 and Gln-125 so it would be unfavourable to displace it with an inhibitor, but favourable to bond to it as is predicted for UNIS00021. Also of note is the phenyl ring of UNIS00021 which is inside the pocket normally occupied by an alkylated base. There is pi-pi stacking between the phenyl ring and Tyr-127. In addition, most of the cyclohexyl group is outside the pocket and in the solvent, so it may be that changing that group to a smaller analogue such as isopropyl will maintain potency but decrease molecular weight which will permit future additions to the molecule. Besides that, the amide N-H of Val-264 acts as a hydrogen bond donor to one of the nitrogen atoms of the core and there is an interaction between His-136 and the thione group.

Figure 1.15: Overlay of εA-oligo (pink)/AAG X-ray crystal structure (PDB: 1EWN) and UNIS00021 (blue) docked into crystal structure from εC-oligo/AAG (PDB: 3UBY, DNA removed).
The aim of this project is to synthesise analogues of UNIS00021 to establish which groups are necessary for potent binding and which can be modified to improve potency, solubility, cell membrane permeability, target selectivity and stability.

### 1.6 The Hit Inhibitor (UNIS00021) and Proposed Analogues

Based on the docking result of UNIS00021 into the AAG crystal structure as described above it was planned to design four synthetic routes to four types of analogue which would establish informative structure-activity relationships (Figure 1.17). It was planned to start by synthesising UNIS00021 and analogues with smaller alkyl groups on the amide including: isopropyl, cyclopropyl, and methyl. This would help to establish whether any alkyl group is necessary considering most of the cyclohexyl group in UNIS00021 is predicted to be exposed to the solvent.

The second synthesis route is to remove the carbonyl in the linkage amide in order to have just the amine. Synthesising the cyclohexylamine analogue of UNIS00021
might increase the strength of hydrogen bonding with the nucleophilic water molecule because the amine would be protonated and hold a positive charge. Another important feature of UNIS00021 is the thione moiety in the triazole-thione core which could be susceptible to oxidation or electrophilic attack. Therefore, it was planned to test replacement of the thione with a different moiety such as a hydrogen atom or a methyl group. Finally, the docking showed the benzyl group inside the enzyme’s base-binding pocket but it is not big enough to fill it. Consequently it was planned to synthesise analogues of UNIS00021 with bigger aryl groups to occupy most of the AAG pocket and pick up extra interactions.

Figure 1.17: Possible points for variation in analogues UNIS00021
1.7 Chemistry of UNIS00021 and Proposed Analogues

1.7.1 Triazole-thione Chemistry

Triazoles are five-membered ring aromatic compounds containing three nitrogen atoms. There are two isomeric forms of triazole: 1,2,3-triazole 18 and 1,2,4-triazole 19 (Figure 1.18).\textsuperscript{65}

![Triazole isomers](image)

Figure 1.18: Triazole isomers

1,2,3-Triazole itself is a colourless solid with a low melting point (23-25 °C) and a boiling point of 203 °C, whereas 1,2,4-triazole is a colourless crystalline solid with a much higher melting point (119-121 °C).\textsuperscript{66} Both exist in tautomeric equilibria: 1,2,4-triazole can be described either as 1,2,4-1H-triazole which means it is a \textit{N}^1-substituted triazole, or by 1,2,4-4H-triazole which describes a \textit{N}^4-substituted triazole.\textsuperscript{65}

The chemistry of 1,2,4-triazoles and their derivatives has been researched extensively over the last few decades.\textsuperscript{67-69} This is mainly due to their important pharmacological activities; 1,2,4-triazole compounds with antifungal\textsuperscript{70} and antiviral\textsuperscript{71} activities have been discovered. Examples of antifungal drugs that contain the 1,2,4-triazole group are Fluconazole \textsuperscript{20,72} and Ravuconazole \textsuperscript{21} (Figure 1.19).\textsuperscript{72}
Figure 1.19: Antifungal drugs with 1,2,4-triazole groups that inhibit the biosynthesis of ergosterol, a major steroid in fungal membranes

1,2,4-Triazole-thiones can exist in two further, major tautomeric forms; thiol (left, Scheme 1.6) and thione (right, Scheme 1.6).\textsuperscript{68,73}

Scheme 1.6: Tautomeric forms of 1,2,4-triazoline thiol (left) and 1,2,4-triazole thiole (left)

### 1.7.2 Triazole-thione Synthesis

A generally used method to synthesise 4-amino-triazole-5-thione involves the reaction of hydrazine with an appropriate electrophile, such as the ester, to give the hydrazide. This is followed by reaction with carbon disulfide then hydrazine hydrate (the mechanism for this reaction is given in \textsuperscript{74,75}. For example, El-Khawass \textit{et al.} synthesised 1-[(4-amino-4\textit{H}-1,2,4-triazole-5-yl)methyl-1\textit{H}-benzotriazole (24) according to Scheme 1.7.\textsuperscript{74}

Scheme 1.7: Synthesis of 1-[(4-amino-4\textit{H}-1,2,4-triazole-5-yl)methyl-1\textit{H}-benzotriazoles
O’Callaghan reported in 1974 that 4-alkyl-5-cyanomethylthiosemicarbazide derivatives (25) can be cyclised in the presence of hydrazine (as base) to yield 4-alkyl-5-cyanomethyl-1,2,4-triazoline-3-thiones (UNIS00073) at room temperature (Scheme 1.8). The proposed mechanism is given in Scheme 1.9.75

![Scheme 1.8: Cyclisation of thiosemicarbazide at RT using hydrazine hydrate as base](image)

![Scheme 1.9: Mechanism of cyclisation of thiosemicarbazide using hydrazine hydrate at RT](image)

Research by Colanceska-Ragenovic et al. has shown that thiosemicarbazides 26 can also be cyclised to 1,2,4-triazoline thiones 27 by sodium hydroxide but not by acid.76 In acidic media, 1,3,4-thiadiazole 28 derivatives were obtained as shown in Scheme 1.10 and Scheme 1.11.77 However, a study by Siwek and Paneth showed that the direction of cyclisation depends also on the nature of substituents and not just on the acidic or alkaline media.78
Scheme 1.10: Cyclisation of thiosemicarbazide by base (NaOH) or acid (H$_2$SO$_4$)

Many publications show that thiosemicarbazide can be cyclised into triazole-thione in alkaline medium by heating the reaction at reflux. **Table 1.1** summarises some products, reaction conditions and yields. All reactions were heated at reflux and worked up by adjusting the pH to 5-6 and removing the resulting precipitate by filtration.
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<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>2</td>
<td>4 h</td>
<td>89%</td>
<td>76</td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td>2</td>
<td>4 h</td>
<td>89%</td>
<td>76</td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td>2</td>
<td>2 h</td>
<td>85%</td>
<td>82</td>
</tr>
<tr>
<td><img src="image8.png" alt="Image" /></td>
<td>2</td>
<td>30 min</td>
<td>62%</td>
<td>83</td>
</tr>
</tbody>
</table>

Table 1.1: Published cyclisations of 1,2,4-triazoline thione using NaOH
2. Synthesis of Amide Alkyl Group Analogues

2.1 Introduction and proposed synthesis

It was important to start this project by synthesising the hit compound UNIS00021, to corroborate the inhibitory activity of the purchased compound, and to vary the alkyl group to smaller groups to maintain its potency but reduce its molecular weight since the docking result showed that most of the alkyl group was outside of the AAG binding pocket.

Based on the literature described in Scheme 1.2, in order to synthesise analogues of UNIS00021 the following route was proposed (Scheme 2.1).

Scheme 2.1: Proposed synthesis of UNIS00021 and analogues with different amide alkyl groups

There is literature precedence for the conversion of esters 29 into hydrazides 30 (Scheme 2.2) at room temperature.\textsuperscript{84}
Scheme 2.2: Conversion of cyanoacetate 30 to cyanoacetohydrazide

Of relevance to the second step of the planned synthesis, O’Callaghan reported the preparation of 1-cyanoacetyl-4-methylthiosemicarbazide (31) by reacting cyanoacetylhydrazine 30 with methyl isothiocyanate (Scheme 2.3).

Scheme 2.3: Synthesis of cyanoacetyl-4-methylthiosemicarbazide by reacting cyanoacetylhydrazine with methyl isothiocyanate

Many syntheses of differently substituted thiosemicarbazides and their cyclisation to form 1,2,4-triazoles and 1,3,4-thiadiazoles have been published. O’Callaghan showed that, on treatment with NaOH at 20 °C, thiosemicarbazide 25 (Scheme 2.4) does not give triazole UNIS00073 but instead pyrazoline 32 by cyclisation onto the nitrile instead of the hydrazide carbonyl group, presumably due to steric factors. However, treatment of 25 with hydrazine hydrate instead of NaOH at 20 °C afforded the triazole-thione UNIS00073.

Scheme 2.4: Cyclisation of thiosemicarbazide into pyrazoline at r.t.
In order to convert nitrile UNIS00073 to the carboxylic acid UNIS00064, it can be hydrolysed using strongly acidic conditions, especially sulfuric or hydrochloric acid. Addition of water to the protonated nitrile gives the primary amide, and hydrolysis of this amide gives carboxylic acid plus ammonia.\(^{85}\)

For the final step of the proposed synthesis, an amide bond must be formed. Amides result from the combination of a carboxylic acid and an amine, but these starting materials do not react at room temperature, only at high temperatures (> 200 °C) with the elimination of water.\(^{86}\) This high temperature is incompatible with the presence of other functionalities,\(^{87}\) therefore, it is important first to activate the carboxylic acid (in which the –OH of the acid is converted into a good leaving group) prior to treatment with the amine.\(^{22}\) Coupling reagents are used to activate carboxylic acids, such as dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Scheme 2.5).\(^{22,88}\)

\[
\text{Scheme 2.5: Amide coupling}
\]

### 2.2 Step 1: Synthesis of 2-Cyanoacetohydrazine (30)

Cyanoacetohydrazide 30 was prepared according to the published procedure\(^{89}\) in 79% yield. The crude product was purified by recrystallization from ethanol. Analysis by \(^1\)H, and \(^{13}\)C NMR showed the compound existed as a mixture of atropisomers due to limited rotation about the amide bond (Scheme 2.6). This was evidenced in the \(^{13}\)C NMR spectra by the presence of two peaks for the 1-C, 2-C and in the \(^1\)H NMR spectrum by two peaks for N-H and N-H\(_2\).
2.3 Step 2: Synthesis of Thiosemicarbazide 25

The preparation of thiosemicarbazide 25 followed the procedure reported by Colanceska-Ragenovic et al.\textsuperscript{76} which involved heating hydrazide 30 with benzyl isothiocyanate in ethanol at reflux for 2 h (Scheme 2.7) and (Scheme 2.8). The product was purified by recrystallization from ethanol to give 83% yield of pure product. The LCMS of this compound showed one peak with a mass spectrum matching a [M-H]\(^{-}\) ion at \(m/z\) 247. The structure of the compound was established by IR, \(^1\)H-NMR, \(^{13}\)C-NMR and CHN analysis. In the IR spectrum characteristic absorption bands were observed: 2265 cm\(^{-1}\) corresponding to the CN group, 1700 cm\(^{-1}\) corresponding to an amide C=O group, 1172 corresponding to the C=S group and 3124-3348 cm\(^{-1}\) corresponding to NH groups. Important peaks in the \(^1\)H-NMR spectrum in DMSO-d6 included three low field singlets at 8.63-10.17 ppm representing the protons of the three N-H groups, and two singlets at 4.74 and 3.66 ppm assigned to the CH\(_2\) groups connected to the benzene ring and CN group, respectively. The \(^{13}\)C-NMR spectrum was in accordance, interestingly exhibiting a characteristic \(-\text{NH-(C=S)NH-}\) signal at 181.9 ppm.

Scheme 2.7: Preparation of thiosemicarbazide 25
2.4 Step 3: Cyclisation to give triazole-thione

Synthesis of triazole-thioneacetonitrile 26 was carried out under the conditions reported by O’Callaghan as described in Chapter 1 using hydrazine hydrate at room temperature for 48 h. Although these conditions afforded the triazole-thione, the yield after silica gel chromatography was only 22% because of other unidentified by-products. Therefore, the reaction was carried out at different temperatures and time (Table 2.1) in order to optimise the yield. Heating the reaction did not improve the yield significantly since some unidentified by-products started to appear as detected by LCMS. There was an increase in the yield by increasing the time at room temperature.

<table>
<thead>
<tr>
<th>Scale (mg)</th>
<th>Conditions</th>
<th>Time (h)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>RT</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>500</td>
<td>Reflux</td>
<td>1</td>
<td>Could not be isolated</td>
</tr>
<tr>
<td>2000 a</td>
<td>50 °C</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>500</td>
<td>RT</td>
<td>64</td>
<td>48</td>
</tr>
</tbody>
</table>

a The reaction was then left at r.t. for 26 h

Table 2.1: Cyclisation of thiosemicarbazide to 1,2,4-triazoline thione using H$_2$N-NH$_2$.H$_2$O
Another concern might have been that the isolated product was pyrazoline 32, formed via cyclisation onto the nitrile instead of the hydrazide carbonyl, and not the triazole-thione UNIS00073. However, this was discounted using HMBC NMR, which detects correlations between carbon and hydrogen atoms which are 2-4 bonds apart. In the pyrazoline, the CH$_2$- of the benzyl group is 6 bonds from carbon ‘e’ so no correlation would be seen by HMBC. Yet, in the actual HMBC there was a correlation between CH$_2$- of the benzyl group and carbon ‘e’ which are three bonds distant in the triazole-thione UNIS00073 (Scheme 2.9).

![Scheme 2.9: Comparison between compound triazole-thione and pyrazoline using HMBC](image)

The alternative published cyclisation method was to use NaOH following the conditions in Table 1.1 in Chapter 1. However, none of compounds reported in the literature contained an acetonitrile group so it was not known if these conditions would work in this case so a test experiment was made. The work up for these reactions involved the addition of 2M HCl to adjust the pH to 5-6 and then extraction with DCM. However examination of the organic layer by LCMS showed no product peak and there was a small peak for the starting material. In a different attempt the pH in the work up was adjusted to 3 and (DCM/MeOH, 9:1) was used for the extraction. Surprisingly, in the LCMS there was one peak with m/z 248 in negative mode which indicated that the thiosemicarbazide had cyclised but in addition the –CN group had hydrolysed into –COOH (Scheme 2.10).
After extracting the compound from the aqueous layer using DCM/MeOH (9:1) the structure of the compound was established by its IR, $^{1}$H-NMR and $^{13}$C-NMR spectra and these results were compared with the nitrile compound UNIS00073. Although the IR spectrum of nitrile UNIS00073 shows a peak at 2265 cm$^{-1}$, it is too small to prove conclusively that nitrile functionality is present. Comparison of the $^{13}$C NMR spectra revealed a quaternary carbon atom at 114.6 ppm for nitrile UNIS00073 but a peak at 167.7 ppm for acid UNIS00064 (Figure 2.1). The CH$_2$ adjacent to the acid was also deshielded (32.0 ppm) compared to that adjacent to the nitrile (15.9 ppm). Also, the $^{1}$H-NMR spectra showed a singlet peak for one proton at 13.88 ppm for the acid UNIS00064 but not for the nitrile UNIS00073 (Figure 2.2).
While these compounds may exist in thione-thiol tautomeric equilibria, the IR spectra of both compounds showed no absorption bands around 2600-2550 cm\(^{-1}\) which would be indicative of the thiol form. The IR absorption due to the C=S group in both compounds appears at 1307-1348 cm\(^{-1}\).\(^{76}\)

Although using NaOH gave directly the acid which was needed for the amide coupling, the yield was only 15%. Therefore an alternative method was used to cyclise the thiosemicarbazide into the 1,2,4-triazole nitrile UNIS00073. It has been reported that heating thiosemicarbazide at reflux in aqueous NaHCO\(_3\) cyclised it into the corresponding 1,2,4-triazole-thione.\(^{90}\) Use of these conditions cyclised 25 and, again, hydrolysed the nitrile into the acid UNIS00064 in 14 h in 86% yield. This represented an efficient one-step synthesis of the desired acid from thiosemicarbazide 25.
In a different attempt this reaction was carried out at the lower temperature of 60 °C for 24 h and gave rise to the cyclisation product with intact nitrile UNIS00073 in 80% yield. The LCMS of this compound gave a [M-H]⁻ ion peak at m/z 229 and its IR, ¹H-NMR and ¹³C-NMR matched the nitrile compound which was obtained from the hydrazine hydrate mediated cyclisation. In this case, the work up involved adding 2M HCl to reach pH 5, rather than 3, since no carboxylate group was present requiring protonation. The nitrile UNIS00073 precipitated after addition of the acid and no further purification was necessary.

![Figure 2.2](image)

**Figure 2.2**: ¹H NMR (500 MHz, DMSO-d6) of acid UNIS00064

### 2.5 Step 4a: Amide coupling

Amides were synthesised from acid UNIS00064 and the appropriate amine using EDC as a coupling reagent. In the first attempt, isopropyl amine was used to give 25% yield of triazole-thione acetamide UNIS00067. Although the literature
reported that a similar reaction to synthesis the amide 35 was carried out for 6 h using the amine 33 and the acid 34 compounds as shown in Scheme 2.11 at room temperature,\textsuperscript{91} both reactions took 26 h and still the yield was low. In an attempt to optimise the reaction it was heated at 42 °C for 6 h and the yield was improved to 50%. The structure of the compound was established by IR, \textsuperscript{1}H-NMR, and \textsuperscript{13}C-NMR. In the IR spectrum the characteristic absorption bands were observed: 3287 cm\textsuperscript{-1} corresponding to N-H of the amide group, 1627 cm\textsuperscript{-1} corresponding to C=O of the amide group, and 1348 corresponding to the C=S group. The \textsuperscript{1}H-NMR spectrum showed a doublet integrating to six protons at 1.1 ppm for the two methyl groups of the isopropyl amide and a septet integrating to one proton at 3.86 ppm for the -CH of the isopropyl amide.

Different amides were made by reacting acid UNIS00064 with the corresponding amine in the presence of EDC as the coupling reagent at room temperature (Table 2.2). The structures of these amides were established by their IR, LCMS, \textsuperscript{1}H-NMR, and \textsuperscript{13}C-NMR spectra. The IR spectra of these compounds all showed the characteristic absorption bands: 3266-3292 cm\textsuperscript{-1} corresponding to N-H of the amide, 1627-1642 cm\textsuperscript{-1} corresponding to C=O of the amide, and 1348-1363 corresponding to the C=S group. There was no absorption for the N-H group for both UNIS00071 and UNIS00070 since, being tertiary amides, they did not have N-H groups. In the \textsuperscript{13}C-NMR spectra characteristic peaks included: 166-169 ppm.
corresponding to C=O of the amide group and 148-149 ppm corresponding to \( \text{N=CH}_2\text{C}=\text{O} \).

<table>
<thead>
<tr>
<th>UNIS code</th>
<th>Amide</th>
<th>Temp.</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
</table>
| UNIS00067 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | RT    | 26 h  | 28%   |
|           |       | 42 °C | 6 h   | 50%   |
| UNIS00021 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | RT    | 21 h  | 20%   |
| UNIS00068 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | RT    | 28 h  | 47%   |
| UNIS00069 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | RT    | 24 h  | 52%   |
| UNIS00070 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | RT    | 29 h  | 40%   |
| UNIS00071 | \[
\text{\begin{array}{c}
\text{N} \\
\text{H} \\
\text{H}
\end{array}}
\] | 21 h at RT then 3 h at 42 °C | 26%   |

Table 2.2: Experimental conditions and yields for alkyl amide analogues of UNIS00021

Examining the molecular weight of UNIS00067 using LCMS gave a [M-H]\(^{+}\) ion peak with \( m/z \) 291 rather than 289 and it was noted that this was observed after leaving it in CD\(_3\)OD for a long time. Similarly, but for UNIS00021, the peak for C\(\text{H}_2\text{C}=\text{O} \) in the \(^{13}\text{C}\)-NMR was not found after leaving it in CD\(_3\)OD for some time but the peak had appeared when the spectrum was taken immediately after dissolution (red circle in Figure 2.3). Also, LCMS of that compound gave a [M-H]\(^{+}\) ion peak at \( m/z \) 331 rather than 329. These observations could be explained as resulting from exchange of the C\(\text{H}_2\text{C}=\text{O} \) protons for deuterons in CD\(_3\)OD as shown in Scheme 2.12.
**Figure 2.3**: 13C NMR (500 MHz, MeOD) of UNIS00021 before and after proton-deuterium exchange

**Scheme 2.12**: Exchange of CH$_2$-C=O protons for deuterons in CD$_3$OD
2.6 Step 4b: Synthesis of primary amide

Several reactions were trialled to synthesise the primary amide UNIS00074 from triazole-thione acetonitrile UNIS00073. The first used sodium borohydride in a mixture of ethanol and water at 80 °C, as published by Praveen who has proposed that NaBH₄ abstracts protons from water to give hydroxide ions, which promote the hydration of nitriles (Scheme 2.13).¹²

Praveen mentioned that the reaction was carried out at 80 °C for between 5 and 12 hours and that excellent yields were obtained for the hydration of 4-cyanopyridine, 2-thiophenecarbonitrile, 3-cyanoindole and 5-cyanophthalide without affecting the heterocyclic ring system. However, carrying out this reaction on nitrile UNIS00073 for 21 hours gave no product, only the starting material was recorded by LCMS.

Benzonitrile gave 94% yield of benzamide when refluxed for 20 min in tert-butyl alcohol containing powdered potassium hydroxide.¹³ Nevertheless, tert-butyl alcohol and powdered potassium hydroxide did not convert nitrile UNIS00073 to the corresponding primary amide, instead LCMS showed a peak for the hydrolysed product, acid UNIS00064 and a peak for the starting material.
There is literature precedent for the use of sodium percarbonate (Na$_2$CO$_3$·3/2H$_2$O$_2$) to convert heteroaromatic nitriles and aromatic nitriles lacking ortho substituents into the corresponding amides.$^{94}$ Using this reagent in a mixture of acetone and water at 50 °C for 1 h, the authors found that benzyl cyanide was converted into benzamide in 95% yield. These conditions were applied to triazole-thioneacetonitrile UNIS00073 and a small peak for the primary amide appeared in the LCMS but about 90% of the starting material was recovered using column chromatography.

Miyazawa et al. established a method to prepare primary amides from nitriles using hydroxylamine derivatives.$^{95}$ The reaction was carried out in an inert atmosphere at reflux using DCM as solvent and 3 equiv. of the hydroxylamine derivative. Yet, the hydration of most nitriles such as heterocyclic nitriles and aliphatic nitriles progressed in low yield after long reaction times. In 2011, Xiao-yun Ma noticed that using water as a solvent in the presence of N,N-diethylhydroxylamine, transformed 3-chloro-4-arylpicolonitrile into 3-chloro-4-arylpicolinomide in quantitative yield at room temperature after 6 h.$^{96}$ He proposed the radical mechanism shown in Scheme 2.14 and reported that in the case of aliphatic nitriles the hydration process was similar to that of aromatic nitriles and occurred in good to excellent yield. For instance, the conversion of benzonitrile to benzamide took 5 hours at reflux and occurred in 92% yield.

Using this method for the conversion of nitrile UNIS00073, a peak for the primary amide was observed by LCMS but it was difficult to separate and purify it. On the other hand, another peak was observed by the LCMS with [M-H] m/z of 319.4, which was isolated in 10% yield. Analysis by NMR showed it to be α-amino amide UNIS00072 and a suggested mechanism for its formation is given in Scheme 2.15.
Another publication reported that hydration of the nitrile could be achieved by acid or base catalysis. Jarugu \textit{et al.} showed that a variety of aromatic, aliphatic and allylic mono- to tetra-nitrile compounds could be converted into their corresponding amides in a single step using a TFA- or an AcOH-H$_2$SO$_4$ reagent system. The reactions of most of these compounds were done at room temperature for 4-8 h, followed by quenching of the reaction mixture with ice-cold water. The conversion to the amide is formally by addition of water, yet the transformation is accomplished in an indirect way. It is the trapping at the imidate stage by the acetate which prevents full hydrolysis to the acid (Scheme 2.16).

\textbf{Scheme 2.15:} Proposed mechanism for the formation of UNIS00072

\textbf{Scheme 2.16:} Proposed mechanism of conversion of nitriles to amides using TFA-H$_2$SO$_4$.\textsuperscript{65}
Applying these condition to nitrile UNIS00073 for 2 h gave the primary amide which was purified successfully using a reverse phase column to give 50% yield (Scheme 2.17).

Scheme 2.17: One-pot synthesis of UNIS00074

2.7 Summary and Conclusion

A robust synthetic method to vary the alkyl group of the amide in UNIS00021 in order to decrease the molecular weight of the inhibitor was established. The core 1,2,4-triazole-thione can be produced by cyclisation of a thiosemicarbazide. The thiosemicarbazide was synthesised in 83% yield by refluxing hydrazide 25 with benzylisothiocyanate in ethanol for two hours. Cyclisation of this into the triazole-thione required optimisation: using hydrazine hydrate at room temperature for 48 h gave a low yield and other by-products after purification using column chromatography. Hence, NaOH was used to cyclise the thiosemicarbazide at reflux and this actually gave the desired acid product UNIS00064, through nitrile hydrolysis, instead of the expected nitrile UNIS00073. Although, this method saved one step, the yield was low at 15%. On the other hand, using 1 M of NaHCO₃ at reflux to cyclise 25 gave the acid product in 86% yield and surprisingly gave the nitrile product UNIS00073 in 80% yield if the reaction was carried out at 60 °C. After discovering how to synthesise acid UNIS00064 in good yield the amide couplings using EDC and the appropriate amines were carried out. Various product amides were purified using column chromatography and characterised by NMR, LCMS and IR. However, the primary amide was not produced by this method but by the hydration of nitrile UNIS00073. The best two methods for this reaction were
to use either water as a solvent in the presence of \( N,N \)-diethylhydroxylamine for 5 hours or TFA-H\(_2\)SO\(_4\) at room temperature for 4 hours. Using the first method the primary amide was obtained but it was difficult to purify and instead another by-product was identified as \textbf{UNIS00072}. On the other hand, the second method gave the product in 50% yield and purification was achieved using reverse phase column chromatography.
3. Synthesis of Analogues Varying at the Linkage Group

3.1 Introduction

It is of interest whether the central amide of UNIS00021 can be replaced with an amine group. Based on the docking result of UNIS00021 into the AAG crystal structure PDB: 3UBY, with DNA oligomer removed, the amide forms a hydrogen bond with the active site nucleophilic water molecule, itself held by 2-3 hydrogen bonds with active site residues. An amine in this position would most likely be protonated and could therefore form a very strong hydrogen bond with the strongly bound water molecule.

To predict the activity of UNIS00021 analogues that lack the amide linkage and replaced with an amine, UNIS00077 was docked into the AAG crystal structure 3UBY as shown in Figure 3.1. In this, the nucleophilic water molecule in the active site makes a hydrogen bond to the amine of UNIS00077. This should be stronger if the amine is protonated than hydrogen bonding to an amide as in UNIS00021. At 3.4 Å, there is also possibly an interaction between this amine and the carbonyl of Val-262 which was not there when UNIS00021 was docked into the same AAG crystal structure.
In this chapter it is planned to design the synthesis by varying the linkage using \( n=1 \) and \( n=2 \). The primary amines can be converted then into different substituted amines using reductive amination. These analogues will help to check the necessity of the carbonyl and whether the protonated amines will add any extra hydrogen bonding. Also, it was planned to check if varying the length of the linkage group will increase the potency of the amine analogues. In this chapter the synthesis of **UNIS00077** (Figure 3.2) and four other analogues varying at the linkage moiety or the amine R group will be discussed.
3.2 Attempted synthesis via reduction of nitrile UNIS00073

3.2.1 Background

Lithium aluminium hydride has been used as a reducing agent to convert nitriles into amines for a long time. The mechanism for this reaction, proposed by Weldon G.,\textsuperscript{97} is shown in Scheme 3.1.\textsuperscript{97-99} For example, Mori \textit{et al.} used LiAlH\textsubscript{4} in dry THF to convert the nitrile group in compound 36 into an amine 37 in 74\% yield (Scheme 3.2).\textsuperscript{100}

![Scheme 3.1: Mechanism for reduction of a nitrile group using LiAlH\textsubscript{4}](image)

Scheme 3.2: Reduction of a nitrile group into an amine using LiAlH\textsubscript{4}
Also, Robert reported that the activity of LiAlH₄ can be improved by the addition of the Lewis acid aluminium chloride. He hypothesised that when 1 equiv. of each of these reagents is used it causes the formation of aluminium hydride and lithium chloride and then further reaction might take place to form aluminium chlorohydride, AlH₂Cl. Robert found that combining these two reagents, rather than just using LiAlH₄, in the reduction of diphenylacetonitrile in dry ether increased the yield from 46% to 91%. However, all their starting materials contained either phenyl, benzyl, or aliphatic groups; none of them bore a triazole. A search using SciFinder revealed no reports of LiAlH₄ being used in the presence of a triazole-thione. Milder NaBH₄ has been used to reduce a more reactive pyridinium side group in the presence of a triazole-thione but the yield was low. NaBH₄ has also been used with triazole-thiones to produce tris(triazole-thione) boron complexes. Based on this literature it was decided to first test access to the desired amine analogues of UNIS00021 via reduction of the nitrile group of triazole-thione acetonitrile UNIS00073 using LiAlH₄ only.

### 3.2.2 Results and Discussion

The reduction of triazole-thione acetonitrile UNIS00073 using LiAlH₄ was carried out following the procedure reported by Mori et al. Three equivalents of LiAlH₄ were added to the starting material at 0 °C using dry THF as the solvent. The reaction was warmed to room temperature for 1.5 h. It was worked-up using Fieser’s method, that avoids aluminium hydroxide gel/emulsion formation, by adding first 0.1 ml water, then 0.2 ml of 2 M NaOH, and finally 0.3 ml water. However, the LCMS of the filtrate did not show any peaks correlating to the expected product ([M-H]⁻ ion at m/z 234) and instead there was a peak for the starting material ([M-H]⁻ at m/z 229), and two other peaks with mass spectra matching a [M-H]⁻ ion with m/z 231, and with 232. It was difficult to purify and isolate these components and attempts to use reverse phase column chromatography was not successful.
Next, the procedure using using LiAlH₄ in combination with Lewis acid aluminium chloride, described above, was trialled.¹⁰¹ 1.3 Equiv. of each was combined and added dropwise to the starting material. After 1.5 h reaction the LCMS showed a small peak corresponding to the starting material and a mixture of other unknown peaks and it did not show the corresponding primary amine. It is hypothesised that this potent mixture also reduced the triazole-thione and led to several further reactions of the products.

It has been found that using Raney Ni / N₂H₄.HCO₂H could transform nitriles to primary amines with a good yield.¹⁰⁴,¹⁰⁵ It was reported that hydrazinium monoformate in the presence of Raney Ni (Scheme 3.3) acts as a hydrogen donor and reduced a wide variety of nitro and nitrile compounds to the corresponding amines in less than ten minutes.¹⁰⁴ However, none of the reported starting materials contained a triazole-thione.¹⁰⁴ It was also reported that hydrazinium monoformate is soluble in solvents like methanol, ethanol, THF, DMF and glycols.¹⁰⁴

\[
\begin{align*}
\text{R-CN} & \quad \xrightleftharpoons{\text{Raney Ni/N₂H₄.HCO₂H, MeOH, r.t.}} \quad \text{R-CH₂-NH₂} \\
\end{align*}
\]

**Scheme 3.3:** Reduction of nitrile using Raney Ni and hydrazinium monoformate. (R=alkyl or aryl residue substituted with –OH, OR, -CO₂H, -CO₂R, -CONH₂, or -NHCOOCH₃.)

The method was applied to nitrile UNIS00073. The hydrazinium monoformate was prepared by slowly neutralizing hydrazine hydrate with 96% formic acid in an ice water bath before adding Raney nickel in ethanol and nitrile UNIS00073. TLC revealed just the starting material even after 24 hours at room temperature and this was corroborated by LCMS. In another attempt the amount of hydrazinium monoformate was increased to 10 equiv. and the reaction was left for 5 hours at room temperature. However, the LCMS again revealed just the starting material so the method was abandoned.
3.2.3 Conclusion

All attempts to produce a primary amine from 1,2,4-triazole-thione acetonitrile UNIS00073 using reducing agents LiAlH₄, LiAlH₄·AlCl₃ and Raney nickel/N₂H₄·HCO₂H were unsuccessful with starting material or over-reduced products being detected in most cases. It was therefore decided to design an alternative synthetic route to triazole-thione alkylamines such as UNIS00077.

3.3 Synthesis via Boc-amino alkyl hydrazides

3.3.1 Background

To permit divergent synthesis of several substituted secondary amines, the triazole-thione alkyl-primary amine was required which could be used in reductive aminations. To mitigate side-reactions of the amine during synthesis of the triazole-thione, it should be protected and the Boc group was chosen. There are relatively few literature and patent reports of syntheses of triazole-thione alkyl(Boc)amines. For example Cowen et. al. reported the synthesis of triazole-thione substituted alkyl(Boc)amines 39 via cyclising thiosemicarbazide that contain the protected amine 38 with ammonia at a temperature ranged between 65 °C to room temperature (Scheme 3.4).¹⁰⁶

![Scheme 3.4: Synthesising triazole-thione substituted alkyl(Boc)amines via cyclising thiosemicarbazide]
Similarly Michaela et. al. synthesised the triazole-thione alkyl(Boc)amines 44 by using the corresponding acid hydrazides 41 in a reaction with benzylisothiocyanate to give the thiosemicarbazide 42 required for cyclisation in 2% NaOH at reflux to give the triazole-thione 43 (Scheme 3.5).\(^\text{107}\)

\[\text{BocN} \overset{\text{NH}_2\text{NH}_2}{\rightarrow} \text{BocN} \overset{\text{PhCH}_2\text{NCS}}{\rightarrow} \text{BocN} \overset{2\text{M HCl}}{\rightarrow} \]

\[\text{40} \quad 87\% \quad \text{41} \quad 68\% \quad \text{42} \quad \text{43} \quad \text{44}\]

\[\text{43} \quad \text{2% NaOH} \quad 82\% \quad \text{44}\]

**Scheme 3.5**: Synthesis of triazole-thione alkyl(Boc)amines 44 via thiosemicarbazide cyclisation.\(^\text{107}\)

Henichart et. al. also reported the synthesis of triazole-thione having dialkylaminoethyl substituent at the 3 position and benzyl in the the fourth position in 85-88% yield by cyclising the corresponding thiosemicarbazide.\(^\text{108}\)

Based on the literature and successful synthesis of triazole-thiones described in Chapter 2, the retrosynthetic analysis shown in **Scheme 3.6** was performed which allows variation of the length of the linker group and seven reactions starting from amino acids 45.
Scheme 3.6: Retrosynthetic plan for analogues of UNIS00021 varying at the linkage group

The proposed forward synthesis is shown in Scheme 3.7.
The synthesis proceeds via the amino acid esters 46 (n=2) which can be easily prepared through activation with thionyl chloride in the presence of methanol. This reaction has been widely used in the literature in yields ranging from 79 to 100%.  

The next step involves protection of the amine group in 46. Boc is one of the most widely used protecting groups in organic chemistry to protect primary or secondary amines as well as amino acids in peptides chemistry. It is ideal to protect amines during the synthesis of multi-functional targets because of its resistance towards nucleophilic and basic attack. Many publications describe Boc amine protection in the presence of a base such as triethylamine (TEA) in excellent yields.

Synthesis of the subsequent hydrazides 49 (n=1) and 50 (n=2) can be achieved by reacting the protected amine compound 47 (n=1) and 48 (n=2) with hydrazine.
hydrate in water at reflux for 2 h,\textsuperscript{119} or for 16 hours using methanol as the solvent.\textsuperscript{115}

Addition of the hydrazide to benzylisothiocyanate should give the thiosemicarbazide 51 (n=1) and 52 (n=2) as was successful for cyanoacetohydrazide 30 as described in Chapter 2.\textsuperscript{75,76,120}

O’Callaghan used hydrazine as the base to cyclise 4-alkyl-5-cyanomethylthiosemicarbazides into the corresponding triazole-thiones.\textsuperscript{75} Other reported methods used (0.5 -2 M) NaOH as the base (see Table 1.1 in Chapter 2). However, the results from this project, described in Chapter 2, found 1 M NaHCO\textsubscript{3} at 60 °C sufficient to give the 1,2,4-triazolothione acetonitrile UNIS00073.

The Boc protecting group can next be easily removed by treatment of the 1,2,4-triazole-thione UNIS00080 (n=1) and UNIS00075 (n=2) with an acid before proceeding to the next step.\textsuperscript{107,121}

This step should be followed by alkylating the primary amine to form the secondary amines 57. Usually, the formation of such secondary amines is achieved by treatment of primary amines with alkyl halides in the Hofmann alkylation.\textsuperscript{122} However, the problem with this type of reaction is overalkylation which is due to the increased nucleophicity of the product amines.\textsuperscript{123} Overalkylation can give rise to mixtures of primary, secondary, tertiary amines, and quaternary ammonium salts (Scheme 3.8).

![Scheme 3.8: Overalkylation of primary amines in the Hofmann alkylation.\textsuperscript{122}](image)

The free 1,2,4-triazol-thione alkyamines UNIS00081 and UNIS00076 can also be converted into the target secondary amines by reductive amination which is the solution to overalkylation problem. In this, the nucleophilic amine attacks the
electrophilic carbonyl to form the intermediate carbinol amine 58 which dehydrates to form an iminium ion (Scheme 3.9). These are then reduced to the alkylated amine product by the reducing agent.

![Scheme 3.9: Reductive amination through the formation of carbinol amine](image)

This reaction can be carried out in one pot by using a milder reducing agent which has selectivity for iminium ions over the starting aldehyde or ketone. Sodium cyanoborohydride (NaBH₃CN) is one such reducing agent. Intrestingly NaBH₃CN reduced aldehydes and ketones effectively at pH 3-4 whereas at pH 6-8 the only the imines are protonated and so are reduced faster than aldehydes or ketones. However, this can produce toxic by-products such as NaCN and HCN during the workup and can contaminate the product with cyanide.

Gordon and Duncan reported the milder reducing reagent sodium triacetoxyborohydride (NaBH(OAc)₃). The electronwithdrawing and the steric effects of the three acetoxy groups are responsible for its mild reducing properties. The pH used with this reagent is ~ 3 in the presence of acetic acid. For example, Ahmed F. et al. used it in their reductive amination reactions of aldehydes and ketones with a variety of aliphatic and aromatic amines and they reported yield between 35-99 %. They found that it reacts faster with a better yields and with fewer byproducts compared to the NaCNBH₃. The reactions of this reagent in DCE as a solvent is faster than those carried out in THF.

The first analogue of UNIS00021 should bear a cyclohexylamine. Use of this substrate using the method above has been reported by Louis L. et al. in the synthesis of pyrrolotriazinone 60 (Scheme 3.10).
It is also of interest to synthesise the monomethyl amine analogue **UNIS00079** to measure the activity against AAG with the smallest alkyl group.

One reported method to methylate the amine was to use a mixture of CO$_2$ and H$_2$ in a ruthenium-catalyzed process (Scheme 3.11).$^{133,134}$ The CO$_2$ acts as the C$_1$ source and H$_2$ as the reducing agent. However, the disadvantages of this method are that it needs high pressure (20-70 bar) and temperature (>140 °C).$^{134}$

A simpler method was reported by LeBleu *et al.* in which a direct N-monomethylation of primary amines was achieved using methyl trifluoromethansulfonate (MeOTf) or dimethylsulfate (Me$_2$SO$_4$), as the electrophilic source of methyl, in the presence of hexafluoroisopropanol (HFIP).$^{135}$ HFIP is thought to prevent overmethylation by selective deactivation of secondary and tertiary amines through hydrogen bonding. The influence of the hydrogen bond donor ability of fluorinated alcohol solvents such as HFIP was examined experimentally by LeBleu *et al.* who proposed that the reaction between a primary amine and MeX should not be affected by HFIP, but the nucleophilicity of the resulting secondary and tertiary amines, with higher energy N lone pairs, would be offset by hydrogen bonding to it and so prevent overmethylation. This proposed
The idea was confirmed by their result of reacting benzyamine (1 mmol) with MeI (1.5 eq.) but the yield after 24 hours was just 8%. However, the monomethyl adduct was the only product. The conversion was improved to 79% by performing the reaction at 60 °C but the monomethylamine was accompanied by tertiary amine (20%) and the quaternary ammonium salt (3%). This was solved by using more powerful methylating agents Me₂SO₄ or MeOTf, which have hydrogen bond acceptors, and the conversion improved to 76-81% with 57-68% selectivity towards secondary amine.¹³⁵

### 3.3.2 Results and Discussion

#### 3.3.2.1 Step 1: Synthesis of β-alanine methyl ester (46 (n=2))

The preparation of β-alanine methyl ester 46 followed the procedure reported by Colak et al., described above.¹¹² Thionyl chloride was added dropwise at 0 °C to the starting material in methanol. The reaction was stopped after eight hours by adding cold diethylether to precipitate the substituted amino acid ester products (Scheme 3.12) in the form of a salt, in good yield (88%). The ¹H-NMR showed a peak at 3.7 ppm which integrated to three and the ¹³C-NMR showed a peak at 52.3 - both representing the new methoxy group. The structure of the compound was established by IR, ¹H-NMR, ¹³C-NMR and GC-MS.

![Scheme 3.12: Preparation of β-Alanine Methyl Ester synthesis](image_url)

65
3.3.2.2 Step 2: Synthesis of N-(tert-butoxycarbonyl)-β-alanine methyl ester (48 (n=2))

N-(tert-Butoxycarbonyl)-β-alanine methyl ester 48 (n=2) was formed by reacting the HCl salt of amino ester 46 with di-tert-butyl dicarbonate (Boc₂O). The product 48 was isolated in 96% yield after removing the triethyl ammonium hydrochloride salt by filtration (Scheme 3.13). The ¹H-NMR showed a singlet peak integrate to nine for the tert-butyl group and ¹³C-NMR revealed a new carbonyl peak at 155.9 ppm assigned to the carbonyl group of Boc and a peak at 79.5 ppm for the (-C-(Me)₃).

\[
\text{Scheme 3.13: Preparation of protecting β-Alanine Methyl Ester using ditert-butyl carbamate}
\]

3.3.2.3 Step 3: Synthesis of N-Boc-aminoalkyl hydrazides with n=1 and n=2

For triazole-thione alkylamines with n=2, Boc-β-alanine hydrazide was required and was synthesised according to S. Poojari et al. from Boc-β-alanine ester 47 and was isolated after aqueous work-up in 67% yield (Scheme 3.14). For triazole-thione alkylamines with n=1, the required starting material, N-Boc-alanine ester 47 is commercially available and was reacted with hydrazine hydrate using the same procedure to give hydrazide 49 in 76% yield (Scheme 3.14).

Analysis by ¹H, and ¹³C NMR for both products showed that there was no longer a peak for the methoxy group. Also, ¹H-NMR in DMSO-d₆ showed a low field singlet at 8.90 ppm (integrating to 1) corresponding to the –NH-NH₂ and another singlet at 6.7 ppm corresponding to the Boc-NH₂.
3.3.2.4  Step 4: Thiosemicarbazide synthesis

The preparation of thiosemicarbazide 51 (n=1) and 52 (n=2) was carried out following the procedure reported by Colanceska-Ragenovic et al. and used in Chapter 2. Owing to impurities observed in the LCMS of the crude products, they were purified by column chromatography, a procedure which was not necessary with the ester products produced in Chapter 2. After this, the reaction yield was 92% for the thiosemicarbazide with n=2 (Scheme 3.15). Whereas the reaction yield with n=1 was 75% and 12% was the triazole-thione as by product (Scheme 3.16). The $^1$H-NMR spectrum in DMSO-d6 showed a new low field singlet at 8.40 ppm representing the protons of the new N-H from the benzyl isothiocyanate. A 5H multiplet in the aromatic region at 7.22-7.29 ppm confirmed the new phenyl group to be present and $^{13}$C-NMR spectrum showed a signal corresponding to the (C=S) group at 182 ppm in the same environment as in spectrum of compound 25 in Chapter 2.

**Scheme 3.15:** preparation of thiosemicarbazides 52
3.3.2.5 Step 5: Cyclisation of thiosemicarbazides into triazole-thiones

The cyclisation of thiosemicarbazides 51 and 52 was carried out using the same conditions used to synthesis UNIS00073 in Chapter 2 which involved heating in 1 M aqueous NaHCO$_3$ at reflux (Scheme 3.17). Both starting thiosemicarbazides cyclised into the desired triazole-thiones. LCMS showed just one peak with $m/z$ 333 matching [M-H]$^-$ for n=2 and 319 for n=1. Purification was not required after work-up and the yields were 79% for n=2 and 80% for n=1. The $^{13}$C-NMR spectra exhibited a signal at 151.3 ppm corresponding to the (-N=C-) group for both products. The signal for (-C=S) moved from 182 ppm in the starting material to 168 ppm. This was also assigned by HMBC and HSQC. The formation of the triazole-thione, as opposed to amino-thiadiazole, was evidenced by IR absorption which showed absorbance of only one carbonyl group at 1666, -C=S at 1356, and -NH-Boc at 3265 cm$^{-1}$ for both products.

Scheme 3.17: Preparation of triazole-thione UNIS00080 and UNIS00075
3.3.2.6 Step 6: Deprotection of the amino group

The most used method that has been reported for N-Boc deprotection is application of mildly acidic conditions\textsuperscript{136} such as HCl in EtOAc, trifluoroacetic acid (TFA) in DCM, H\textsubscript{2}SO\textsubscript{4} in tert–BuOAc, or aqueous phosphoric acid in THF.\textsuperscript{137,138}

The first method that was tested was TFA in DCM (1:1). This was added to the Boc-amine \textbf{UNIS00075} and left at room temperature for 2 h.\textsuperscript{121} The LCMS of the crude product showed a peak with a mass spectrum matching a [M-H]\textsuperscript{+} ion at \textit{m/z} 233 and other unknown impurities. The reaction was stopped by adding saturated NaHCO\textsubscript{3} and extracted with DCM. That was followed by reverse phase column since the TLC and LCMS showed that the product was very polar. However, impurities remained. In a repeat of the reaction 1 M NaHCO\textsubscript{3} was added to make the solution pH~8 and then it was extracted with DCM/MeOH (9:1) to give the product in 40\% yield. The TLC of this showed one spot but the \textsuperscript{1}H- and \textsuperscript{13}C NMR still showed impurities. This problem is associated with use of TFA to remove Boc groups and has been reported previously along with the fact that the yields after aqueous extraction can be poor.\textsuperscript{139}

A different reported deprotection method employs HCl in dioxane or MeOH/EtOAc at room temperature.\textsuperscript{140,141} HCl/EtOAc (1:1) was trialled as the solvent\textsuperscript{142} with Boc-amine \textbf{UNIS00075}, however, the starting material did not dissolve in the HCl acid mixture even after leaving it for 2 h at room temperature. The TLC showed just starting material so it was decided to heat the reaction at reflux. After one hour at reflux there was no starting material by TLC and there was a new spot. At that point the solvent was evaporated to dryness to give the product as a white powder in 86\% yield (\textbf{Scheme 3.18}). The LCMS of the isolated product \textit{n}=1 showed a peak at 1.23 retention time with a mass spectrum matching a [M-H]\textsuperscript{+} ion at \textit{m/z} 235.

Since the product was in the salt form and not the free amine which was required for the next step (reductive amination), it was decided to repeat the reaction but
quench it using NaHCO$_3$ to pH~8 followed by extraction with DCM/MeOH (9:1). In this, although the extraction process was carried out ten times and NaCl was added to the aqueous layer, the yield was only 11%. It was therefore decided to try to use UNIS00076 (n=2) in the salt form for the reductive amination step (see next Section).

The same conditions were used to deprotect the shorter Boc-alkylamine UNIS00080 in 92% yield (Scheme 3.18).

![Scheme 3.18: Deprotection of the amino group in triazole-thione UNIS00081 and UNIS00076](image)

The structure of the two compounds were established by IR, $^1$H-NMR, $^{13}$C-NMR. In the IR spectrum characteristic absorption bands for NH$_2$, were observed at 3028 cm$^{-1}$. In the IR spectrum characteristic absorption there was no band observed for the removed Boc carbonyl group. The $^1$H-NMR spectrum showed the disappearence of the singlet of the tert-butyl group.

### 3.3.2.7 Step 7a: Reductive amination

The reductive amination reaction, described in Section 3.3.1, involves reacting the appropriate aldehyde or ketone with the amine and NaBH(OAc)$_3$ in the presence of acetic acid.$^{132}$ This procedure was followed with cyclohexanone and 1,2,4-triazole-thione ethylamine UNIS00076 (n=2). A 24 h reaction time was required for full conversion of the starting material then, after aqueous work-up and flash silica column chromatography the product was obtained in 63%. The yield would
have been higher but some product remained trapped in the aqueous layer as detected by LCMS.

Using this method, the four triazole-thione alkylamines shown in Scheme 3.19 were synthesised, all in moderate yield. The structures of these amines were confirmed by LCMS, IR, and NMR. The IR spectra all showed the characteristic absorption bands: 3228-3233 cm\(^{-1}\) corresponding to N-H of the amine and 1350-1362 corresponding to the C=S group.

Scheme 3.19: Reductive amination reaction using NaBH(OAc)\(_3\) in the presence of acetic acid.

### 3.3.2.8 Step 7b: Monomethylation of triazole-thione alkylamines

The method that was used to monomethylate the triazole-thione alkylamines was that reported by LeBleu et al. using HFIP and Me\(_2\)SO\(_4\).\(^{125}\) In the first attempt using triazole-thione methylamine UNIS00081 (n=1) and 1.5 equiv. of Me\(_2\)SO\(_4\) (Scheme 3.20), the mixture formed a non-stirrable sludge so an extra 3 equiv. HFIP was added. Regardless, after 1 h, TLC showed just starting material. Addition of DCM to better bring the mixture into solution also led only to the detection of starting material by LCMS.

Scheme 3.20: Attempted synthesis of methylamine triazole-thione 61
It was thought that this reaction failed because amine UNIS00081 was in the salt (protonated) form and therefore non-nucleophilic. Hence, it was decided to add one equiv. of anhydrous Na$_2$CO$_3$ to deprotonate the amine into the same reaction. However, this did not dissolve and again, after 2 h LCMS still showed only starting material. At that point it was decided to add one equivalent of 1 M aqueous NaHCO$_3$. After 30 min, LCMS showed a peak with a mass spectrum matching a [M-H]$^-$ ion at m/z 233 for the desired product. The reaction was stopped after 24 hours by adding 2 M HCl until the mixture reached pH~7 and extracting with DCM/MeOH (9:1). However, after purification using flash silica column chromatography, the desired secondary amine was not isolated. Instead, LCMS of the isolated product showed it to be a mixture of tertiary amine and quaternary ammonium salt which were not present before the work up based on the LCMS. It was hypothesised that overmethylation occurred during the extraction process in which Me$_2$SO$_4$ was still present and HFIP partitioning into a different layer to the amines so it was rendered less effective at inhibiting the nucleophilicity of the secondary amine.

The reaction was repeated, albeit with longer chain amine UNIS00076 due to availability, including the 1 M aqueous NaHCO$_3$ as a base. Once Me$_2$SO$_4$ was added the mixture turned from a milky solution into a transparent solution. After 5 h, LCMS showed a peak with a mass spectrum matching a [M-H]$^-$ ion of the desired product at m/z 247 and there was no peak of the starting material. This time the crude material was purified directly using a reverse phase column, thus avoiding addition of 2 M HCl and the extraction process in which the remaining Me$_2$SO$_4$ can overmethylate the product. Interestingly, the $^1$H-NMR of the isolated compound showed the product but also Me$_2$SO$_4$ as a major contaminant which revealed that quenching the reaction with acid was necessary to trap the remaining Me$_2$SO$_4$ in the aqueous layer.

In a final attempt to synthesise UNIS00079 the reaction was stopped first by adding 2 M HCl to achieve a pH~2-3 and then extracting with DCM three times to remove
all of the Me$_2$SO$_4$ from the aqueous layer but leaving the protonated product amine there. Next, the aqueous layer was basified using 1 M NaHCO$_3$ to pH~8 and this was extracted with DCM/MeOH (9:1) three times. The combined organic layers were purified using reverse phase silica column chromatography to give the desired secondary amine in 78% yield with no Me$_2$SO$_4$ detectable in $^1$H-NMR (Scheme 3.21). The $^1$H-NMR in MeOD showed a 3H singlet peak at 2.79 ppm representing the new methyl group.

\[ \text{Scheme 3.21: Synthesis of the 1,2,4-triazole-thione ethyl methylamine (UNIS00079)} \]

3.4 Summary and Conclusion

The synthesis of analogues of UNIS00021, with a free amine in place of the amide, by reduction of 1,2,4-triazole-thione nitrile UNIS00073 using LiAlH$_4$ and other reducing agents and over-reduced products was not successful and probably over-reduced products were obtained. Instead, a robust synthetic method which also allowed variation of the length of the alkyl linkage group was developed. Similar to the amides synthetic method (Chapter 2), the 1,2,4-triazole-thione core can be produced by cyclisation of thiosemicarbazides which were synthesised with $n=1$ and $n=2$ in 75% and 92%, respectively, by refluxing hydrazides 50 and 49 with benzylisothiocyanate in ethanol for 2 h. As for the amides described in Chapter 2, heating these thiosemicarbazides in 1 M of NaHCO$_3$ induced cyclisation to give the 1,2,4-triazole-thione protected amines in 79-80% yields. After removing the Boc group using HCl/EtOAc (1:1), because TFA gave too many impurities, reductive amination using NaBH(OAc)$_3$ in the presence of acetic acid was carried out. Cyclohexyl and isopropyl amines, each with two lengths of linkage, were
produced and fully characterised. The methyl amine triazolothione with n=1 was synthesised in 78% yield using Me₂SO₄ as the electrophile with HFIP additive in 1 M aqueous NaHCO₃. The use of aqueous base with the known HFIP methodology to produce only monomethylated secondary amines has not previously been reported.
4. The Synthesis of UNIS00021 Analogues Varying at the Core

4.1 Introduction

One of the important features of triazole-thiones is that they can exist in two major tautomeric forms: the thione and the thiol. Thiols are one of the most reactive groups found in cells and are susceptible to electrophilic attack and oxidation to form stable disulfides. UNIS00021 in the thiol form could react with electrophilic biomolecules or cysteine residues and cause off-target effects, although its nucleophilicity and oxidation potential should be attenuated by the conjugated N-atoms in the ring. It is not known which tautomeric form binds to AAG and each has different hydrogen bonding patterns in which thion can act as the hydrogen bond acceptor (HBA) and the NH of the core act as the hydrogen bond donor (HBD) and show in Scheme 4.1.

Nevertheless, it was important to synthesise the triazole core lacking the thiol to potentially remove reactivity complications and investigate the role of the thiol/thione and hydrogen bonding of the ring so the analogues shown in Figure 4.1 were desired. These consist of methyltriazole UNIS00088, with a methyl group in place of the thiol, and triazole 62 lacking any group (except a proton) in its place. The activities of these compounds should provide insights into both the role of the sulfur atom and the active tautomeric form. If methyltriazole is of comparable
activity to **UNIS00021** it would suggest that the thiol tautomer is the active one, or that NH of the thione tautomer is not hydrogen bonding, and that the methyl rests in a hydrophobic area originally filled by the S-atom. If triazole **62** maintains activity, it might suggest that the S-atom is superfluous and again, N-H is not required for a significant interaction.

![UNIS00088 and 62](image)

Figure 4.1: Target analogues of UNIS00021 varying at the core

The absence of a thiol or thione in **UNIS00088** (compared to **UNIS00021**) means that the N-atoms of the triazole ring can act as hydrogen bond acceptors but not donors. **Figure 4.2** shows the predicted binding of **UNIS00088** in AAG’s active site using MOE docking. Similar to the **UNIS00021** docking, the catalytic water molecule of AAG, held by Val262, Arg182, and Glu125, hydrogen bonds with the amide N-H of inhibitor. In addition, for UNIS00088 the phenyl ring appears inside AAG’s nucleobase-binding pocket forming a pi-pi stacking interaction with Tyr127. The docking result did not show any interaction between the methyl group **UNIS00088** and His136 which was noticed between the thiol/thione of UNIS00021 His136 in MOE predicted binding (Chapter 1 **Figure 1.16**).

In this chapter the synthesis of **UNIS00088** and attempts towards triazole **62** will be discussed.
4.2 Proposed Syntheses and Background Literature

3,4,5-Trisubstituted 1,2,4-triazoles 69 are usually synthesised by cyclisation of N-acylamidrazones 67 which can be accessed in a variety of ways and are often reaction intermediates which are not isolated.67 They have been synthesised through coupling of hydrazides with a range of activated amides including chloromethylene amides 63145, imidates 64146, thioamides 65147, and thioamidates 66148 as shown in Scheme 4.2. This reaction can also proceed via the N-acylhydrazonate 68 which can be cyclised into the target 1,2,4-triazoles by reacting it with an amine.149,150 However, there is little information available in the literature
on which intermediate, \(N\)-acylamidrazones 67 or \(N\)-acylhydrazonate 68, will be formed and why. However, it was noticed from the reported literature reactions that if the activated amide is an imidate 64 with \(X=\text{OMe}, R_2=\text{H}\) then the \(N\)-atom is displaced, not the alkoxy group and so proceeds via the hydrazonoate and a new amine can be added for the cyclisation.

\[
\begin{align*}
\text{R}_1\text{N}^+\text{R}_2 & \rightarrow \begin{array}{c} \text{X} \end{array} \\
\text{R}_1\text{N}^+\text{R}_2 & \rightarrow \begin{array}{c} \text{H}_2\text{N}^+\text{R}_3 \\
N\text{-Acylhydrazonate (68)}
\end{array}
\end{align*}
\]

**Scheme 4.2:** Synthesis of 3,4,5-trisubstituted 1,2,4-triazoles via activated amide intermediates.\(^\text{151}\)

For example, Brook et al. synthesised the 5-unsubstituted triazole 73 by using formic acid hydrazide in a reaction with imidate 71 to give the hydrazonoate 72 required for cyclisation with benzylamine to give the triazole 73 (Scheme 4.3). It is worthy of note, due to use in this project, that the imidate 71 was formed by reacting [2-nitro-4-(trifluoromethyl)phenyl]acetonitrile 70 with the anhydrous HCl solution produced by reacting AcCl with methanol.
In another example for the formation of N-acylhydrazonate 69 instead of N-acylamidrazone 67, Ashton et al. reported the synthesis of 3-alkyl-5-aryl-(or heteroaryl)-4-(arylmethyl)-4H-triazoles such as 76. Here, the imidate hydrochloride 74 was reacted with 2-furoic acid hydrazide 75 to give furanoyl hydrazonate 76 which was converted to the triazole 78 upon heating with 4-nitrobenzylamine 77 (Scheme 4.4).  

On the other side, Olson S. et al. synthesised adamantyl triazole 82 via acylamidrazone 67 in 80% yield by condensing the imidate imino ether 80 of caprolactam 79 with adamantyl-1-carboxyamide 81 in toluene. The imidate 80 was formed by reaction with Meerwein’s salt as shown in Scheme 4.5.
In this project, using this methodology, there are two possible disconnections of desired product 83 (Scheme 4.6). The first (red) requires the starting hydrazide to be attached to the acetoester (86) and the triazole C5-atom to come from the activated amide 85 whereas the second (blue) requires the starting hydrazide to be attached to include the C5-atom (88) and the activated amide to incorporate the acetoester (89).

**Scheme 4.6**: Two possible disconnections of 1,2,4-triazole ester 83

### 4.3 One pot synthesis via activation of future triazole C5-containing amide

The one-pot synthesis is one of the effective synthetic method to synthesis the 3,4,5-trisubstituted1,2,4-triazoles using the idea of activating the amide with chlorinating agent. Many research groups have reported the formation of such
heterocycles via the substitution of the activated amide derivatives with hydrazides.$^{145, 153, 154, 67, 146}$

Lindström and Johansson reported a one-pot synthesis of 3-aryl-5-methyl-4-substituted 1,2,4-triazoles 93 involving activation of $N$-substituted acetamides (90) to form imidoyl chlorides 91 using oxalyl chloride as the activating reagent.$^{145}$ The imidoyl chlorides reacted readily with aryl hydrazides 92 to form stable intermediates which could be easily cyclised to the triazole by heating in the presence of base as shown in Scheme 4.7 and Scheme 4.8. Yields of 32-87% were reported for most examples, however, the importance of an electron-donating group on the aryl group of the acetamide to activate it for reaction with oxalyl chloride was noted. For example, when $N$-phenyl acetamide and $N$-4-nitrophenyl acetamide were used there was no gas evolution (CO$_2$ and CO from reaction with oxalyl chloride) and 1,2-dibenzoylhydrazine was the major isolated product when benzhydrazide was added to this mixture. Interestingly, one of their examples had a methyl group at C$^5$ of the triazole, but none of their examples had a benzyl or acetoester in the 4- or 3-positions of the 1,2,4-triazole as required in the UNIS00021 analogues.

Scheme 4.7: Synthesis of 3-aryl-5-methyl 4-substituted [1,2,4] triazoles$^{145}$
It was hoped that benzyl amides would be sufficiently electron rich to react with oxalyl chloride and so this method was applied to the desired 1,2,4-triazole ester with a methyl group at C₅ (Scheme 4.9). In the first instance, this required synthesis of hydrazide acetoester 96.
4.3.1 Step 1: Synthesis of hydrazide acetoester (96)

Park et al. synthesised acetyldrazide-\textit{d}_3 by reacting acetyl chloride-\textit{d}_3 with hydrazine at 0 °C.\textsuperscript{155} This procedure was followed in an attempt to synthesise hydrazide acetoester 96 by reacting methyl malonyl chloride (1 equivalent) with hydrazine (2 equivalents). After two hours the LCMS in the positive mode showed a peak with mass spectra showing \textit{m/z} 232.9 but no sign for the product mass. The reaction was quenched at that point by diluting it with ethanol and extracted with ethylacetate. The crude product was purified using flash column chromatography but the only product isolated was shown to be dimer 104 (Scheme 4.10) by LCMS ([M+H]\textsuperscript{+} 232.9 and IR where no –NH\textsubscript{2} stretch between
3550-3200 cm\(^{-1}\) was seen. Unfortunately, there was no signal for NH in \(^1\)H NMR even in DMSO-d6.

\[
\text{MeO}\text{-COCl} \rightleftharpoons \text{NH}_2\text{-NH}_2 \rightleftharpoons \text{MeO}\text{-CONHNHCONMe}
\]

\[0 \degree C, \text{H}_2\text{O}\]

**Scheme 4.10:** The formation of ethoxycarbonylacetoxydrazide dimer from reaction of malonyl chloride with hydrazine at 0 \degree C

It was hypothesised that the dimer formed because the addition of the methyl malonyl chloride was done at 0 \degree C and so there was enough time for it to accumulate in the presence of the desired product and react with it. It was therefore decided to add the methyl malonyl chloride into the hydrazine at room temperature. However, the LCMS again showed the same peak correlate to the dimer [M+H]\(^+\) 232.9 and there was no sign of the product.

Metwally *et al.* reported the synthesis of hydrazide acetoester 106 in 34% yield by reacting hydrazine with diethyl malonate (105) at room temperature for 24 h (**Scheme 4.11**).\(^{156}\) This procedure was followed exactly. After stopping the reaction by filtration, it was purified by trituration with diethyl ether to give the product in 38% yield. The first filtration before trituration was malonohydrazide (107) which was isolated as a byproduct in 24% yield. LCMS of the purified 106 revealed one peak with a mass spectrum matching a [M+H]\(^+\) ion at \(m/z\) 147. This reaction was repeated several times with the same conditions and the yield ranged between 33-46%.

\[
\text{O} \text{-CO} \text{-CO} \text{-EtOH} \rightleftharpoons \text{NH}_2\text{-NH}_2 \rightleftharpoons \text{O} \text{-CONHNHCONH}_2
\]

\[\text{r.t., 24 h}\]

**Scheme 4.11:** Synthesis of hydrazide acetoester 106
4.3.2 Step 2a: One-pot conversion into 5-methyl triazole

Following the procedure described above, \( N \)-benzyl acetamide was used so that after subsequent steps, a methyl group would be incorporated on \( C^5 \) of the target 1,2,4-triazole. Thus, oxalyl chloride was added to a mixture of benzyl acetamide and 2,6-lutidine as base followed by addition of hydrazide acetoester 106 after 40 minutes. After 3 h, LCMS showed one peak at 0.49 minutes retention time with a mass spectrum matching a \([\text{M+H}^+]\) ion of the desired intermediate acyl amidrazone 108 with \( m/z \) 278.

After evaporating the solvent, saturated NaHCO\(_3\) was added and the mixture heated at reflux for 3 h to facilitate the cyclodehydration. Unfortunately, LCMS showed no peak corresponding to the desired triazole. Analysing the extracted organic product after 3 h by \(^1\text{H}-\text{NMR}\) showed a quartet and triplet at 4.1 and 1.2 representing the ethyl ester group which did not integrate correctly compared to the benzyl group as shown in Scheme 4.3.

![1H (500 MHz, CD\(_3\)OD)](image)

**Figure 4.3:** The result of the cyclisation step of the intermediate 34 in 0.5 M NaHCO\(_3\)**
Interestingly, it was noticed that heating the acyl amidrazone at reflux with saturated NaHCO₃ for 24 hours gave an LCMS peak at 2.6 minutes retention time with a mass spectrum matching a [M+H]⁺ ion at m/z 232 for the acid version of triazole **UNIS00085**. After 48 h this reaction was quenched using 2 M HCl to pH~5.5–6 and was extracted with DCM/MeOH 9:1 but nothing was present by TLC. However, by ¹H-NMR the isolated organic product (**Figure 4.4, b**) did not show the expected acid since there was no signal for the -CH₂-COOH, and there appeared to be two peaks for -CH₂-Ph and two peaks for methyl groups. Furthermore, examining the same ¹H-NMR after some time (~two months, c) showed a change in the peaks of the benzene signals. Also, it now showed single peaks corresponding to the -CH₂-Ph and methyl groups and no sign of a peak for -CH₂-COOH. As will be discussed in section 4.7.3, the acid was found to be unstable, decarboxylating to dimethyl triazole **UNIS00086**, and this is what is thought to have partially occurred in this reaction.

**Figure 4.4:** ¹H-NMR spectrum showing instability of the triazole acid 103
The reaction was repeated but with heating at reflux in toluene only to promote the cyclodehydration, thus avoiding aqueous NaHCO₃ which could cause hydrolysis and contribute to by-product formation (Scheme 4.12). Surprisingly, after one hour at reflux the LCMS showed a peak at 4 minutes retention time with a mass spectrum matching a [M+H]⁺ ion with m/z 260 corresponding to the product. However, the intermediate was not fully soluble in toluene causing most of it to form a black, sticky sludge at the bottom of the flask. It was decided to leave the reaction longer so the sticky sludge of starting material might dissolve and go into the solution. Yet, after six hours of reflux there was still black sludge and so it was decided to stop the reaction at that point. The product was isolated in just 5% yield by filtering the crude and evaporating all the solvents (Scheme 4.12). Interestingly, the ¹H-NMR in CD₃OD revealed the product with the correct integration for the ester and benzyl groups. The ¹³C-NMR spectrum showed three quaternary carbon atoms at 169.1, 154.5, and 151.0 ppm representing the (C=O), (CH₃-C=N), and –CH₂-C=N-) respectively.

![Scheme 4.12: Cyclodehydration of intermediate acyl amidrazone 108 into 1,2,4-triazole acetoester UNIS00085](image)

Based on this result different solvents were tested to improve the yield of the cyclisation at reflux either for 24 h or 43 h and all were followed by LCMS (Table 4.1). The best solvent to use was a mixture of CH₃CN and toluene (2.5:1).
<table>
<thead>
<tr>
<th>Solvents</th>
<th>Intermediate acyl amidrazone</th>
<th>Intermediate acyl amidrazone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% area by LCMS (24 h)</td>
<td>% area by LCMS (43 h)</td>
</tr>
<tr>
<td>DMF/toluene (1:1)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>CH$_3$CN/toluene (2.5:1)</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Toluene$^b$</td>
<td>2</td>
<td>98$^b$ (5%)$^d$</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>saturated NaHCO$_3$$^c$</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>H$_2$O/toluene (1:1)</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Isopropanol/toluene (1:1)</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>Pyridine</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CH$_3$CN/toluene (1:2)</td>
<td>42</td>
<td>58 (49%)$^d$</td>
</tr>
</tbody>
</table>

$^a$% of total LCMS UV chromatogram peaks by area  
$^b$Most of the intermediate formed black sticky sludge at the bottom of the round bottom flask which give a false representation of the reaction mixture.  
$^c$40% represent a new peak with a mass spectrum m/z 232  
$^d$isolated yield after purification  

Table 4.1: Testing the cyclisation using different solvent followed by LCMS

This cyclisation of the intermediate using a mixture of CH$_3$CN and toluene (2.5:1) at reflux was followed by $^1$H-NMR to check the time that needed to stop it by checking the crude of the reaction after 24 h and 48 h as shown in Figure 4.5. It was interesting to observe the formation of the benzyl group in the crude reaction compared to the intermediate spectrum after 24 h and also the change in the shape of the coupling of the ester group.
This time the reaction was stopped by cooling it in a cold water bath and then filtering off the 2,6-lutidine salt (as evidenced by NMR shown in Figure 4.6). After purification by repeated column chromatography the final yield of pure triazole acetoester UNIS00085 was 49%. The rest of the product contained other impurities from which separation was too difficult.

Figure 4.5: The ¹H-NMR spectrum of the intermediate 108 compared to the cyclised 1,2,4-triazole ester UNIS00085 after 24 h and 48 h in toluene/CH₃CN at reflux.

Another two attempts were made to improve the yield and this time the reactions were scaled up to 2 and 3 g. However, the yield remained around 50-53% and multiple column chromatography was necessary.
4.3.3 Step 2b: Attempted one-pot conversion into 5-H triazole (110)

This one-pot reaction was also tested with benzyl formamide to put a proton at C5 of 1,2,4-triazole ester instead of a methyl group (Scheme 4.9). The same conditions were applied, however this time the mass of the intermediate acyl amidrazone 109 (molecular weight 263) was not detected by LCMS. The mixture was heated at reflux in CH3CN/toluene (2.5:1) anyway and after 20 h the 1H-NMR spectrum of the crude was compared with that of the 'intermediate' which revealed no difference between them. The reaction was left for 44 h but the 1H-NMR still showed no difference. The reaction was stopped and purified using flash column chromatography. Examining the isolated product by 1H-NMR (DMSO-d6) revealed a compound with structure similar to the intermediate acyl amidrazone 109.
(Scheme 4.13) but with several anomalies in the NMR spectra (which included COSY, HSQC and HMBC for assignments).

There were two singlets at 10.6 and 10.2 ppm both integrating to one and a triplet at 9.4 ppm which also integrated to one all of which represent three –NHs. This was further evidenced by treating the same ¹H-NMR sample with D₂O and seeing all three resonances disappear. Moreover, the –CH₂ in the benzyl group was a doublet before the D₂O shake and turned to a singlet after the treatment. This indicates a proton is present on the benzylic nitrogen atom which couples to the benzylic CH₂; it is unlikely that the compound is in its protonated form because it passed silica chromatography and the benzylic CH₂ is seen at 4.34 ppm. The ¹³C NMR spectrum displayed four low field carbon resonances at 167, 164, 159 and 158 ppm, which indicate the two carbonyl signals, and two more sp²-carbon atoms bound to electronegative atoms, of which there is only one (C=N) in intermediate 109. Despite these observations, the structure of the compound could not be identified.

4.4 Synthesis via acyl hydrazonoates

Based on the survey of the literature described in the introduction it was decided to design an alternative route to synthesize the 5-H triazole (113). The route was designed to access the 5-H triazole 100 via the N-acylhydrazonate 112 as shown in (Scheme 4.14).
The synthesis proceeds via the imidate 111 which can be easily synthesised through addition of methanol to methyl cyanoacetate 29 in the presence of anhydrous HCl which can be generated by reacting acetyl chloride with methanol at 0 °C. For example, preparation of compound 111 was reported by Uneo et al with 77% yield (Scheme 4.15).

The methylhydrazoneate 112 is formed by reaction of imidate 111 with formic or acetic hydrazide at room temperature. This intermediate can then be reacted with benzylamine and cyclised into the 1,2,4-triazole ester 100. The 1,2,4-triazole ester 100 can be converted into the target analogues using direct amidation or ester hydrolysis followed by amide coupling of the resulting carboxylic acid.
4.4.1  Step 1: Synthesis of imidate (111)

As described above, synthesis of the imidate salt 111 involves reaction of the appropriate nitrile with the required alcohol in the presence of HCl.\textsuperscript{157,158,160} However, this procedure requires the use of HCl lecture bottles and their associated hazards. Instead, HCl gas can be generated using either concentrated H\textsubscript{2}SO\textsubscript{4} and NaCl salt\textsuperscript{161} or by adding acetyl chloride to alcohol as reported by Brooks et al.\textsuperscript{150} Both methods were trialled in this project.

The synthesis of 111 was carried out following the procedure reported by Brooks et al (Scheme 4.3) in which HCl gas was generated by adding acetyl chloride into anhydrous methanol at 0 °C. The methanol was dried using molecular sieves which were activated by heating under high vacuum and kept under nitrogen gas. After generation of the HCl solution, methyl cyanoacetate 29 was added and the reaction was kept at 0 °C overnight. In the first and second attempts NMR analysis of the filtrate revealed dimethyl malonate 114, and ammonium chloride salt. The dimethyl malonate was hypothesised to result from overhydrolysis of the starting material which could result from a second addition of methanol followed by loss of MeCl, as shown in Scheme 4.16, or more simply from the presence of water in the reaction mixture. The ammonium chloride salt obtained from the reaction appeared as a triplet in the proton NMR spectrum at a chemical shift of ~7.5 ppm (in DMSO-d\textsubscript{6}) which agreed with that of a purchased sample (Figure 4.7).
Scheme 4.16: Possible mechanism for overhydrolysis of methyl cyanoacetate

Figure 4.7: Formation of NH₄Cl from the imidate synthesis
In the third attempt the reaction was repeated using the same conditions but using purchased anhydrous methanol supplied in a bottle with a septum. However, NMR showed a new product 115 resulting from self-condensation of the desired product (Scheme 4.17). The $^1$H NMR spectrum in DMSO showed two methyl group proton resonances at 3.60 (6H, s, -CO-OMe), and 3.59 (3 H, s, -OMe). The $^1$H NMR spectrum in DMSO-d$_6$ showed two methyl group proton resonances at 3.60 (6H, s, -CO-OMe), and 3.59 (3 H, s, -OMe). The $^{13}$C NMR spectrum showed the presence of three low field carbon resonances at 168.4 (-N=C-OMe), 167.2 (-CH$_2$-C=NH), and 167.0 (-CO-OMe). The structure of this compound was almost fully assigned using HMBC and HSQC as shown in Figure 4.8 but, unfortunately, the correlation between –C=NH and its next neighbouring –CH$_2$- was not seen although this could be due to overlap with the HMBC peaks for -COOMe.

![Scheme 4.17: Self-condensation of product imidate](image)

![Figure 4.8: HMBC correlations of self-condensation product 115](image)

As an alternative, HCl gas was generated by adding concentrated H$_2$SO$_4$ to sodium chloride and using a series of bubblers (one containing concentrated H$_2$SO$_4$) to remove moisture before entry into the reaction vessel and also to
quench excess HCl (saturated sodium carbonate solution) on exit from the reaction vessel (Figure 4.9).\textsuperscript{161}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Apparatus for generation of HCl gas using concentrated H\textsubscript{2}SO\textsubscript{4} and sodium chloride (left) and bubbling into reaction mixture (right)}
\end{figure}

The reaction temperature was kept at 0 °C overnight as described in the literature.\textsuperscript{158} Unfortunately, using this method, only starting material was recovered as shown by NMR (Figure 4.10), perhaps indicating insufficient HCl dissolved into the reaction solution or insufficient temperature.
Figure 4.10: Recovering starting material by using H$_2$SO$_4$ and brine salt to generate HCl gas

Synthesis of the analogous ethyl imidate 117 has been reported by Michael et al using ethyl cyanoacetate, ethanol and anhydrous HCl gas but their reaction was warmed from 0 °C to room temperature and stirred for 20 h to give the product in 99% yield. Following these temperature conditions and also using ethyl cyanoacetate as the starting material, but acetyl chloride and dry ethanol to form HCl, the desired product was obtained as a powder (Scheme 4.18).
Scheme 4.18: Synthesis of ethyl 3-ethoxy-3-iminopropanoate

It was purified by trituration with diethyl ether to give 60% yield of the product. LCMS analysis of this compound showed one peak with a mass spectrum matching a [M+H]$^+$ ion at $m/z$ 160. The structure of the compound was established by IR, $^1$H-NMR, $^{13}$C-NMR and compared with the literature. The IR spectrum was key to characterising the product, showing peaks at 1741 and 1615 cm$^{-1}$ representing the C=O and C=N, respectively.

The same temperature profile (0 °C addition then RT for 20 h) was applied to the original reaction using methyl cyanoacetate, methanol and HCl generated by acetyl chloride and dry methanol but no solid was formed. Instead an oily product was obtained after evaporating and triturating with diethyl ether. The $^1$H-NMR spectrum revealed this oil to contain neither product nor starting material. However, a white precipitate had been observed in the reaction after three hours. Therefore, it was supposed that the reaction needed less time than the ethyl cyanoacetate to generate the product and longer time led to by-products instead. The same reaction was repeated at room temperature for four hours and the work up was similar to the previous reaction. The methyl imidate salt was obtained as a white powder in 75% yield.

The LCMS of this compound showed one peak with a mass spectrum matching product cation at $m/z$ 132. The structure of the compound was established by IR, $^1$H-NMR, $^{13}$C-NMR and compared with the literature. Two singlets at 4.34 and 3.76 ppm were found corresponding to the two $-\text{CH}_3$ of the methoxy groups. The IR
spectrum was again key to characterising the product, showing peaks at 1732 and 1614 cm\(^{-1}\) representing the C=O and C=N bonds, respectively.

In conclusion, the synthesis of imidate 111 required careful control of temperature and time with successful reaction requiring warming to room temperature but for a limited time so as not to lead to by-products. Limited investigation of this showed optimum time to be dependent on the alcohol used (methanol or ethanol).

### 4.4.2 Step 2: Synthesis of Acylhydrazonoates

The synthesis of the methyl and ethyl hydrazonoates requires substitution of the imidate with formic hydrazide at room temperature as reported by Brook et. al.

Following this procedure formic hydrazide was added to imidate 117, which was stored at room temperature, at room temperature (Scheme 4.19). Unfortunately, in two attempts, only starting material could be isolated in 84% yield.

![Scheme 4.19: Synthesis of ethylhydrozonate 118](image)

Interestingly, in these two attempts it was noticed that leaving the starting material at room temperature caused it to decompose into a product that was no longer soluble in CDCl\(_3\). It was thought that the reaction did not work for that reason. It was therefore decided to synthesise 111 and 117 from scratch but keep them at -20 °C under nitrogen gas.

The new, carefully-stored 117 was reacted with formic hydrazide following the same conditions. The reaction was monitored by TLC until starting material was no longer present (4 h). It was quenched by adding saturated NaHCO\(_3\) solution,
as described in the literature, and extracted with DCM/MeOH (9:1) several times. Once again, no product could be isolated but only the neutral form of imidate 117, in 84% yield, as shown by a change in the chemical shifts of the two –CH₂ of the ethyl.

Ashton et al. synthesised furanoyl hydrazonoate 76 by preparing a solution of 2-furic acid hydrazide 75 in dry EtOH and adding it dropwise into a solution of the imidate hydrochloride in dry EtOH at -10 °C followed by holding it at 5 °C for three days to give the product in 58% yield (Scheme 4.4).

Following this procedure using imidate 117, after three days the LCMS showed two broad peaks adjacent to each other, each with a mass spectrum matching a [M+H]⁺ ion at m/z 203-03-17-3rd day 9am. At that point the reaction was stopped and the crude mixture was purified using flash column chromatography (DCM/MeOH 9:1) following the procedure by Ashton et al. However, it was challenging to separate the two spots shown by TLC and the ¹H-NMR spectrum showed that it was still a mixture of either the starting material and the product or product and something else. Also, the ¹³C-NMR spectrum showed an excess of peaks (Figure 4.11)
It was thought the acetylhydrazonoate 119, which would later be converted to the desired 5-methyltriazole analogue UNIS00085, may be less reactive and prone to
by-product formation than formic hydrazonoate 118 so acetylhydrazide was subjected to the same reaction conditions (Scheme 4.20).

![Scheme 4.20: Synthesis of ethylhydrazonoate 119](image)

The LCMS of the crude mixture showed two broad peaks adjacent to each other whose mass spectra correlated to the product with a m/z 217 matching a [M+H]+. There was also another unknown peak at 0.47 min retention time that increased in area as the reaction progressed (Figure 4.12).

![Figure 4.12: LCMS of crude mixture resulting from reaction of 117 with acethydrazide at 5 °C for 4 d](image)

The reaction was stopped after four days by filtering off the NH4Cl salt followed by flash column chromatography (DCM/MeOH 9:1). The 1H-NMR of desired hydrazonoate 119 should show two peaks for each of the –CH2 groups and two for
each of the $-\text{CH}_3$ groups of the ethoxy groups. However, the isolated product revealed three $-\text{CH}_2$ peaks and three $-\text{CH}_3$ peaks as shown in Figure 4.13. Also, the $^{13}$C-NMR spectrum showed a large number of peaks which could not be correlated with the $^1$H-NMR peaks. It was hypothesised that the product existed as a mixture of atropisomers and/or tautomers (Scheme 4.21). It was too challenging to prove this using HSQC and HMBC so instead it was thought to see if the NMR peaks would coalesce as the NMR temperature was increased (r.t., 40, 60, 70, 90°C) in DMSO and the rate of interconversion between atropisomers approached the NMR timescale. Unfortunately, there was no large change observed in this experiment (Figure 4.14). Another thought was to separate the atropisomers by reverse phase column chromatography, based on the LCMS result. However, this attempt failed and both peaks came off together. Therefore, it was assumed that the isolated mixture was the product and it was used directly in the next step.

Figure 4.13: $^1$H-NMR of the product mixture resulting from reacting 117 with acetyldihydrazide

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In a different attempt the reactions were repeated using the formic hydrazide and acetylhydrazide but this time the reaction was warmed from -10 °C to room temperature to try to improve the yield of the product. Surprisingly, the LCMS of
the crude after 10 hours showed just the two broad peaks of the products without impurities.

In order to quench any remaining hydrazide it was preferred to use the sodium bicarbonate again. Interestingly, using just 0.1 M sodium bicarbonate gave a better result without the need to carry out column chromatography and the yield was improved to 90%.

4.4.3 Step 3: Amination and cyclisation of N-acylamidrazono

to form 1,2,4-triazole

As described in Section 4.2, amination and cyclisation of acylhydrazonoates 118 and 119 can be achieved by reacting them with benzylamine at room temperature for three days.\(^{150}\) This procedure was followed using ethyl acetylhydrazonoate 119 in ethanol as solvent (Scheme 4.22).

\[ \text{Scheme 4.22: Attempted amination and cyclisation of 119 into 1,2,4-triazole UNIS00085} \]

Unfortunately, there was no sign of the product mass by LCMS of the reaction mixture but GCMS and \(^1\)H-NMR spectroscopy revealed the presence of benzyl acetamide suggesting transamidation of the starting material by benzylamine. After three days the reaction was quenched by adding 0.1 M HCl and extracting with DCM/MeOH (9:1). The isolated product after flash column chromatography (DCM/EtOAc 4:6) was shown to be the neutral form of the imidate 117 by \(^1\)H-NMR (Figure 4.15),
The same reaction conditions were applied to the ethyl formylhydrazonoate 118 (Scheme 4.23). GCMS of the crude product mixture revealed the formation of benzyl formamide, again suggesting transamidation of the starting material.

**Scheme 4.23**: Attempted amination and cyclisation of ethyl formylhydrazonoate 118 into 1,2,4-triazole 110

It was thought that the problem could have arisen from use of the protic solvent ethanol as proton transfers are involved in the transamidation mechanism. Therefore, it was decided to repeat the reaction of ethyl formylhydrazonoate 118 in aprotic solvent (THF, DMF, and CHCl₃) and in a protic solvent (EtOH). Following
the reactions by LCMS it was observed with all solvents that the starting material was converted into a new peak similar to that which was found previously at 0.81 minutes retention time. It was therefore decided to heat the reactions at 50 °C for six more hours but no change was observed. The ¹H-NMR spectrum of the isolated peak again revealed the free imidate. It was a challenge to isolate the benzyl formamide. However, the presence of benzyl formamide was proved by comparison with the ¹H-NMR of purchased benzylformaldehyde and by its addition into the crude reaction (Figure 4.16).

![Figure 4.16: ¹H-NMR spectrum of the crude reaction mixture, from reaction of 20 with benzylamine, spiked with purchased benzylformaldehyde showing a large increase in size of peaks at 8.14 (-CHO), 7.29 (-Ph), and 4.39 (-CH₂-Ph) ppm](image)

In summary, the proposed synthetic route to triazole analogues of UNIS00021 could not be completed. The challenges were the difficulty in characterising the acyl hydrazonoates 118 and 119 and aminating/cyclising them into 1,2,4-triazoles. In all attempts the benzylformamide or benzylacetamide was formed indicating transamidation of the starting material instead of condensation.
4.5 One pot synthesis via activation of acetoester amide

4.5.1 Introduction and background literature

As mentioned in Section 4.2, N-acylamidrazone 67 are cyclisation precursors to triazoles and can be prepared from chloroimidates 63. These, in turn, can be prepared by activating amides with a dehydrating/chlorinating agent such as oxalyl chloride.\textsuperscript{163}

William et al. have developed a practical one-pot synthesis to access multiple 3,4,5-trisubstituted 1,2,4-triazoles by activating secondary amides with trifluoromethanesulfonic anhydride (Tf\textsubscript{2}O).\textsuperscript{151} The reaction began with the amide and Tf\textsubscript{2}O at 0 °C in either DCE or DCM in the presence of 2-fluoropyridine (2-FPyr) as a base for 10 minutes. 2-FPyr was necessary to deprotonate the amide. That was followed by addition of the hydrazide followed by heating at 110 °C for one hour under microwave (μW) irradiation to promote the cyclodehydration (Scheme 4.24 and Scheme 4.25). One of the example products in the paper, triazole 120, included \textsuperscript{N}\textsuperscript{4}-benzyl substitution and another, triazole 121, included \textsuperscript{C}\textsuperscript{3}-CH\textsubscript{2}-CO\textsubscript{2}Et, both of which are present in precursors to the desired triazole analogues of UNIS00021. However, no examples were unsubstituted at the \textsuperscript{C}\textsuperscript{5} position (Scheme 4.24).
**Scheme 4.24:** One-pot synthesis of 3,4,5-trisubstituted 1,2,4-triazoles using trifluoromethanesulfonic anhydride and 2-fluoropyridine\(^{164}\)

Based on the above literature a synthetic route was proposed to synthesise triazole acetamides 100, unsubstituted at the C\(^5\) position, and discover whether the Tf\(_2\)O-mediated condensation/cyclisation described above is applicable when only a proton is present on the C\(^5\) atom. The route is shown in **Scheme 4.26** and begins with synthesis of N-benzyl malonamic acid methyl ester 123 from methyl malonyl chloride.\(^{165}\) This is followed by one-pot activation using Tf\(_2\)O, substitution with formic hydrazide and cyclodehydration to give 1,2,4-triazole ester 100 which can then be converted into the target analogues 125 using direct amidation or ester hydrolysis followed by amide coupling of the resulting acid.
4.5.2 Step 1: Synthesis of N-benzyl malonamic acid methyl ester (123)

The synthesis of amide 123 was carried out following the procedure reported by Alessandra (Scheme 4.27). After work-up and purification by flash column chromatography, the product was isolated in 34% yield. Before trying to improve this yield, the material was tested in the planned one-pot synthesis to test its viability (next section). The $^1$H-NMR spectrum in CDCl$_3$ exhibited a doublet at 4.50 ppm representing the protons of the $–$CH$_2$ of the benzyl group. IR, $^1$H-NMR and $^{13}$C-NMR data all correlated with that in the literature.
4.5.3 Step 2: Attempted one-pot conversion into triazole (100)

Following the procedure reported by William et al., Tf₂O was added to a mixture of amide 123 and 2-FPyr in DCE at 0 °C. After ten minutes, the formic hydrazide was added and the reaction was heated in the microwave at 140 °C for two hours. The reaction was stopped by adding saturated NaHCO₃ and purified using flash column chromatography. Unfortunately, LCMS of the only isolated product showed no sign for the mass of the product and the ¹H-NMR spectrum showed a great many peaks which could not be used to identify any products.

4.6 Attempted direct amidation of 1,2,4-triazole acetoester UNIS00085

There are many reported methods for direct amidation of esters using efficient inorganic catalytic systems such as (Zr(O'Bu)₄-HOAt)¹⁶⁶, and also organic catalysts such as DBU¹⁶⁷, triazabicyclo[4.4.0]dec-5-ene¹⁶⁸, and N-heterocyclic carbenes.¹⁶⁹

Price et al. reported a mild, high yielding direct amidation of alkyl cyanoacetates in the presence 0.25 equivalents of DBU without the need to activate the cyanoacetic acid or use temperatures higher than 40 °C (Scheme 4.28).¹⁶⁷ They hypothesised that DBU acts as a nucleophilic (Lewis base) catalyst in which DBU displaces the alkoxy moiety and activates the carbonyl for attack by the amine as shown in Scheme 4.29 However in all their examples the α-cyano group was present so this may be necessary for the reaction to take place.

![Scheme 4.28: Direct amidation using DBU¹⁶⁷](image-url)
This method was used in an attempt to convert 1,2,4-triazole acetoester UNIS00085 to isopropylamide UNIS00089 (Scheme 4.30) using n-BuOH as solvent. After 15 h, LCMS analysis showed a very small peak at 3.6 minutes retention time with a mass spectrum showing a product [M+H]+ ion at m/z 273 along with a major peak at 4.1 minutes retention time with a mass spectrum showing m/z 288. After 21 h the reaction was acidified, worked-up and purified using flash column chromatography to give the product of n-BuOH transesterification.

Price et al. also reported the use of 2-methyltetrahydrofuran as solvent so the reaction was repeated using this solvent but with all other conditions the same. After 24 h the UV chromatogram in LCMS analysis of the crude showed a peak for
the product but with just 25% conversion based on the integral chromatogram. The same result was obtained after heating at reflux for 14 h.

It has been reported elsewhere that ester aminolysis can be catalysed with anionic nucleophiles, in particular the anion of 1,2,4-triazole generated when it is treated with DBU. It was mentioned in this paper that the aminolysis of unactivated esters in the presence of DBU alone does not work even at elevated temperatures under solvent-free conditions and that the addition of 1,2,4-triazole resulted in a dramatic rate acceleration. This corresponds to the experience with acetoester UNIS00085 described above and suggests an α-cyano group is necessary for successful use of the method reported by Kristin et al.

The anionic nucleophile direct amidation of UNIS00085, described above, was applied following the authors’ protocol which involved solvent free conditions. However, the reaction became very dry and it was decided to add 2-methyltetrahydrofuran as solvent. Only starting material was detected by LCMS after 24 h reaction so direct amidation was abandoned in favour of hydrolysis and amide coupling (next section).

4.7 Hydrolysis of 1,2,4-triazole acetoester UNIS00085

4.7.1 Attempted hydrolysis with 1 M NaOH

The synthesis of 1,2,4-triazole acetamides can also potentially be done by hydrolysing the 1,2,4-triazole acetoester and carrying out an amide coupling. Most similar to acetoester UNIS00085 in the literature is the example reported by Soares et al. involving the hydrolysis of imidazole-thione ester 127 into the imidazole acid 128 using 1 M NaOH in MeOH at room temperature in a yield of 72%.
This procedure was applied to 1,2,4-triazole acetooester UNIS00085. After two hours, LCMS showed nearly complete conversion to the product. The reaction was quenched using 2 M HCl to pH~2 and extracted with DCM/MeOH (9:1). However, very little organic compound was extracted and the rest was trapped in the aqueous layer confirmed by LCMS which showed [M+H]$^+$ ion with m/z 232. Unfortunately, the $^1$H-NMR spectrum of the isolated product in DMSO-d$_6$ (Figure 4.17, a) did not correspond to the desired acid, and repeated $^1$H-NMR of the sample after leaving it at room temperature for some time (~4 d) showed instability of the compound (Figure 4.17, b). The decomposition product spectrum matched that of the product described in section 4.2.1 resulting from heating acyl amidrazone 108 at reflux with saturated NaHCO$_3$ (Figure 4.17, c) which was identified as dimethyltriazole UNIS00086 resulting from spontaneous decarboxylation.

Figure 4.17: The $^1$H-NMR spectrum of hydrolysing UNIS00085 with 1M NaOH

### 4.7.2 Hydrolysis with LiOH

An alternative reported ester hydrolysis uses LiOH in THF. It was thought that the lithium ions may coordinate to, and activate, the ester in preference to the triazole of triazole acetooester UNIS00085 and so it was applied using 3 equiv. of LiOH.H$_2$O at room temperature (Scheme 4.32).
After 3 h, LCMS analysis showed only starting material so 1 ml of water was added and after 30 min, LCMS showed the product and there was no sign of the starting material. The reaction was stopped by adding 2 M HCl to pH ~2 and extracting with DCM/MeOH (9:1) to the product as a sticky oil in 34% yield. The low yield was attributed to the difficulty of extract the compound from the aqueous layer so several trials were performed to improve this by: 1. adding saturated NaCl in the extraction step; 2. carrying out reverse phase chromatography on the mixture after addition of HCl; and 3. freeze-drying the mixture after HCl addition followed by trituration with DCM/MeOH (9:1). Regardless, the yield remained 15-34%.

4.7.3 Decomposition of 1,2,4-triazole acetic acid (103)

It was noticed that the peak representing -CH$_2$-COOH of acid 103 in the $^1$H-NMR was not found after leaving the sample in DMSO-d6 for some time and the methyl group of the triazole no longer integrated to three but instead to six. Similarly the peaks for -CH$_2$-COOH and –COOH in the $^{13}$C-NMR were not found. Also, the mass spectrum [M+H]$^+$ ion of this sample changed to be at m/z 188. It was suspected that decarboxylation of the acid occurred at room temperature to give the dimethyl triazole (UNIS00086). To prove this hypothesis a newly synthesised acid sample was analysed by $^1$H-NMR in DMSO-d6 every two hours for 24 hours. This showed the appearance, and increase in area over time, of a new peak for the dimethyl
group (Figure 4.18). Based on this result, it was concluded that the acid should be used directly for the next step or be kept at -20 °C.

![Graph and NMR spectra showing spontaneous decarboxylation of 1,2,4-triazole acetic acid into dimethyl triazole over time](image)

Figure 4.18: Graph and NMR spectra showing spontaneous decarboxylation of 1,2,4-triazole acetic acid 103 into dimethyl triazole over time

### 4.8 Amide coupling

As reported previously in Chapter 2, formation of the amide bond requires activation of the carboxylic acid using a coupling reagent such as EDC, DCC, or DIC. One of the challenges in applying coupling reactions to triazole acetic acid 103 was the amount available was either small or it was combined with LiCl salt (if the extraction with DCM/MeOH was done after freeze drying of the reaction...
mixture). Following the same conditions used in chapter 2, cyclohexylamine and EDC were used with 1,2,4-triazole acetic acid 103 to give 50% yield of the desired cyclohexylamide UNIS00088 after 24 h. In this attempt heating was avoided to prevent decarboxylation.

![Chemical Reaction](image)

**Scheme 4.33:** Preparation of cyclohexylamide UNIS00088 using triazole acetic acid 103 and EDC

It was decided to synthesise the isopropyl amide using the same coupling reagent and conditions. In the first attempt it was a challenge to purify the product. Although the crude material was purified twice using the flash column chromatography and preparative TLC the $^1$H-NMR spectrum still showed a mixture of the product and starting material. In a different attempt the crude product was purified using preparative TLC but the $^1$H-NMR spectrum did not show the desired product but the methyl ester triazole UNIS00087 instead. This was evidenced by a 3H singlet at 3.63 ppm as well as a mass spectrum which showed a [M+H]$^+$ ion at m/z 246. It was hypothesised that the presence of methanol in the starting material which was purified by extraction with DCM/MeOH (9:1) led to this product in preference to the sterically hindered isopropylamine.

The reaction was repeated with a dry starting material using the same conditions. Unfortunately, after purification methyl ester UNIS00087 was again isolated in 16% yield and just 1% yield of the desired isopropylamide triazole (UNIS00089) was obtained. The structure of UNIS00089 was established by IR, $^1$H-NMR, and $^{13}$C-NMR. The $^1$H-NMR spectrum showed a doublet integrating to six protons at 1.01
ppm for the two methyl groups of the isopropyl amide and a septet integrating to one proton at 3.76 ppm for the -CH of the isopropyl amide.

It was next thought that the formation of methyl ester UNIS00087 could have arisen from the use of EDC as the HCl salt as it may protonate the isopropylamine of which 1.2 equiv. was used. Therefore, in an attempt to improve the yield trimethylamine was added to the reaction along with EDC at 0 °C followed by warming to rt for 24 h. However, the isolated product from this reaction was the dimethyl triazole resulting from decarboxylation even though the starting material had been kept at -20 °C after synthesis and prior to use.

In a parallel work (described in Chapter 5) the triazole-thione acetic acid UNIS00091 was purified using dowex resin to give the acid in quantitative yield without the presence of the salt as described in Chapter 5. This method was applied to triazole acetic acid 103 and allowed it to be isolated in quantitative yield. This was followed by amide coupling reaction using EDC as the coupling reagent but this time in the presence of HOBT and DIPEA. After purification by flash chromatography followed by reverse flash chromatography, the desired isopropyl amide UNIS00089 was isolated in 60% yield (Scheme 4.34).

Scheme 4.34: Preparation of isopropylamide UNIS00089 using triazole acetic acid 103 and EDC and HOBT
4.9 Conclusion

The synthesis of an analogue of hit triazole-thione UNIS00021 bearing a C₅-methyl group instead of thiol/thione was achieved using a one-pot synthesis involving activation of benzyl acetyl amide, using oxalyl chloride, to form an imidoyl chloride which was reacted hydrazide acetoester to give an intermediate acyl amidrazone which could be cyclodehydrated to give a triazole acetoester. This was successfully hydrolysed to the acid using LiOH in THF/water where use of NaOH in water had instead hydrolysed the core triazole. The resulting acid undergoes decarboxylation at room temperature so must be used straightaway or stored at low temperature. It could be coupled with cyclohexylamine or isopropylamine using EDC as the coupling agent to give the desired analogues for future testing as inhibitors of AAG.

The same route could not be used to synthesise the C₅-H analogue due to a failure in cyclising the intermediate acyl amidrazone 109. Attempts to access this compound with alternative methods including: 1. Tf₂O activation of N-benzyl malonamic acid methyl ester (123) and reaction with formic hydrazide; and 2. Synthesis of hydrazonoates followed by amination and cyclisation with benzylamine; also failed so this compound was not obtained.
5. Synthesis of UNIS00021 Analogues Bearing N\textsuperscript{4} Substituents Alternative to Benzy1

5.1 Introduction

The docking result of UNIS00021 into the Aag crystal structure using MOE showed the phenyl ring occupies the pocket which normally accepts the damaged base of substrate DNA (Figure 1.15 and Figure 1.16, Chapter 1). In this pocket, a parallel displaced pi-pi stacking interaction is made between the phenyl ring and Tyr127. However, this phenyl ring does not fill the whole pocket compared to the ethenoadenine- or ethenocytidine-containing oligomers. Therefore, it was important to synthesise analogues of UNIS00021 bearing different aryl groups to assess the possibility of finding additional and/or stronger binding interactions in this pocket.

In this chapter the attempted development of a divergent synthesis towards analogues of UNIS00021 varying at the N\textsuperscript{4}-substituent will be discussed. This would allow preparation of a range of analogues in one step from a common precursor.

5.2 Proposed Synthesis and Background Literature

Molander and Maxwell have reported the use of potassium organotrifluoroborates in palladium-catalyzed cross-coupling reactions with aryl halides.\textsuperscript{174} They report high yields with low catalyst loadings and broad reaction scopes. The potassium organotrifluoroborates have been used widely due to their air and moisture stability and also their greater nucleophilicity.\textsuperscript{175} Of relevance to the synthesis of analogues of UNIS00021, Molander et. al. have produced amidomethyl- and tertiary
aminomethyl-trifluoroborates and used them in Suzuki-Miyaura cross-coupling reactions with aryl halides (Scheme 5.1) and Scheme 5.2. \(^{176, 177}\)

![Scheme 5.1: Aminomethylations via cross-coupling of potassium organotrifluoroborates with aryl bromides.](image)

Based on these successes, the same group also reported the synthesis of a potassium Boc-protected aminomethyltrifluoroborate \(^{129}\) and its use in Suzuki-Miyaura cross-coupling reactions with various aryl and heteroaryl chlorides (Scheme 5.3). The trifluoroborate \(^{130}\) was prepared in a one-pot process over four steps in 75% yield as shown in Scheme 5.4.

![Scheme 5.3: Suzuki-Miyaura cross-coupling reactions using potassium Boc-protected aminomethyltrifluoroborate](image)

![Scheme 5.4: The preparation of the potassium Boc-protected aminomethyltrifluoroborate](image)
It was hypothesised that Molander’s methods could be applied to analogues of UNIS00021 by synthesizing a precursor in which a potassium aminomethyltrifluoroborate takes the place of UNIS00021’s phenyl ring allowing it to be later subjected to Suzuki-Miyaura cross-coupling reactions to afford a range of arylmethyl triazole-thiones. Therefore, the retrosynthesis shown below (Scheme 5.5) was devised. It was planned to synthesise the 3,5-disubstituted cyclohexylamide triazole-thione UNIS00092 first and add the potassium methyltrifluoroborate group last. This was to avoid the need to synthesise the amide after Suzuki-Miyaura cross-coupling reaction of each analogue. Also, potassium organotrifluoroborate salts can be prone to hydrolysis so it would be difficult to carry it through the earlier steps of the synthesis, especially ester hydrolysis.

![Scheme 5.5: Retrosynthesis of analogues of UNIS00021 varying at the N^4 benzyl group](image)

Based on the retrosynthetic analysis, a six step synthetic route was proposed (Scheme 5.6).
The synthesis proceeds via base-mediated cyclisation of acyl thiosemicarbazide 133 which can be synthesised through the addition of ethyl-3-chloro-3-oxopropionate 131 to the thiosemicarbazide 132.\textsuperscript{179} The 1,2,4-triazole-thione ester UNIS00090 is prepared by reacting 133 with strong base, either aqueous sodium hydroxide solution or sodium ethoxide in ethanol.\textsuperscript{179,180}

Alternatively, Elnagdi \textit{et al} reported the synthesis of the 1,2,4-triazole-thione UNIS00090, in 32\% yield, through reaction of thiosemicarbazide 132 with imidate 117, which was synthesised in Chapter four in 70\%, (Scheme 5.7).\textsuperscript{181} This yield is low and there is no spectral data found for UNIS00090 in this reported method.

\textbf{Scheme 5.6:} Proposed synthesis of analoges of UNIS00021 varying at the $N^4$ benzyl group

\textbf{Scheme 5.7:} The synthesis of 1,2,4-triazolinethiones ester UNIS00090.\textsuperscript{181}
Noel et al. also reported the formation of 1,2,4-triazole-thione acetic acid UNIS00091 by heating 1-aminobarbituric acid 137 at reflux in aqueous acid (Scheme 5.8).\textsuperscript{182}

![Scheme 5.8: Synthesis of 1,2,4-triazole-thiones acetic acid UNIS00091\textsuperscript{182}]

In all the reported methods the \textsuperscript{1}H-NMR spectrum of the $-\text{NH}$ of the 1,2,4-triazole-thione core was not reported and different tautomeric forms were drawn in the schemes; all possibilities are shown in Scheme 5.9

![Scheme 5.9: Possible tautomeric forms of 1,2,4-triazole-thione ester UNIS00090\textsuperscript{5}]

In order to convert ester UNIS00090 to the carboxylic acid UNIS00091, it can be hydrolysed using basic conditions, such as NaOH or NaHCO$_3$. Of closest relevance to the synthesis of UNIS00091, although not bearing the thione group, Nara et al. hydrolysed ethyl 1H-1,2,4-triazol-3-ylacetate (138) using 4 M aqueous sodium hydroxide solution in a mixture of water, methanol and THF at reflux for 2 h.

![Scheme 5.10: Hydrolysis of the 1,2,4-triazole ester 138 into 1,2,4-triazole acid 139\textsuperscript{4}]

The synthesis of the 1,2,4-triazole-thione cyclohexylamide \textit{UNIS00092} could be achieved by activating the acid \textit{UNIS00091} using a coupling reagent such as EDC prior to treatment with amine as done previously to synthesise \textit{UNIS00021} analogues in previous chapters. However, these amides were obtained in low yields and that was hypothesised to be due to the absence of a base such as Et$_3$N or DIPEA while purchased EDC is in the acid form which may protonate and deactivate the substrate amine. It has also been reported that the use of EDC in the presence of HOBt and a base such as DIPEA promotes amide couplings in excellent yields.$^{173,183}$ Alternative amide coupling reagents are also available, such as HATU, an example of whose use includes coupling of pyrrole carboxylic acid (140) with isopropylamine (\textit{Scheme 5.11}).$^{184}$

\begin{equation}
\begin{array}{c}
140 \\
\text{HATU, NEt$_3$} \\
-30 \text{ °C, 2 h}
\end{array}
\rightarrow
\begin{array}{c}
141 \\
\text{-30 °C, 30 minutes, 90%}
\end{array}
\rightarrow
\begin{array}{c}
142 \\
\text{NH$_2$, NEt$_3$}
\end{array}
\end{equation}

\textit{Scheme 5.11:} Amide coupling of pyrrole carboxylic acid with isopropylamine using HATU coupling reagent

The aminomethyltrifluoroborate 1,2,4-triazole-thione 134 will be formed by reacting the triazolothione amide \textit{UNIS00092} with potassium (bromomethyl) trifluoroborate, commercially available,$^{185}$ in the presence of a base to deprotonate the target NH of the 1,2,4-triazole-thione core. Selectivity between the various acidic protons on the core is an issue at this point but there is precedence for selectivity for N$^4$: Kumudha \textit{et al.} alkylated the same position with ethyl chloroacetate using K$_2$CO$_3$ as a base in 72% yield (\textit{Scheme 5.12}).$^{186}$
5.3 Results and Discussion

5.3.1 Step 1: Synthesis of 1,2,4-triazole-thione acetoester

The synthesis of ethyl 2-[(carbamothioylamino)carbamoyl]acetate 133 was performed in one step according to the existing literature procedure. After 2 h at reflux, the reaction was stopped by filtering off the precipitate, and recrystallizing from ethanol. The product was isolated in poor yield (23%) (Scheme 5.13) and most of the thiosemicarbazide remained unreacted as shown by LCMS.

Scheme 5.13: Acylation of thiosemicarbazide.
Cameron et al. synthesised 133 by adding ethyl malonyl chloride dropwise into a mixture of thiosemicarbazide and anhydrous pyridine as solvent at -5 °C and then warming to room temperature. This method was carried out instead, but again, after 24 h 99% thiosemicarbazide was recovered from the filtrate.

It was decided then to add just one equivalent of pyridine to a mixture of ethyl malonyl chloride and thiosemicarbazide in CH₃CN and it was left at reflux for 2 h. However, this time the yield was even lower (13%).

As described in the introduction to this chapter, Elnagdi et al. reported that reaction of thiosemicarbazide with imidate 117 yielded a mixture of 1,2,4-triazole-thione UNIS00090 in 32% yield and amino-1,3,4-thiadiazole in 49% yield. Since imidate 117 was synthesised previously in good yield, as described in Chapter 4, it was decided to try this method. The reaction was first performed in CHCl₃ at 0 °C and, after 2 h and silica chromatography the desired 1,2,4-triazole-thione UNIS00090 or isomer amino-1,3,4-thiadiazole 136 (by LCMS) was isolated in 55% yield (Scheme 5.14). The remainder was was a mixture of thiosemicarbazide and triethylamine salt. This reaction was repeated with three different solvents: CHCl₃, CH₃CN, and EtOH and the best yield obtained was 60% using EtOH.

![Scheme 5.14: The synthesis of UNIS00090 using the imidate 117 and thiosemicarbazide](image-url)
It was a challenge to assign the structure of the isolated product as that of the desired 1,2,4-triazole-thione UNIS00090 and not amino-1,3,4-thiadiazole 136 since as shown in Scheme 5.15 both could possibly show an HMBC correlation (2-4 bond distance) could show a correlation between the NH- of ‘a’ and quarternary carbon ‘e’.

![Scheme 5.15: HMBC correlation between carbon ‘e’ and the triazole or thiadiazone core protons](image)

However, IR spectrum could be used to differentiate between the three groups (C=S, -S-H, C-S-C). Based on the literature the C-S-C stretching vibration frequency is around 690-725 cm⁻¹ and the NH₂ stretching frequency is 3080-3300 cm⁻¹.¹⁸⁷,¹⁸⁸ On the other hand, C=S in unsubstituted 1,2,4-triazole-thione has a stretching frequency of 1200 cm⁻¹ and the S-H and C-S IR absorption frequencies in 3-mercapto-1,2,4-triazole are at 2565 and 944 cm⁻¹ respectively.¹⁸⁹,¹⁹⁰ Unfortunately, all these absorption frequencies could be seen in the IR spectrum of the isolated product and it was difficult to assign the actual structure (Figure 5.1).

Kiseleva et al. mentioned that refluxing thiosemicarbazide 133 in AcOH affords not the 1,2,4-triazole-thione UNIS00090 but amino-1,3,4-thiadiazole 136 and that the amino group nitrogen is less reactive than sulfur during cyclisation under these conditions.¹⁹¹ However, they only reported melting points of their products (158-160 °C for amino-1,3,4-thiadiazole and 192-194 °C for 1,2,4-triazole-thione) and there were no proton NMR or IR in their paper with which to compare.¹⁹¹ The melting point of the isolated product was between 179-180 °C which was closer to
the reported melting point of the desired 1,2,4-triazole-thione UNIS00090. This product was therefore taken forward to the next step.

Figure 5.1: IR spectrum of isolated product from reacting imidate 117 with thiosemicarbazide

5.3.2 Step 2a: Attempted direct amidation of 1,2,4-triazole-thione acetoester UNIS00090

Subsequent sections will describe the difficulties encountered in converting acetoester UNIS00090 into the carboxylic acid ready for amide couplings. During that work, some attempts were made to amidate the ester directly instead. These are described in this section.

As described in Section 4.6, Chapter 4, Price et al. reported the use of 0.25 equiv. of DBU as a nucleophilic (Lewis base) catalyst at 40 °C for direct amidation of esters.\textsuperscript{167} This method was applied to ester UNIS00090 with cyclohexylamine (Scheme 5.16). However, LCMS after 12 and 24 h showed just starting material and no sign of the product. In a different attempt, DBU was combined with 1,2,4-triazole in a bifunctional catalytic, solvent free system (described in Section 4.6, Chapter 4).\textsuperscript{170} However, after 24 h LCMS again showed only starting material. It
was decided then to heat the reaction at 70 °C after adding 0.5 ml of 2-Me-THF. Yet, after 24 h of starting heating the LCMS showed just the starting material.

![Scheme 5.16: Failed DBU-catalysed amidation of UNIS00090](image)

In contrast to basic/nucleophilic catalysis, Hiroyuki et al. used lanthanum trifluoromethanesulfonate as a Lewis acid catalyst for amidation of esters under solvent free conditions at 70 °C; they reported excellent yields for both aliphatic and aromatic esters. These conditions were applied to triazole-thione acetoester UNIS00090 with isopropylamine **Scheme 5.17**. Care was taken to achieve anhydrous conditions heating the catalyst under high vacuum before adding the starting material and amine. Again, after 24 h, LCMS showed only starting material with no sign of the product (**Scheme 5.17**). The reaction was repeated but using dry ethanol as a solvent, but again, LCMS after 3 h and 24 h revealed only starting material. It was hypothesised that the lanthanum catalyst preferentially coordinates to the thione group rather than the carbonyl group it is required to activate since it the higher energy level of sulfur orbitals matches better those of lanthanum.

![Scheme 5.17: Failed La(O Tf)₃-catalyzed amidation of UNIS00090](image)

Moving back to basic catalysis, the use of potassium tert-butoxide (t-BuOK) to convert aliphatic and aromatic esters into amides in good to excellent yield has also been reported in the literature. However, an attempt using 2 equiv. of t-
BuOK at room temperature for 24 h to convert triazole-thione acetoester UNIS00090 into the desired amide 145 again showed only starting material by LCMS.

Ohshima et al. developed the use of NaOMe as a catalyst and showed access to a wide variety of functionalised amides through direct amidation of esters. They also tested the reaction with various solvents and it to proceed in non-polar and polar solvents including THF, 1,4-dioxane, hexane, and NMP, but not in acetonitrile or alcohols. This method was applied to acetoester UNIS00090 using 1.3 equiv. of cyclohexylamine in dry THF at 50 °C (Scheme 5.18). After 24 h, LCMS showed a very small peak corresponding to the [M+H]+ product m/z 241. Another 5 mol% NaOMe was added but after another 24 h there was no significant change in the LCMS UV peak of the product.

It was hypothesised that NaOMe deprotonated the triazole-thione core proton (pK_a ~ 8.4) instead of the amine (pK_a ~ 10.6) and that the reaction might require 2 equiv. of NaOMe. so if one equivalent of it deprotonate the triazole proton then the other equivalent should be capable to deprotonate the amine after it attack the carbonyl group. Based on this, the reaction was repeated with 2 equiv. of NaOMe. However, after 24 h only ~7% conversion (by LC-UV chromatogram) was observed. Addition of another 1.3 equiv. of cyclohexylamine and 24 h stirring increased this to ~23%. Finally, heating at reflux for 24 h, followed by use of the microwave at 110 °C for 1 h improved the apparent LC-UV conversion to ~42%.

It was decided to stop the reaction by adding saturated NH_4Cl as reported, followed by adding 2 M HCl to reach pH~ 5-6 to re-protonate the triazole-thione. After extraction with DCM/MeOH (9:1) and flash silica chromatography, the product cyclohexylamide UNIS00092 was isolated in just 4% yield and the starting material was recovered in 22% yield (Scheme 5.18). The structure of UNIS00092 was confirmed by ^1H-NMR, IR, and LCMS.
Since the yield for direct amidation of acetoester \textbf{UNIS00090} was so low, the method was abandoned in favour of hydrolysis to the acid and amide coupling (next section).

### 5.3.3 Step 2b: Hydrolysis 1,2,4- triazolothione ester \textbf{UNIS00090}

The hydrolysis of 1,2,4-triazole-thione ester \textbf{UNIS00090} into acid \textbf{UNIS00091} was carried out using LiOH in THF and water (Scheme 5.19), conditions used previously, and described in Chapter 4, for the hydrolysis of methyl-1,2,4-triazole \textbf{UNIS00085}.\textsuperscript{196} LCMS showed the disappearance of the staring material and the appearance of a new peak which corresponded to the product [M+H]\(^+\) \(m/z\) 160.

The reaction was stopped by adding 2 M HCl to reach pH~2 and extracted with DCM/MeOH (9:1). The \(^1\)H-NMR (DMSO-d6) of the organic layer showed the disappearance of the ethyl group and a broad peak at 12.9 ppm, integrating to one, which might correspond to the acid proton. However, the product was isolated in
very low yield 10% even after several extractions and the addition of saturated NaCl to the aqueous layer.

An attempt was made to evaporate most of the aqueous layer, filter off the salt and then extract the filtrate with DCM/MeOH (9:1) but comparing the mass of the isolated product (349 mg) with the theoretical yield (84 mg) indicated the presence of high amount of LiCl salt. This was run through a reverse phase column in order to elute the salt first using water before ramping up the ratio of organic solvent. Unfortunately, both the product and the salt eluted at the same time and again the mass was higher than the theoretical yield.

Continuous liquid-liquid extraction is a method to extract very polar compounds from aqueous solution using an organic solvent that is heavier than water.197 This method was used in an attempt to extract the acid UNIS00091 from the pH~2 aqueous mixture using DCM. Although the process was left for 24 h, there was no product at all in the organic layer by TLC or LCMS.

An SCX column was used in another attempt to purify the acid UNIS00091 from the salt. This is a strong cation exchange sorbent which could extract the lithium cations from the triazole acid (Scheme 5.20). The evaporated reaction mixture was dissolved in methanol and passed through the SCX column. After evaporation, 10 mg of the eluted product was examined by NMR using an internal standard (1mg/ml hexamethyl-cyclotrisiloxane in DMSO-d6, 0.5 ml).198 Disappointingly, the calculated mass of the acid in the mixture was only 2.07 mg out of 10 mg as shown in Figure 5.2.

Scheme 5.20: LiCl salt removal using an SCX column
After repeated failures to purify triazole-thione acetic acid UNIS00091, it was decided to try the amide coupling reaction using the impure mixture containing the LiCl salt.

5.3.4 Step 3a: Attempted amide coupling of LiCl-contaminated 1,2,4-triazole-thione acetic acid

As described in previous chapters, the amide coupling agent EDC had successfully provided the desired amides but in low yields. This time, to improve the yield, HATU was tested in a coupling between cyclohexylamine and the LiCl-contaminated 1,2,4-triazole-thione acetic acid UNIS00091. The reaction was started at -30 °C and after LCMS showed starting material only, was warmed to RT and stirred overnight. Regardless, LCMS showed only starting material.
Following this, several coupling reagents and conditions were tested as shown in Table 5.1. Unfortunately, in all only starting material was detected by LCMS. It was thought that the problem was the presence of the LiCl salt.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Solvents</th>
<th>Time</th>
<th>Temperature</th>
<th>% Yield by LCMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td>EtOAc</td>
<td>24 h</td>
<td>60 °C</td>
<td>0</td>
</tr>
<tr>
<td>DCC</td>
<td>DMF</td>
<td>24 h</td>
<td>60 °C</td>
<td>0</td>
</tr>
<tr>
<td>DIPEA, TBTU</td>
<td>DMF</td>
<td>24 h</td>
<td>r.t.</td>
<td>0</td>
</tr>
<tr>
<td>DIPEA, HOBT, EDCI</td>
<td>DMF</td>
<td>48 h</td>
<td>r.t.</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.1: Testing of different amide coupling reagents and conditions with LiCl-contaminated 1,2,4-triazole-thione acetic acid UNIS00091 and cyclohexylamine

In conclusion, the amide coupling of 1,2,4-triazole-thione acetic acid UNIS00091 mixture containing LiCl salt contaminant was not successful so it was deemed essential to find a method of purifying the acid as will be described in the next section.

5.3.5 Purification of 1,2,4-triazole-thione acid UNIS00091 using Dowex ion-exchange resin

It was decided to purify LiCl-contaminated 1,2,4-triazole-thione acetic acid UNIS00091 (resulting from hydrolysis using LiOH in THF and water, as described in Section 4.7.2) using anion exchange chromatography. It was proposed to use Dowex® resin in the hydroxide ion form. Triazole-thione acetic acid UNIS00091 would form carboxylate ions which have higher affinity for the resin and so would exchange for hydroxide ions and become bound to the resin. Since neutral species and cations do not interact with the resin, the Li cations would be eluted at the beginning. After elution of impurities, the sample ions can then be eluted by
introducing an ion with a higher affinity for the resin or a high concentration of an ion with equivalent or lower affinity.\textsuperscript{199}

Thus, Dowex\textsuperscript{®} resin in the chloride ion form was first exchanged with hydroxide ions by washing the resin with 20 equiv. of 1 M NaOH followed by adding 4 equiv. of deionised water to remove any unexchanged OH anions (Scheme 5.21). Then a new LiOH hydrolysis reaction was completed and the entire reaction mixture containing 1,2,4-triazole-thione acetic acid was added to the resin. The column was first washed with MeOH to elute any neutral impurities and unbound LiCl. This was followed by eluting the column with 0.1 M formic acid in MeOH and then with 0.1 M HCl in MeOH. Since the product was yellow, it was noticed that once the formic acid was added the product began to elute. It was isolated in quantitative yield. LCMS showed a peak with a mass spectrum matching a [M-H]\textsuperscript{-} ion at $m/z$ 158. \textsuperscript{1}H-NMR using an internal standard (described in Section 5.3.4) revealed the absence of any salt by mass and clear N-H peaks of the triazole-thione.

Scheme 5.21: Purification of 1,2,4-triazole-thione acetic acid UNIS00091 using Dowex 1X2 resin
5.3.6 Amide coupling of 1,2,4-triazole-thione acetic acid

Many papers report the use of EDC as amide coupling reagent in the presence of HOBt and a base such as DIPEA. With the pure acid UNIS00091 in hand, it was decided to try the combination of EDC, HOBt, and DIPEA for the amide coupling reaction (Scheme 5.22). After 3 h, LCMS showed a peak for the desired product UNIS00092 and it was in apparent 61% conversion by the UV chromatogram. After 24 h this increased to 81% and the reaction was worked-up and purified by flash column chromatography to give the product in 33% yield. The low yield of the product was due to loss to the aqueous layer during work-up as shown by LCMS.

![Scheme 5.22: Preparation of cyclohexylamide UNIS00092 using triazole acetic acid UNIS00091 and EDC](image)

Unfortunately, due to time limits, conversion of the triazole-thione into methyltrifluoroborate 134 and coupling with a range of aryl halides could not be completed.

5.4 Summary and Conclusion

The synthesis of analogues of hit triazole-thione UNIS00021 varying at the phenyl group using Suzuki-Miyaura cross-coupling of a trifluoroborate precursor could not be completed due to time limits. However, the first three steps on this route were established: reaction of imidate 117 with thiosemicarbazide gave the triazole-
thione ester UNIS00090 in one step in 60% yield. Direct amidation of the ester was not possible but it could be hydrolysed to the acid using LiOH in THF/water. Purification required significant experimentation and success was achieved using ion exchange chromatography with a Dowex resin to give the acid in quantitative yield. Amide coupling of this with cyclohexylamine using EDC in the presence of HOBT and DIPEA gave 33% yield of UNIS00092. The low yield was due to loss of product in the aqueous layer during extraction and this could be improved in the future using a direct reverse phase column chromatography.
6. Biochemical Assays of AAG Activity and Inhibitor Potency

6.1 Introduction

Biochemical assays to test enzyme activity in the absence and presence of inhibitors are essential for comparison of inhibitor potencies. Many sensitive enzyme assays have been developed for use with high throughput screening (HTS) to enable testing of millions of compounds in a short time. However, designing a cell-free enzyme assay requires an understanding of the enzyme mechanism of action and its initial test conditions such as buffer, salt concentration and pH.

As discussed in Chapter 1, the AAG enzyme’s mechanism of action is similar to other DNA repair enzymes. It gains access to DNA damaged bases, in double stranded DNA, by flipping the nucleotide 180° into its active site where it is made a better leaving group through hydrogen bonding with the backbone carbonyl oxygen of Ala-134. This is followed by hydrolysis of the N-glycosidic bond using an active site water molecule held in its active site which releases the free damaged base into solution and leaves an abasic (apurinic/apyrimidinic) site for repair by downstream enzymes. Given this information, a biochemical assay for AAG activity must use a substrate duplex DNA oligomer containing a damaged base which is a substrate for AAG and must provide a means for quantifying either the substrate DNA oligomer, the product DNA oligomer containing an abasic site, or the product excised free base.
6.2 Microplate surface-bound three-component fluorescent DNA oligomer assay

6.2.1 Introduction

Elliott has developed a microplate-based assay which uses a duplex DNA oligonucleotide substrate which is bound covalently to the surface of functionalised 96-well plates. Thermo Scientific use a patented photochemical method for their construction of surface-activated ‘Nunc™ Immobilizer Amino’ plates which bear an electrophilic group that reacts with amines. The substrate oligonucleotide consists of three parts (Figure 6.1): INC01, a single stranded oligomer which is attached to the plate via a 5’-amino modification; HX01, which contains an internal hypoxanthine (H) residue, to act as a substrate for AAG, and ends at the 3’-end with a fluorescein molecule and the 5’-end with a phosphate for ligation to INC01; and REP04, which is the complementary strand to ligated INC01 and HX04.

![Figure 6.1: DNA oligomer structure used for AAG surface-bound plate assay. Lower case letters indicate the presence of phosphorothioate linkages to help protect the complex from non-specific exonucleases.](image)

After INC01 is bound to the plate, REP04 and HX01 are allowed to anneal with it and each other to give a Watson-Crick base-paired complex. The complex is then treated with DNA ligase to covalently bind INC01 to HX01 (Figure 6.2). The 3’-fluorescein is now attached to the plate via the INC01-HX01 oligonucleotide. By incubating this with AAG, abasic sites are produced by the enzyme’s excision of hypoxanthine. At this point, after a set time, the wells can be emptied and washed to remove AAG and any inhibitors being tested; this is a major advantage of this assay which should prevent inhibitor interference with fluorescence. Subsequent
treatment of the oligonucleotide with sodium hydroxide solution (i) generates a break in the INC01-HX01 strand, at the alkali-susceptible abasic site only, and; (ii) denatures the complex leading to release of fluorescein where a stand break has been produced. There are three approaches which may be used to quantify the extent of action of AAG on the oligonucleotide complex: (i) direct detection of eluted fluorescence; (ii) direct detection of retained fluorescence and; (iii) by detection of retained fluorescence with an anti-fluorescence antibody-horseradish peroxidase (HRP) conjugate whose action leads to a colour change when its substrate 3,3',5,5'-tetramethylbenzidine (TMB) is added. To begin with, eluted fluorescence was measured.

![Diagram](image)

**Figure 6.2:** General procedure for the AAG activity biochemical assay

Compounds were assessed for inhibitory activity by incubating with a fixed concentration of AAG. It was important that this concentration was one which, within the incubation time of the assay, produced an output where inhibition could easily be detected, i.e. that this AAG concentration was in the linear range of an AAG standard curve. For this reason, every assay assessing inhibitors included a standard curve of AAG activity generated by incubating the substrate oligonucleotide with varying concentrations of AAG. This was done for two further
reasons: (i) to check the enzyme was functioning correctly; and (ii) to provide a curve equation for the interpolation of absorbance values resulting from inhibitor assays to give apparent AAG activities and, thereby, percentage inhibition values.

Chu used this assay (quantifying eluted fluorescence) to test synthesised lone ethenocytidine nucleoside along with three phosphate derivatives but found them to be completely inactive (IC$_{50}$ > 1 mM) against AAG. He also used this assay to test the activity of 49 purchased compounds predicted to be inhibitors from the virtual screen using MOE software. Only UNIS00021 was found to inhibit AAG and its IC$_{50}$ was ~65 μM.$^{10}$

6.2.2 Results and Discussion

6.2.2.1 Failed attempts to reproduce bioassay results

A reliable biochemical assay for assessment of the activity of the synthesised analogues of UNIS00021 was required. To begin with, reproduction of the original assay, used to identify UNIS00021 as an inhibitor and measuring eluted fluorescence, was attempted.

In the first assay, the normal standard curve shown in Figure 6.3 was produced, which verified the use of AAG at a concentration of 0.1 U/100 μl for testing the inhibitors as inhibition from this point is easily detectable on the approximately linear portion of the curve.
**Figure 6.3:** AAG standard curve generated using the surface-bound DNA oligomer assay and measuring eluted fluorescence. Values are averages of n=3, error bars show standard deviation.

On the same plate, several purchased analogues of **UNIS00021** were tested at a fixed concentration of 0.1 mM and 0.1 U/100 µl AAG. Although the standard curve produced in this experiment was normal, some of the compounds appeared to show inhibition while others (**UNIS00064**, **UNIS00065**, and **UNIS00066**) apparently increased the eluted fluorescence signal (corresponding to an apparent activation of AAG) compared to the blank (no inhibitor) (**Figure 6.4**). It was hypothesised that either AAG was being 'activated' by these compounds, an error in enzyme concentration with inhibitors had been made or something was interfering with the fluorescence values.
Initially, it was thought that the well washing protocol used after incubation with AAG may not have been sufficient to remove all traces of test compounds and that the latter compounds were themselves fluorescent thus increasing the signal in the eluate after treatment with sodium hydroxide and showing a corresponding apparent ‘activation’ of AAG. To test this hypothesis, compounds UNIS00064-UNIS00066 were dissolved at a range of three concentrations in 0.1 M sodium hydroxide solution and their fluorescence measured in the same type of plate and plate reader. No compound showed significant fluorescence relative to blank 0.1 M sodium hydroxide solution so this explanation was eliminated.

This was followed by several assays in which the inhibitory εC-containing oligomer (εC oligo) was tested as a positive control. Also as mentioned previously, it has been reported that morin (UNIS00084) can act as an inhibitor against AAG with an IC₅₀~2.6 µM using 2.3 nM of AAG in a gel-based excision activity bioassay.⁶² Since
morin is a small organic inhibitor, we examined its potential for use as a positive control. Some other compounds were also tested in these assays: UNIS00021, a newly purchased batch and synthesised, UNIS00067, UNIS0007, and UNIS0008 as shown in Table 6.1. The results showed that the bioassay was working well based on good standard curves and that εC oligo did inhibit the enzyme in all the assays with an IC₅₀~10 nM which was close to that obtained in the previous work by Chu using 0.05 U/100 µl Aag. However, the candidate inhibitor results were not consistent and not reliable even when the same concentration of inhibitors, DMSO and AAG were used (e.g. compare Assay 1 with Assay 2, column 2). Moreover, morin (UNIS00084) should show 100% inhibition at 100 mM based on the result (IC₅₀ = 2.6 µM) from Dixon et al. Of greater concern were the large negative inhibition values observed in some instances.

<table>
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<tr>
<th></th>
<th>Assay 1</th>
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<th>Assay 3</th>
<th>Assay 4</th>
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<tr>
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<tr>
<td>[Inhibitor] /mM</td>
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<td>0.19</td>
<td>0.1</td>
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<td><strong>% inhibitions</strong></td>
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<tr>
<td>UNIS00021 (new purchase)</td>
<td>10 ± 18</td>
<td>56 ± 2</td>
<td>-26 ± 8</td>
<td>28 ± 14</td>
</tr>
<tr>
<td>UNIS00021 (synthesised)</td>
<td>19 ± 11</td>
<td>26 ± 4</td>
<td>-37 ± 20</td>
<td>19 ± 12</td>
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<tr>
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<td>31 ± 3</td>
<td>-71 ± 18</td>
<td>-17 ± 37</td>
</tr>
<tr>
<td>UNIS00067</td>
<td>3 ± 10</td>
<td>31 ± 7</td>
<td>19 ± 14</td>
<td>2 ± 11</td>
</tr>
<tr>
<td>UNIS00008</td>
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</tr>
<tr>
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<td>21 ± 9</td>
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<td>85 ± 1</td>
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<tr>
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<td>n.d.</td>
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</table>

**Table 6.1:** Comparison of % inhibition values from repeated AAG surface-bound oligomer assays showing inconsistent results and anomalous, apparently negative, values. Values are averages of n=3, errors are standard deviations.
It was thought that three factors could be responsible for the anomalous results:

- **Use of DMSO as the solvent to dissolve the compounds:** It was found previously by Chu that AAG is sensitive to DMSO and shows lower activity at higher concentrations. It was thought that any small change in DMSO amount might cause these inconsistent results. Therefore it was thought to try a different organic solvent such as DMF to prepare the standard curve and the inhibitor dilutions.

- **The incubation time of the compounds with the enzyme before addition to the substrate oligomer (inhibitor pre-incubation) was insufficient.** The incubation was done at 0 °C. M. Dixon et al. incubated inhibitors with AAG for ten minutes at room temperature before reaction with the substrate oligomer. Therefore, it is important to check the effect of the incubation time and temperature.

- **The compounds might not be completely soluble at 100 µM in the glycosylase buffer/2% DMSO from which the rest of the dilutions were prepared.** If so then the dilutions might sometimes include precipitate and sometimes not and therefore be of differing concentrations. Consequently, it was important to develop a method to measure the kinetic solubility of the test compounds in 2% DMSO-glycosylase buffer.

These possibilities were investigated through the following experiments.

### 6.2.2.2 Investigation of the effect of using DMSO as test compound solvent

To check if the inconsistent and negative inhibition of the inhibitors was caused by dissolving the inhibitors in DMSO, published inhibitor morin was chosen to be tested in the absence of DMSO. The reason for this was that it possesses five hydroxyl groups that makes it more likely to be soluble in water and the published aqueous solubility is (>151 µg/ml). Also, from the result in Table 6.1 it was clear that εC oligo inhibited AAG either in DMSO or buffer only, so it could be used as a
positive control. In this assay all the dilutions were kept in ice and then AAG enzyme was added to them to give 0.025 U/100 µl. The result shown in Figure 6.5 B revealed that the εC oligo worked well even without DMSO. However, the result of the different concentrations of morin did not give gradual inhibition and the highest inhibition was 42% at 0.0125 mM (Figure 6.5 C). Interestingly, it was noticed in this assay that the apparent AAG inhibition values did not show overactivity of AAG indicating that, in the previous assays, this may have been due to a systematic error in dilutions for the AAG standard curve leading to more inhibitory DMSO being present than planned.

Figure 6.5: AAG standard curve generated using the surface-bound oligonucleotide assay. % Inhibition values were calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve. For inhibitor testing, 0.025 U/100 µl AAG in aqueous buffer was used. Values are averages of n=3-4, all dilutions were kept in ice, error bars show standard deviation.
Changing the solvent of the inhibitors from DMSO to a different one was one of the suggested solutions. DMF is another polar solvent with low volatility so it was tested in the assay in place of DMSO. **Figure 6.6** shows that DMF was not as good a solvent for this assay since the standard curve shape was abnormal, **UNIS00084** showed, at low concentrations, apparent AAG activity greater than that in the absence of inhibitors (illustrated as negative % inhibition) in some of its dilutions and even the εC oligo did not inhibit AAG. From these two bioassays it could be concluded that this assay was very solvent sensitive. However, all of the synthesised **UNIS00021** analogues were not water soluble and an organic solvent was required to prepare the stock solution of these inhibitors. It was decided to continue using glycosylase buffer/ 2% DMSO but to try to ensure no variability in DMSO concentration was produced.

**Figure 6.6**: AAG standard curve and **UNIS00084** (morin) inhibition curve generated using the surface-bound DNA oligonucleotide, % inhibition was calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve. 0.05 U/100 µl AAG in 2% DMF-aqueous buffer was used with the inhibitor, values are averages of n=4, all dilutions were kept in ice, error bars show standard deviation.
6.2.2.3 Investigation of the effect of pre-incubation time of inhibitors with AAG enzyme

In all the previous assays the test compound dilutions were pre-incubated with AAG on ice before adding them into the reaction plate with substrate DNA oligomer bound. It was decided to check the effect of applying this pre-incubation at room temperature as reported by M. Dixon et al. For this, an assay was designed to check the effect of the pre-incubation of AAG dilutions at room temperature on the standard curve. These pre-incubations were done for five, ten, and twenty minutes in a water bath at 30 °C and on ice for 10 min. Interestingly, there was no significant difference between any of the standard curves.

This experiment was followed by testing the effect of incubating the AAG enzyme with UNIS00021, UNIS00067, and morin at room temperature for ten minutes using 0.025 U/100 µl of AAG and 0.1 mM of the inhibitors in 2% DMSO-glycosylase buffer. The result was promising since UNIS00021, both synthesised and newly purchased, gave the same inhibition results (70 and 73% respectively). In this assay morin was also tested in both 2% and 1% DMSO-glycosylase buffer and it seemed that with higher DMSO concentration the inhibition decreased (from 75% to 56%). UNIS00067 also showed 97% inhibition.

Based on this successful assay, the same conditions were applied to generate an IC$_{50}$ curve for morin (2% DMSO-glycosylase buffer, and 10 min pre-incubation with AAG at room temperature). Unfortunately, as shown in Figure 6.7, the two highest concentrations (0.2 and 0.1 mM) were less effective than lower concentrations and the standard deviations were large compared to those of the εC oligo positive control, which incidentally inhibited AAG completely at 20 nM.
**Figure 6.7:** AAG standard curve and UNIS00084 (morin) inhibition curve generated using the surface-bound duplex oligonucleotide. % inhibition was calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve. Using 0.1 U/100 µl AAG in 2% DMSO-aqueous buffer, values are averages of n=4, all dilutions were pre-incubated with AAG at r.t. for ten minutes before incubation at 37°C with the substrate oligomer, error bars show standard deviation.

The same assay was applied to UNIS00021 to generate an IC<sub>50</sub> curve but the results were similar to those with morin lower concentrations appearing to inhibit AAG more than higher concentrations. Also, the standard deviations were large. Nevertheless, given the original success, it was decided to pre-incubate inhibitors with AAG at room temperature for 10 min in all subsequent experiments. Attention next turned to the solubility of UNIS00021 and morin as a cause for the anomalous IC<sub>50</sub> curves.

### 6.2.2.4 Development of a solubility assay

**Introduction**

The last factor to be checked for causing reproducibility problems in the bioassay was the solubility of the test compounds. Since the above results showed a higher inhibition results with lower concentrations of the tested inhibitors compared to the higher concentrations, it was thought that this might be due to poor solubility.
In addition, solubility is an important physicochemical property which affects the absorption of oral drugs so early determination in the drug discovery process is important.\textsuperscript{204}

Thermodynamic solubility can be measured by mixing the compound with the appropriate solvent and shaking it for at least 24 h to ensure equilibrium is reached. The mixture can then be filtered and the concentration of the dissolved compound determined by a suitable analytical method.\textsuperscript{205} However this method is not ideal for this project because the different dilutions of the inhibitors in 2\% DMSO-glycosylase buffer are used directly in the bioassay without leaving time to equilibrate with the solvent. An assessment of kinetic solubility would therefore be preferred.

Kinetic solubility can be measured using laser nephelometry in which precipitated compound can be detected by measuring the intensity of scattered light when light is applied to the suspension.\textsuperscript{203,205} Solubility has been measured using this method by preparing a stepwise dilution of a fine suspension and then the concentration at which turbidity just disappeared is determined using UV spectrophotometry.\textsuperscript{206}

More recently, Bevan and Lloyd measured the kinetic solubility of several drugs using laser nephelometry in microtiter plates.\textsuperscript{203} They prepared their dilutions from 10 mM DMSO stock solution of the drug into an aqueous PBS buffer at constant 5\% DMSO to lessen the gradual increase in DMSO and detected the concentration at which precipitate formed and scattered light as the solubility. Based on this literature a method was developed to measure the solubility of some of the synthesised compounds using a Zetasizer Nano instrument which can quantify light scattering (mean count rate) as well as providing the ability to measure particle size over a wide range of concentrations using Dynamic Light Scattering (DLS).
Measuring the solubility of the inhibitors

Bevan and Lloyd used 5% DMSO in PBS as the solvent for all the compounds so initially, the method was tested on two of their reported compounds which included prednisone and morin hydrate. The highest concentration for each of them was prepared from 100 mM % DMSO stock solution added to 5% DMSO in PBS which then was diluted several times in 5% DMSO in PBS. Light scattering of each solution was measured using the Zetasizer Nano instrument as mean count rate. The mean count rate was then divided by the % transmission which is read from the attenuator index table in the manual as the instrument automatically adjusted this to optimise detection. On a plot of light scattering against concentration, the intersection between the precipitate concentrations and the dissolved concentrations would be equivalent to the point of compound solubility. The result of the prednisone solubility was 91.50 µg/ml (0.30 mM) which is comparable to that found by Boven (> 179 µg/ml) (from DMSO solution by HPLC) but the mean count rate/% transmission of the lower concentration did not give the expected result where there should be almost no scattering. The same procedure was applied to morin hydrate but this time the two lines intersected at a point and the solubility was found to be 88 µg/ml which was also comparable to that found by Boven (>151 µg/ml).

Unfortunately, it was not possible to measure the solubility at 0 °C, which would match plate setup in the bioassay, because of the condensation that formed in the cell. However, to improve the result and to avoid any precipitation it was decided to prepare each dilution of the inhibitor by making separate stock solutions at decreasing concentrations in 100% DMSO and then diluting them to 2% DMSO-glycosylase buffer to resemble the bioassay setup conditions. This was applied to test the solubility of morin hydrate in bioassay conditions and the results are shown in Figure 6.8. Solubility was found to be 1.2 mM (366 µg/ml).
The same method was applied to measure the solubilities of UNIS00021 and UNIS00067 and these were found to be 0.1 mM (32 µg/ml) and 0.4 mM (116 µg/ml), respectively.

In conclusion, theoretically solubility should not be a problem with morin since all dilutions in previous bioassays were prepared from 0.2 mM in 2% DMSO-glycosylase buffer – well below its determined solubility of 1.2 mM. However, for UNIS00021, solubility (0.1 mM) is lower than that of the starting dilution (0.2 mM). To address this, the following assays were designed to test whether the use of a lower concentration stock solution of the inhibitor would give more consistent results.
Addressing solubility through modification of dilution sequence in the bioassay

In a parallel work with the solubility assay the surface-bound DNA oligomer bioassay was repeated by preparing morin dilutions from two different stock solutions: 10 mM (100% DMSO) to prepare final plate 2% DMSO/glycosylase buffer dilutions of 0.1 and 0.05 mM; and 5 mM (100% DMSO) to prepare final plate 2% DMSO/glycosylase buffer dilutions of 0.05, 0.025, 0.0125 and 0.0031 mM. This was done so that any precipitation problems would become apparent from the higher concentration stock solution results compared to the lower. The results are shown in the Figure 6.9. The dilutions prepared from the 5 mM stock solution showed high inhibition starting from 74% and there was a gradual decrease in AAG activity as the morin concentration increased (Figure 6.9 B). The curve showed the IC$_{50}$ to be 10 µM which was close to that reported by Dixon et al. (2.6 µM). Yet the same two dilutions (0.1, and 0.5 mM) that were prepared from the 10 mM showed just slight inhibition (Figure 6.9 C). This result did not match the solubility result which showed that morin should be completely soluble at 100 mM in 2% DMSO and thus starting from a different stock solution should not affect the outcome of the bioassay.
Figure 6.9: AAG standard curve (A), UNIS00084 (morin) inhibition curve (B) and bar chart showing effect of diluting inhibitor from different DMSO stock concentrations (C) generated using the surface-bound duplex oligonucleotide, % inhibition was calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve. Using 0.1 U/100 µl AAG in 2% DMSO-aqueous buffer, values are averages of n=3, all dilutions were pre-incubated with AAG at r.t. for ten minutes, error bars show standard.

A further anomalous result was obtained when the bioassay of morin was repeated following exactly the same conditions, starting from the 5 mM (100% DMSO) stock solution and using 0.1 U/100 µl of AAG: No IC$_{50}$ curve was obtained and some of the dilutions showed negative inhibition of the enzyme. Similarly, preparing UNIS00021 dilutions in glycosylase buffer/ 2% DMSO from a 5 mM (100% DMSO) stock solution and using 0.1 U/100 µl of AAG also did not give an IC$_{50}$ curve and
there was a higher % inhibition with the lower concentrations compared to the higher concentrations.

In an alternative method to reduce the likelihood of compound precipitation during dilution from stock solution, it was thought to prepare all the dilutions of the inhibitor in 4% DMSO-glycosylase before adding them to an equal volume of the AAG enzyme dilution, prepared in glycosylase buffer only, to obtain a final concentration in 2% DMSO-glycosylase buffer. **UNIS00021** was used for this assay. As in the previous experiment, 10 and 5 mM (100% DMSO) stock solutions were prepared and used to make 0.4 and 0.2 μM solutions in glycosylase buffer/4% DMSO so any effect of precipitation would be limited to individual dilutions only. All lower dilutions between 50 μM and 0.25 μM were prepared from a 2.5 mM (100% DMSO) stock solution. The dilutions were mixed with AAG to give **UNIS00021** in glycosylase buffer/2% DMSO and 0.1 U/100 μl of AAG. Unfortunately, there was no improvement in the consistency of the activity of each dilution (**Figure 6.10**) with the two highest concentrations giving equal inhibitions and the third highest showing a negative inhibition.

![Figure 6.10: UNIS00021 inhibition curve from AAG assay using the surface-bound DNA oligonucleotide, % inhibition was calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve. 0.1 U/100 μl AAG in 2% DMSO-aqueous buffer was used with inhibitors, values are averages of n=4, all dilutions were pre-incubated with AAG at r.t. for ten minutes, error bars show standard deviation.](image-url)
Overall, it was decided that factors other than solubility and precipitation were causing inconsistent and irreproducible bioassay results.

6.2.2.5 Checking the possibility of inhibitors interfering with fluorescence

Some purchased analogues of UNIS00021 had already been tested for fluorescence (Section 6.2.2.1) and shown not to fluoresce thus eliminating the possibility of residual compounds affecting bioassay results. However, it was subsequently thought that there might be an interaction between the inhibitors and fluorescein, after their action on AAG, which quenches the fluorescence to an extent depending on how much compound remained on the plate.

To test for fluorescence quenching, an assay was designed to use just free (not surface-bound) DNA-fluorescein complex (HX01/REP04) and mimic the alkali-mediated DNA-fluorescein release step without the need to do the ligation step or the AAG treatment. The concentration of the HX01/REP04 was chosen according to the fluorescence reading that gave an ‘eluted fluorescence signal’ which is corresponded to approximately 50% AAG inhibition and that was found to be 50 nM as shown in Figure 6.11. HX01/REP04 was mixed with morin and UNIS00021 at several concentrations in alkaline denaturation buffer then incubated at 37 °C for 2 h to mimic the final step of the assay. Fluorescence was then measured in the plate reader and the results are shown in Figure 6.11. It was clear that there was no effect of UNIS00021 on fluorescence since the same fluorescence level was detected for each concentration. Also, there was no quenching of fluorescence by morin but morin was itself fluorescent and increased fluorescence corresponding to its concentration.
Figure 6.11: Measured fluorescence from different concentrations of fluorescent oligo complex (left) and the effect of the presence of inhibitors on fluorescence (right). All dilutions in 100% alkaline denaturation buffer (0.1 xSSC, 0.1 M NaOH), [HX01/REP04]=50 nM, values are averages of n=3, error bars show standard deviation.

6.2.3 Summary of surface-bound 3-component fluorescent DNA oligomer bioassay

In summary, although considerable effort was made to explain anomalous results from this bioassay and improve it, IC\textsubscript{50} curves remained inconsistent and irreproducible. Furthermore, apparent overactivity of AAG was observed with some dilutions of (even published) inhibitors. At this point it was decided to try a different microplate-based assay which was giving consistently good results on another project investigating DNA repair enzyme activities. It also used a duplex DNA oligonucleotide substrate bound covalently to the surface of functionalised 96-well plates but consisted of only 2 components due to the inclusion of a hairpin loop. The fluorescein molecule was also held closer to the plate. In parallel work, a third bioassay was designed to use LCMS to assess AAG activity by quantifying the excised base in solution. These will be described in the following sections.
6.3 Microplate surface-bound hairpin loop fluorescent DNA oligomer assay

Given the ongoing problems with the 3-component fluorescent DNA oligomer assay, it was decided to try a slightly different experimental method, which had already been used successfully by another student investigating DNA repair enzyme activities. In this assay, in place of the linear double stranded oligonucleotide complex, a hairpin oligonucleotide structure is immobilised to the microplate wells. Construction of the hairpin loop is achieved by coupling single stranded oligonucleotide HX02 to the surface of the wells and then hybridizing and ligating oligonucleotide Loop01 to it (Figure 6.12).

In addition to this change, a more sensitive fluorescein detection method was employed, again also discovered in parallel work. This used antifluorescein-horse radish peroxidase (HRP) conjugate colorimetric detection system whereby the antibody conjugate binds to remaining fluorescein on the plate and the conjugated HRP enzyme converts an added substrate 3,3',5,5'-tetramethylbenzidine (TMB) to a product which is blue in colour. This amplifies the signal originally resulting from retained fluorescein-linked oligomer.

Initial experiments were performed to determine appropriate concentrations of the oligonucleotides to use in the assay. The desired AAG standard curves were successfully obtained and a concentration of 0.1 pmol/well oligonucleotide HX02 was selected for subsequent experiments.
Next, an experiment was designed to test the effects of some of the candidate compounds on AAG activity, using a final microplate concentration of 0.05 mM of each compound and 0.05 U/100 µl AAG. The compounds tested both synthesised and purchased UNIS00021. The standard curve obtained with serial dilutions of AAG was as expected and 0.05 U/100 µl AAG was at an appropriate position on the linear portion of the curve to allow inhibition to be quantified. However, both synthesised and purchased UNIS00021 and some other inhibitors again showed apparent overactivity of AAG with large error bars (Figure 6.13).
Figure 6.13: AAG standard curve generated using the surface-bound duplex oligonucleotide (Loop01) assay and measuring absorbance (left). % Inhibition values from candidate inhibitors (calculated from apparent AAG activity obtained by interpolation of absorbance values into the AAG standard curve). Inhibition reactions were performed using 0.05 U AAG/100 µl reaction in 2% DMSO-aqueous buffer. Values shown indicate averages of \( n = 3 \) and error bars indicate standard deviation. *Synthesised UNIS00021

This assay was repeated twice using 0.1 mM of each candidate inhibitor and again the level of inhibition of the inhibitors were not the same in all the assays with also apparent overactivity of AAG with some of the inhibitors.

Further assays suggested that UNIS00021 at 0.1 mM only marginally inhibited AAG, while purchased UNIS00021 showed 37% inhibition (Figure 6.14). Hypothesising that an impurity could be responsible for the apparent activity of the purchased UNIS00021, the purity of the same samples was analysed using LCMS. The result showed that the synthesised version was pure and there was a very small impurity peak in the purchased one with a mass of 659 \( m/z \) in the positive mode corresponding to the mass of the disulfide dimer of UNIS00021. However, it was not possible to isolate it given the small sample size. Hence an effort was made to synthesise the disulfide dimer of UNIS00021 but it was unsuccessful.
Figure 6.14: AAG standard curve generated using the surface-bound duplex oligonucleotide (Loop01) assay and measuring absorbance (left): % Inhibition values (right, calculated from apparent [AAG] obtained by interpolation of absorbance values into the AAG standard curve) of purchased analogues of the original hit UNIS00021. Using 0.1 U/100 µl AAG in 2% DMSO-aqueous buffer, values are averages of n=4, error bars show standard deviation. * Synthesised UNIS00021

In most AAG activity assays described in the literature, bovine serum albumin (BSA) is included as one of the components of the buffer. Elliott had not found this necessary for the surface-bound assays but O’Brien et al. found that low concentrations of AAG enzyme are susceptible to inactivation within minutes to hours under the standard reaction conditions. However, they found that this problem could be overcome by adding BSA (0.1 mg/ml) or ovalbumin (0.1 mg/ml) which can stabilise even very low concentrations of AAG (~1 nM) for more than 24 h at 37 °C. It was hypothesised that AAG being unstable and its stability affected by changes in the solution, such as the concentration of candidate inhibitors, could have been leading to the inconsistencies between bioassays.

An assay was designed to check the effect of BSA on stabilising the AAG enzyme and whether its incorporation would lead to more consistent standard curves and consistent inhibitor IC₅₀ curves. The BSA concentration used in most of the
literature is 100 µg/ml so this was used in the hairpin loop bioassay alongside an assay without BSA on the same plate. UNIS00021, morin and εC oligo were tested in this assay 2017-05-23- Aag Hairpin loop Colorimetric Assay - BSA coat test PROTOCOL morin+UNIS00021 (1). The colour development of the standard curve and so the absorbance readings were better when BSA was included (Figure 6.15 A). The signal difference between the Aag concentration used with the inhibitors (0.1 U/100 ul) and the 0 U/100 µl was very small with no BSA (0.09) which can not give an indication of the inhibitors’ effect. Yet, the difference in in the absorbance signals when BSA was included was 0.33 which was enough to show inhibition. Interestingly, repeating this assay to check the consistency of the standard curves gave approximately the same curves. The εC oligo curve showed an IC$_{50}$~ 5 nM (Figure 6.15 B) which was close to that reported, and both UNIS00021 and morin showed increases in inhibition with concentration but the curves were shallow and showed negative inhibition at low concentrations and that was more negative in the assay with BSA (Figure 6.15 C and D).
Figure 6.15: The effect of adding 0.1 mg/100 µl BSA into the AAG glycosylase buffer on the standard curve and inhibition curves ([AAG] = 0.1 U/100 µl, values of average n=3, all [AAG] dilutions were pre-incubated with AAG at r.t. for 10 min before incubation at 37 °C with the substrate oligomer. Error bars show standard deviation.

It was also thought to coat the plate with 0.1 mg/100 µl BSA as an extra step after loading of the first oligomer and either before hybridisation or after for 1 h or overnight. This was mentioned in the Thermo Scientific Nunc™ Immobilizer Amino instruction protocol as an additional recommendation to quench the remaining reactive groups on the plate surface. This step was tested keeping also 0.1 mg/100 µl BSA in the glycosylase buffer. The results are shown in Figure 6.16 and reveal little difference between including the BSA coating step and not. Interestingly, in these assays, εC oligo showed good inhibition curves with IC₅₀ again ~5 nM, but UNIS00021 showed no inhibition and only a small amount of negative inhibition.
at the start of the curve. Morin only began to show inhibition at very high concentration (200 µM) and also still showed negative inhibition at lower concentrations.

**Figure 6.16:** The effect of adding 0.1 mg/100 µl BSA into the AAG glycosylase buffer on the standard curve and coating the plate with 0.1 mg/100 µl BSA. [AAG] = 0-0.4 U/100 µl, values of average n=3, all [Aag] dilutions were pre-incubated with Aag at r.t. for ten minutes before incubation at 37 °C. error bars show standard deviation

In summary, the presence of BSA in the assay buffer was found to make standard curves more robust but negative inhibition was still observed at low concentrations of candidate inhibitors. It was also looking more likely that **UNIS00021** was not a real, specific inhibitor of AAG.
6.4 A free substrate oligomer assay using LCMS

Liquid chromatography coupled with single stage and tandem mass spectrometers (LCMS) is increasingly used in pharmaceutical research and development. This is due to its high-throughput determinations of drug concentrations in biological samples.\textsuperscript{208-210}

It was thought that designing an LCMS-based assay would permit the use of label-free, non-bound substrate DNA oligomer would dramatically simplify the assay and could solve the problems of inconsistent and irreproducible inhibitor curves encountered with the surface-bound fluorescein-linked DNA oligomer assays.

In this assay, AAG enzyme would be added to a solution of a free substrate oligo containing the damaged base hypoxanthine (Hx). On incubation at 37 °C, AAG should excise the Hx into solution and heating the plate to 65 °C after a set time should denature AAG and halt the reaction. Excised Hx could be quantified using a Q-Exactive Plus Orbitrap Mass Spectrometer combined with UltiMate™ 3000 LC.

In order to design this assay the following points needed to be studied:

1. Optimisation of an LC method and column to separate the buffer components, DNA oligomers, AAG enzyme and inhibitors from Hx and an internal standard. This is important to avoid ion suppression which might cause a low signal for the target components peaks. This should be followed by reducing the period of time to run each sample to minimize the time required to run a whole 96-well plate based assay. For this, a shorter LC column should be used to maintain sensitivity.

2. The limit of detection (LOD) of hypoxanthine by the instrument to inform the concentration of substrate oligomer and AAG that should be used to give a detectable signal at a fraction of AAG’s full activity in order to detect the action of inhibitors.
3. Find the best internal standard that can be added to the assay samples that does not affect AAG’s activity itself. The presence of internal standard in the calibration curve and the unknown samples of the assay is important since there will be 96 samples running overnight in which variations in the sensitivity of the instrument could alter the peak sizes of detected ions. This should be normalized by considering the ratio of peaks for Hx and the internal standard at constant concentration. The internal standard should have a molecular weight and structure that is close to the target analyte in an attempt to match ionization and detection levels.

6.4.1 Development of an LC Method

One property which affects the separation of sample components by LC is their pKₐ (along with the pH of the mobile phase) since ions should have a lower affinity for the reverse phase than neutral molecules and hence a shorter retention time. The pKₐ values of proposed sample components of the LCMS bioassay are shown in Figure 6.17 and represent an aim to select those which would be ionized while Hx remained neutral. Tris(2-carboxyethyl)phosphine (TCEP) is a reported alternative, odorless reducing agent to β-mercaptoethanol and is stable in acidic and basic solutions.²¹¹ Although, no literature reported its use in the AAG assays, it was decided to check its use here since it would be easily ionized compared to β-mercaptoethanol.

During LC method development, it was found that the main buffer component used previously, Tris, remained on the column for a long time, giving a long-tailing peak on the chromatogram which overlapped other peaks. Interestingly, a buffering compound 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) has also been reported for use in AAG assay buffer and it was found to elute with a sharper peak shape.⁶²
Several attempts were made to separate the buffer components and hypoxanthine using a Phenomenex Onyx Monolithic C18 (100 mm × 2 mm) column, different LC solvents (MeCN and MeOH) and aqueous buffers, such as formic acid 0.1% (pH~2.7) or ammonium acetate 10 mM (pH~3.8-5.8) and normal LC water, and different LC gradients. However, all these attempts failed to separate them and they were eluted very quickly from the column along with the hypoxanthine.

It was decided to change the column to the Hyperclone ODS (C18) 150 mm x 4.6 mm column containing 5 µm silica. Interestingly, this column showed a slight separation. This was followed by several attempts to find the best separation conditions using a 7 minute run. These conditions included changing the gradient of the solvents, buffers, flow rate and the mode of electrospray (positive or negative mode). The best conditions found for separation was to use the mobile phase acetonitrile in aqueous ammonium acetate using the positive mode of the MS and flow rate of 1 ml/min. The gradient was as follows: 0-1 min, 5% MeCN, 1-7 mins, 5-100% MeCN, 7-8 mins, 5% MeCN (Table 6.2 and Figure 6.18).
<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>Retention time /min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine</td>
<td>136.12</td>
<td>2.10</td>
</tr>
<tr>
<td>TCEP</td>
<td>250.19</td>
<td>1.25</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.1</td>
<td>1.60</td>
</tr>
<tr>
<td>EDTA</td>
<td>292.24</td>
<td>1.28</td>
</tr>
<tr>
<td>Tris</td>
<td>121.14</td>
<td>1.35</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>78.13</td>
<td>1.83</td>
</tr>
<tr>
<td>UNIS00021</td>
<td>330.45</td>
<td>5.30</td>
</tr>
</tbody>
</table>

**Table 6.2**: Retention time of glycosylase buffer components by LCMS using MeCN in ammonium acetate (pH 7); 0-1 min, 5% MeCN, 1-7 mins, 5-100% MeCN, 7-8 mins, 5% MeCN

Since the retention time of β-mercaptoethanol was very close to hypoxanthine it was decided to substitute it for TCEP. As discussed above, tris showed a long, trailing peak so was substituted for HEPES.

**Figure 6.18**: Chromatogram with the retention time of the glycosylase buffer components using the Q-Exactive Plus Orbitrap Mass Spectrometer
These conditions were used to determine the limit of detection for hypoxanthine (LOD) in this instrument. Several dilutions of hypoxanthine were tested (25 - 0.382 nM). Unfortunately, the results showed that the LOD of the hypoxanthine was only 25 nM. A lower LOD was required so an affordable concentration of substrate oligomer and AAG enzyme could be used in the assay.

One way to increase the sensitivity to hypoxanthine was to use a smaller column and shorter run time with a lower flow rate to give taller peaks.

On advice from the Phenomenex company a new column, Luna Omega PS, was tested. It has a positively charged surface in between the C18 chains which would help to repel the hypoxanthine’s delta-positive nitrogen functional groups helping to minimise tailing and give a better peak shape.

Therefore, this column was used to check first its ability to separate the components. Again, many attempts were made to optimise conditions as shown in Table 6.2. Entry 6 showed the best separation in the shortest time.

A new assessment of the LOD using these conditions and several dilutions of hypoxanthine (195 - 0.76 nM) prepared in 2% DMSO / 20 mM HEPES (pH 7.8) / 5 mM EDTA / 5 µM TCEP / 100 mM KCl as the new AAG glycosylase buffer. In this test 12 nM of caffeine was added as the internal standard which showed a retention time at 4.3 minute. This gave the calibration curve shown in Figure 6.19 which could be used to determine the LOD of hypoxanthine by calculating the standard deviation, multiplying it by 3 and adding the result to the apparent concentration derived from (the noise of) a blank sample. The LOD was determined in this way to be 6 nM. Unfortunately, the chromatogram did not show a defined peak for the 12 nM caffeine although it was detectable when run at 50 nM.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Component</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ESI Negative, MeCN in water: 0-2 min, 0%; 2-5 min, 0-100%, 5-7 min, 0%</td>
<td>EDTA</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>4.75</td>
</tr>
<tr>
<td>2. ESI Positive, MeCN in ammonium bicarbonate (pH 8): 0-2 min, 0%; 2-5 min, 0-100%, 5-7 min, 0%</td>
<td>EDTA</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>4.68</td>
</tr>
<tr>
<td>3. ESI Positive, MeCN in water, 1% formic acid: 0-2 min, 0%; 2-5 min, 0-100%, 5-7 min, 0%</td>
<td>EDTA</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>4.72</td>
</tr>
<tr>
<td>4. ESI Positive, MeCN in ammonium acetate (pH 7): 0-2 min, 0%; 2-5 min, 0-100%, 5-7 min, 0% (Q-Exactive Plus Orbitrap Mass Spectrometer)</td>
<td>EDTA</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>4.78</td>
</tr>
<tr>
<td>5. ESI Positive, MeCN in ammonium acetate (pH 7): 0-2 min, 0%; 2-5 min, 0-100%, 5-7 min, 0% (Q-Exactive Plus Orbitrap Mass Spectrometer)</td>
<td>EDTA</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>4.6</td>
</tr>
<tr>
<td>6. ESI Positive, MeCN in ammonium acetate (pH 7): 0-1 min, 0%; 1-3 min, 0-100%, 3-4 min, 100-0%, 4-5 min, 0% (Q-Exactive Plus Orbitrap Mass Spectrometer)</td>
<td>EDTA</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>UNIS00021</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 6.3: Retention times of glycosylase buffer components by LCMS using Luna Omega PS column
Several compounds of similar structure to hypoxanthine were tested for use as internal standard (Figure 6.20). Theobromine (180.164 g/mol) was found to have a retention time of 3.30 min using the same condition in Table 6.3 entry 5 so it was used in the first LCMS-bioassay.

![Chemical Structures]

**Figure 6.20:** Different compounds tested as internal standard

### 6.4.2 Design of the LCMS-based bioassay

After developing an LCMS method to separate proposed new components of an AAG assay buffer system from hypoxanthine, the new buffer of 20 mM HEPES, 5 mM EDTA, 5 uM TCEP and 100 mM KCl were tested in the former bioassay to
generate a standard curve which was usually reproducible. The new HEPES-based buffer showed a comparable standard curve to the original tris buffer.

The next step was to design a simple substrate DNA oligomer. The shortest used with AAG in an assay (³²P-labelled, gel-based) and reported in the literature is a 25-mer so a 25-mer equivalent of the oligomer used in the surface-bound assay was used to decrease costs and hypoxanthine was incorporated as the damaged base (Figure 6.21).

\[5' - \text{CATACACAAACAG} \text{GTCCTACTCCT} - 3'\]

\[3' - \text{GTATGTGTTGTCAGGTGAGGTA} - 5'\]

**Figure 6.21:** 25-mer DNA oligomer designed for use as substrate in the LCMS assay

Since the LOD of the hypoxanthine was 6 nM, the AAG enzyme and substrate oligomer concentrations used needed to be calculated so that the 6 nM would be a fraction (or high % inhibition) of the maximum produced by AAG in the specified time at the fixed concentration of AAG used to test inhibitors. The chosen fraction was 3% maximum AAG activity which would correspond to release of hypoxanthine at 200 nM concentration for 100% AAG activity. According to AAG manufacturer’s specifications, one unit (U) of the enzyme was defined as the amount of enzyme required to release 1 pmol of hypoxanthine per hour assuming there is excess substrate. To reduce expense, a reaction volume of 50 µl and an incubation time of 2 h was chosen thus requiring 5 U of AAG (10 U/100 µl) to produce a 200 nM hypoxanthine solution. An excess of substrate oligomer was required for this not to be a limiting factor and this excess was chosen to be 5-fold which was 1 µM.

After 2 h of incubation, the reaction would be quenched by 15 minutes incubation at 65 °C to deactivate the enzyme. It was decided to add the internal standard, theobromine into the buffer to be one of the components during all steps. This was to decrease the pipetting error since the reaction volume was very small. However,
theobromine had not been tested against the AAG enzyme and so several dilutions of it were also made up for testing of its effect on AAG activity.

6.4.3 Results of the LCMS-based assay

The first experiment was to generate the AAG standard curve so AAG concentrations either side of the fixed 10 U/100 µl chosen for use with inhibitors (0-40 U/100 µl). Standard curves were tested with and without BSA and a calibration curve of hypoxanthine vs theobromine was included in every LCMS run. Standard curves were generated successfully as shown in Figure 6.22. The concentration of BSA prepared was accidentally 10-fold too dilute (10 µg/ml) so the standard curves almost look the same. Surprisingly, despite the calculations of how much AAG to use to release 200 nM hypoxanthine at 10 U/100 µl, this concentration actually produced almost 900 nM hypoxanthine. Hence it was reduced to 1.25 U/100 µl in subsequent assays.

Figure 6.22: Standard curves generated using free substrate oligomer and the Q-Exactive Plus Orbitrap Mass Spectrometer, all dilutions were in 2% DMSO-glycosylase buffer with or without BSA, 50 nM theobromine, values of average n=3, all dilutions were pre-incubated with AAG at r.t. for ten minutes before incubation at 37 °C with the substrate oligomer. LC-MS conditions: acetonitrile in 10 mM ammonium acetate (pH 6.9) 0-1 min: 0%, 1-3 min: 0-100%, 3-4 min: 100%, 4 min: 100-0%, 4-5 min: 0%. Error bars show standard deviation.
However, the extracted ion chromatograms of theobromine showed high levels of noise (Figure 6.23) which gave high standard deviations. Therefore, it was decided to increase its concentration in the buffer to 300 nM since it did not show any inhibition result against AAG in this assay up to 2.5 µM.

![Theobromine](image)

**Figure 6.23:** The chromotgram of 50 nM theobromine obtained from Q-Exactive Plus Orbitrap Mass Spectrometer

The next two assays were planned to test inhibitors εC oligo, UNIS00021, UNIS00067 and morin using 300 nM of theobromine and 1.25 U/100 µl AAG with the inhibitors dilutions. Unfortunately, in these two attempts there were a dramatic reduction in peak areas and that was clear in the calibration curves as shown in **Figure 6.24**.
This could be due to a reduction in the cone temperature (inlet temperature) of the MS instrument compared to the last experiment (300 °C). Another factor could be contamination of the cone and this was hypothesised to be due to the high concentration of buffer components passing into the mass spectrometer. Therefore, it was deemed necessary to use the divert valve, which can be programmed to switch at specified run times, after the LC to direct everything but hypoxanthine and internal standard to waste. A summary of the divert valve schedule and assay component concentrations and retention times is shown in Table 6.4. Based on this data the KCl, TCEP, EDTA and HEPES were eluted earlier than the analyte and theobromine, and the DNA, BSA, and UNIS00021 were eluted much later than the analyte and theobromine so the divert valve timings were proposed as shown in Table 6.4.
<table>
<thead>
<tr>
<th>Component</th>
<th>Concn (uM)</th>
<th>MW</th>
<th>Concn (mg/mL)</th>
<th>RT (min)*</th>
<th>Proposed divert schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>100000</td>
<td>74.5</td>
<td>7.45</td>
<td>0.2</td>
<td>0.0-0.7 to waste</td>
</tr>
<tr>
<td>HEPES</td>
<td>20000</td>
<td>238.30</td>
<td>4.77</td>
<td>0.2-0.6</td>
<td>0.7-1.1 to MS</td>
</tr>
<tr>
<td>TCEP</td>
<td>5000</td>
<td>286.65</td>
<td>1.43</td>
<td>0.2-0.3</td>
<td>1.1-1.7 to waste</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1</td>
<td>136.11</td>
<td>0.14</td>
<td>0.8-1.0</td>
<td>1.7-2.0 to waste</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td>66399.00</td>
<td>100.00</td>
<td>3.3-3.6</td>
<td>2.0-5.0 to waste</td>
</tr>
<tr>
<td>DNA 25-mer</td>
<td>1</td>
<td>7839.10</td>
<td>7.84</td>
<td>2.8-3.2</td>
<td></td>
</tr>
<tr>
<td>Theobromine (int. std.)</td>
<td>0.3</td>
<td>180.16</td>
<td>54.05</td>
<td>1.8-2.0</td>
<td></td>
</tr>
<tr>
<td>Inhibitors (e.g. UNIS00021)</td>
<td>200</td>
<td>290.39</td>
<td>58.08</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

*acetonitrile in 10 mM ammonium acetate (pH 6.9) 0-1 min: 0%, 1-3 min: 0-100%, 3-4 min: 100%, 4 min: 100-0%, 4-5 min: 0%.  

**Table 6.4:** Concentrations and retention times of the buffer and assay components using the LCMS

Using this method ten samples of just the buffer components and 1 uM of the DNA oilgo were tested and no contamination of the MS instrument was detected. However, the extracted ion chromatogram of theobromine still showed lots of noise. Therefore, it was decided to search for a different internal standard which should be eluted close and after the hypoxanthine. Two interesting compounds were considered to check their retention time: mercaptopurine (pKa= 7.71) and 3-methylxanthine (pKa= 2.3) (**Table 6.5**). Mercaptopurine was chosen since it eluted earlier and would allow a single switch of the divert valve to MS.
Compound

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mercaptopurine</td>
<td>1.46</td>
</tr>
<tr>
<td>3-methylxanthine</td>
<td>2.85</td>
</tr>
</tbody>
</table>

Table 6.5: The retention time of other internal standard, LC-MS condition: LC-MS condition: acetonitrile in 10 mM ammonium acetate (pH 6.9) 0-1 min: 0%, 1-3 min: 0-100%, 3-4 min: 100%, 4 min: 100-0%, 4-5 min: 0%.

Using this method the last assay was repeated using the same conditions. The standard curve was successful but the calibration curve was poor. Closer inspection of the chromatograms showed some hypoxanthine extracted ion chromatogram peaks to be bimodal as shown in Figure 6.25 indicating carryover of hypoxanthine and that was more noticeable in the last replicate of the calibration curve. This caused incorrect integration of the hypoxanthine peaks.

Figure 6.25: The chromatogram of the hypoxanthine bimodal peak and accurate mass spectrum proving it is hypoxanthine.
It was first thought that the hypoxanthine was trapped in the MS after switching to the waste. However, that should not be correct because the hypoxanthine chromatogram peak was normal in the standard curve and also with the inhibitors results. Subsequently it was hypothesised that the internal standard, mercaptopurine was hydrolysing into hypoxanthine since it was the last compound to enter the MS before the divert valve switched to waste. Residual mercaptopurine might sit in electrospray chamber at high temperature and be hydrolysed to hypoxanthine which is then detected when flow returns to the MS on subsequent run and divert valve switch to MS. This problem could be solved by running the last 1.5 minutes of the run (which should be pure MeCN then pure aqueous mobile phase) to MS to wash the electrospray chamber.

Interestingly, this time the result in all the runs showed the hypoxanthine as a single peak and there was no sign of the bimodal peak, indicating that all the internal standard residues had been washed off before the next run as shown in Figure 6.26. Also, the standard curve was much better compared to the previous LCMS assay standard curves (Figure 6.27 A).

![Figure 6.26: The total ion count and extracted ion chromatogram peaks of hypoxanthine and mercaptopurine obtained from LCMS-Orbitrap](image-url)
Another concern about the result of this assay was that there was no inhibition by any candidate inhibitors, even the εC oligo and, strangely, the assay showed that hypoxanthine was produced by 1.25 U/100 µL AAG in the presence of inhibitors to a greater extent than the 1.25 U/100 µL point on the standard curve. It was hypothesised that inhibitors stabilise AAG against denaturation during heat treatment reaction quench at 65 °C and a higher temperature was needed to denature the enzyme in the presence of inhibitors.

Therefore, this assay was repeated but the enzyme was deactivated at 80 °C for 15 minutes. This time the hypoxanthine produced by 1.25 U AAG/100 µl in the presence of the ethenocytidine and UNIS00021 was not greater than that produced by the 1.25 U/100 µl point on the standard curve, indicating that the enzyme was completely deactivated. Yet both inhibitors showed about the same amount of hypoxanthine production which did not decrease with inhibitor concentration Figure 6.27 B and C). It was hypothesised that this could be due to the high amount of both enzyme and substrate used compared to inhibitor. The IC50 of the εC oligo in the surface-bound fluorescein-linked oligomer assay was about 5 nM using 0.05-0.1 U/ 100 µl of AAG and 0.1 pmol/ 100 µl (1 nM) substrate oligomer. The amount of the enzyme used in LCMS was 1.25 U/ 50 µl, which is 50-25 times more concentrated, and the substrate was 1 µM which is 1000-fold higher than 0.1 pmol/ well (assuming that is all coupled to the plate and forms complete ligation complex). Correlating this to the εC oligo concentration of 320 nM, the highest concentration used in both assays, it was around 320-fold excess over substrate in the surface-bound assay whereas in the LCMS-assay it was in only 0.32-fold excess. Combining this with the 50-fold increase in the enzyme concentration in the LCMS assay meant that the inhibitor was at a concentration far lower than that required to show inhibition.
Figure 6.27: AAG standard curve (A) using the free substrate oilgo and the Q-Exactive Plus Orbitrap Mass Spectrometer; inhibition curves for εC oligomer (B) and UNIS00021 (C). All dilutions were in 2% DMSO-glicosylase buffer containing 0.1 mg/100 ul BSA, values are averages of n=3, error bars show standard deviation.

Unfortunately, time constraints prevented any further research into optimising the LCMS-based assay.

6.5 Conclusion: Artifactual inhibition and aggregation

Three assays were investigated for the assessment of the potency of candidate inhibitors of AAG. Although all could be used to generate an appropriate standard curve for increasing AAG concentrations and the surface-bound hairpin loop oligomer assay generated correct inhibition curves for εC oligo, the curves for
UNIS00021 and morin were inconsistent and irreproducible. Such characteristics of a candidate inhibitor can be attributed to non-specific inhibition of AAG by aggregation.\textsuperscript{212,213} Shoichet reported that many drug-like organic molecules that passed Lipinski rules aggregate into colloid-like particles in aqueous media at micromolar and submicromolar concentrations.\textsuperscript{212} These types of inhibitors can sequester protein targets and inhibit them in a non-specific manner. He found that these types of inhibitors were reversible by dilution and they can inhibit multiple enzymes. Also, Shoichet mentioned that increasing the amount of the target enzyme or adding large amounts of BSA diminished the potency of these compounds. This was noticed in our results in the AAG hairpin loop assay in which there were almost no inhibition with all the dilutions in the presence of BSA. Shoichet proved their hypothesis by measuring the particle size of aqueous mixtures of these inhibitors using DLS and transmission electron microscopy. They found that the particles were larger than the target enzymes by up to two orders of magnitude, being hundreds of nanometers in diameter DLS. The aggregation reduces the concentration of monomer and leads to false potencies especially if the actual monomer concentration is much less than the total compound concentration.\textsuperscript{213} Shoichet concluded that these type of compounds were non-competitive, their inhibition was time-dependent with steep dose-response curves, high sensitivity to enzyme concentration and to detergent. Interestingly, one of his reported compounds was morin which might explain its inhibition behaviour against the AAG enzyme in the Dixon paper but not in the assays reported here.\textsuperscript{62,212} This could also be the case for UNIS00021. In the future, to prove this, assay conditions should be found which show these molecules to have inhibition curves and then the assay should be repeated in the presence of a detergent to examine whether all inhibitory activity is lost, as would be the case if inhibition was through non-specific aggregation.
7. Conclusion and Future Work

The research described in this thesis involved the design and development of synthetic routes to small molecule candidate AAG inhibitors. These were based on a hit triazole-thione-based compound UNIS00021 that had been shown to have an IC$\text{}_{50}$ of $\sim 68 \mu \text{M}$ against AAG in a biochemical assay.

In summary, a robust synthetic method to vary the alkyl group of the amide in UNIS00021 has been developed. The key step was cyclisation of a thiosemicarbazide and it was found that the most successful conditions were to heat it at reflux in 1 M aqueous NaHCO$_3$ because then it cyclised into the desired triazole-thione acetic acid in 86% yield. Out of this synthesis five different novel amide analogues of the hit compound have been synthesised successfully using amide coupling reactions: iPr (UNIS00067, 50%), Me(UNIS00068, 47%), (cyclopropyl (UNIS00069, 52%), N-Methyl-N-cyclohexyl (UNIS00070, 40%) and also the hit compound UNIS00021 (20%). Also, the equivalent primary amide (UNIS00074) was made (50%) by reacting the corresponding 1,2,4-triazole-thione nitrile with TFA-H$_2$SO$_4$ at RT.

To synthesise analogues of UNIS00021 with a free amine in place of the amide and with variation in the length of the alkyl linkage group a robust synthetic method was developed. Similarly, this was achieved by synthesising the corresponding thiosemicarbazides, after protecting the amine using a Boc group, and cyclising them in 1 M NaHCO$_3$ at reflux. Removing the Boc group to provide the free amine was a challenge in this synthesis and it was solved using HCl/EtOAc (1:1). Out of this synthesis novel cyclohexyl (UNIS00077, UNIS00082) and isopropyl amines (UNIS00078, UNIS00083), each with two lengths of linkage, and methyl amine triazole thione with n=1 linkage (UNIS00079) were produced using reductive amination of the free primary amines.
A one-pot synthesis was also developed to access an analogue of UNIS00021 bearing a C⁵-methyl group instead of thiol/thione. The key step in this synthesis was to cyclodehydrate the intermediate acyl amidrazone, synthesised by activating benzyl acetylamide and reacting it with hydrazide acetoester, to give a triazole acetoester which was then hydrolysed to the acid using LiOH in THF/water. This was then used to produce the isopropyl amide UNIS00089 in 60% yield in an amide coupling reaction.

A synthesis of analogues of UNIS00021 varying at the phenyl group was designed to use Suzuki-Miyaura cross-coupling of a trifluoroborate precursor but it was not completed due to time limits and only the first three steps of this route were established successfully. The triazole-thione ester was synthesised in one step by reacting thiosemicarbazide with an imidate. This was followed by hydrolysis into the acid using LiOH in THF/water. This could be then used to synthesis the cyclohexylamide triazole-thione.

To test the activity of these synthesised compounds and other purchased analogues of the original hit UNIS00021 against the AAG enzyme, two types of AAG bioassay using a surface-bound fluorescein-linked duplex DNA oligonucleotide substrate were investigated and optimisation of a novel LCMS-based assay was also begun. The two types of AAG surface-bound oligomer bioassays revealed that the hit UNIS00021 is most likely not a specific inhibitor of AAG and this could also be the case for published inhibitor morin. No inhibition was observed by the analogues of UNIS00021. It is hypothesised that the original UNIS00021 inhibition result was artifactual and due to aggregation. Despite this, during assay optimisation, it was concluded that adding BSA into the AAG buffer gives a more robust bioassay and the enzyme linked immunosorbent colorimetric detection method is more sensitive than the eluted fluorescence detection method. However, dilutions involving inhibitors often showed negative inhibition and there was still inconsistent curves with morin and UNIS00021 and both might be due to aggregation effects. In future, the free substrate oligomer assay using LCMS could
be improved and used in future screens of novel candidate inhibitors based on new virtual screens or substrate based design. To fully establish whether \textbf{UNIS00021} and morin are aggregators, assay conditions should be found which show them as inhibitors and this should be repeated in the presence of surfactant to break up any aggregates. This might require resort to replication of the $^{32}\text{P}$-radiolabelled oligomer gel-based assay reported in the literature which showed morin to be an inhibitor.
8. Experimental Procedures

8.1 General Procedures

All reactions were carried out under nitrogen atmosphere in glassware dried under high vacuum by a heat-gun unless otherwise stated.

Reagents and solvents

Reagents and solvents for reactions were reagent grade and were used without further purification unless otherwise specified.

Chromatography

Flash column chromatography was carried out using silica gel 40-60u 60A. Thin layer chromatography (TLC) was performed using precoated aluminum backed plates (silica gel 60 F254) and visualised by UV radiation at 254 nm, or by staining with potassium permanganate solution ($K_2CO_3$ (13.3 g), KMnO$_4$ (2 g), water (200 mL), NaOH solution (10% w/v, 1.7 mL, added last)).

IR spectroscopy

Infra-red (IR) spectra were recorded in the range 600-4000 cm$^{-1}$ using an Agilent Clary 640 FTIR spectrometer with MKII Golden Gate Single Reflection ATR System or as a thin film between sodium chloride plates.

Gas chromatography mass spectrometry (GC-MS)

GC-MS was carried out on an Agilent 7890A-5975C with an electron ionisation (EI) detector. Column length was 30 m, injection volume was 2 µL, temperature was
50 °C for 3 minutes followed by an increase of 10 °C per minute to 250 °C and held for 2 minutes.

**Mass Spectrometry (MS)**

LCMS was carried out on a Waters 2695 LC coupled to a Waters 2487 Dual λ Absorbance Detector and a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer using electrospray ionisation (positive or negative mode). LC conditions were as follows: inj. vol. 10 μL, column Phenomenex Onyx Monolithic C18 (100 mm × 3 mm), column temp. 30 °C, flow rate 1 mL min⁻¹, mobile phase (positive mode) MeCN (containing 0.1% formic acid) in water (containing 0.1% formic acid), mobile phase (negative mode) MeCN in water, gradient 0-0.4 min, 5%; 0.4-2 min, 5-20%; 2-3.5 min, 20-100%; 3.5-5.5 min, 100%; 5.5-6 min, 100-5%; 6-7 min, 5%.

GCMS was carried out on an Agilent 7890A GC system connected to an Agilent 5975C inert XL EI/Cl with Triple-Axis Detectors and the conditions were as follows: inj. Vol. 1μL, inj. Temp. 250 °C, column Zebron ZB-5MS (30 m × 0.25 mm), oven temperature gradient 0-3 min, 50 °C; 3-23 min, 50-250 °C (10 °C ramp per minute); 23-35 min, 250 °C.

High-resolution mass spectrometry (HRMS) was performed at the Institute of Cancer Research, Sutton, UK on a Waters QTOF Premier with electrospray ionisation.

**Nuclear Magnetic Resonance Spectroscopy (NMR)**

¹H- and ¹³C-NMR spectra were recorded using a Bruker 500MHz spectrometer at 500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR. ¹H-NMR spectra were referenced either to TMS at 0 ppm or to residual protonated solvent: 2.50 ppm for DMSO-d6, 3.31 ppm for CD₃OD and 7.26 for CDCl₃. ¹³C-NMR spectra were referenced to
deuterated solvent peaks: 39.0 ppm for DMSO-d6, 77.16 for CDCl₃ and 49.00 for CD₃OD. The data is given as follows: chemical shift (δ) in ppm, integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet), coupling constant(s) (J in Hz accurate to 0.5 Hz), assignment.

8.2 Experimental Detail

2-Cyanoacetohydrazide (29)

\[ \text{atropisomerisation} \]

To a mixture of methyl cyanoacetate (1.00 g, 10.0 mmol) and hydrazine hydrate (0.51 g, 10.1 mmol) was added ethanol (10 ml). The mixture was heated at reflux for 1.5 h and then evaporated to dryness. The resulting white powder was recrystallized from ethanol to give the title compound as white crystals as a mixture of atropisomers (0.79 g, 79%); m.p. = 95-97 °C; IR (cm⁻¹) 3346 (N-H), 3281, 3188 (N-H₂), 3029, 2978, 2928, 2260 (CN), 1685 (C=O), 1619, 1527, 1467, 1389, 1342, 1255, 1101, 1007, 956, 912, 738; δH (500 MHz; DMSO-d6) 9.28, (0.9H, s, N-H), 8.79 (0.1H, s, N-H atrop.), 4.50 (0.2H, s, NH₂ atrop.), 4.35 (1.8H, s, NH₂), 3.83 (0.2H, s, 2-C atrop.), 3.53 (1.8H, s, 2-C); δC (126 MHz; DMSO-d6) 167.36 (1-C), 161.76 (1-Catrop.), 116.71 (3-C), 116.16 (3-Catrop.), 24.0 (2-C). Reference: sigmaaldrich NMR database.

N-[(Benzylcarbamothioyl)amino]-2-cyanoacetamide (25)
Cyanoacetoxydrazide (29)(520 mg, 5.20 mmol) and benzyl isothiocyanate (938 mg, 6.30 mmol) were dissolved in ethanol (10 ml) was heated at reflux for 1.5 hours. The reaction mixture was left to cool and a solid product precipitated. This was filtered off and recrystallized from ethanol to give the title compound as a white solid (1 g, 83%); m. p. 167-168 °C; IR (cm⁻¹) 3348 (N-H), 3240, 3124, 3029 (CH arom.), 2265 (CN), 1699 (C=O), 1223, 1172 (S=C); δH (500 MHz; DMSO-d6) 10.17 (1H, br s, NH-CO-), 9.50 (1H, br s, NH-NH-CS-), 8.63 (1H, br s, -NH-CH2Ph), 7.31 (2H, br t, J 7.2 Hz, 3',5'-H), 7.26 (2H, d, J 7.2 Hz, 2',6'-H), 7.22 (1H, t, J 7.2 Hz, 4'-H), 4.74 (2H, d, J 5.9 Hz, CH2-NH-), 3.66 (2H, s, CH2-CN); δC (126 MHz; DMSO-d6) 181.9 (C=S), 162.5 (C=O), 139.1 (1'-C), 128.1 (3',5'-C), 126.9 (2',6'-C), 126.7 (4'-C), 115.71 (CN), 46.64 (PhCH2-NH-), 24.36 (-CH2-CN); LCMS m/z (ESI-) 247 ([M-H]-, 100%, 206 (11%), 180 (39%), 495 (7%); Elemental analysis; calculated: C, 53.21; H, 4.87; N, 22.56; found: C, 53.21; H, 4.84; N, 22.48.

2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)acetonitrile (UNIS00073)

Method 1

A mixture of 4-benzyl-1-cyanoacetylthiosemicarbazide 25 (500 mg, 2 mmol), hydrazine hydrate (104 mg, 2 mmol, 0.1 ml) and ethanol (20 ml) was stirred at RT for 48 h. The solution was then acidified with hydrochloric acid until the mixture was pH 5 (pH paper) and then extracted with DCM (3 x 7 mL). Flash silica column chromatography, gradient elution (DCM in ethylacetate ethylacetate gave the title compound as a yellow solid powder (140 mg, 28%) m. p. = 186 °C; IR (cm⁻¹) 3096, 3049, (C-H arom.), 2265 (CN), 1583, 1496, 1456 (CH aliph.), 1356, 1307 (C=S), 766; LCMS m/z (ESI-) 229 ([M-H]-, 100%, 138 (2%), 155 (5%), 197 (21%), 481
Method 2

4-Benzyl-1-cyanoacetylthiosemicarbazides (500 mg, 2.01 mmol) was dissolved in 1M NaHCO₃ (20.16 ml) and the reaction mixture was heated at 60 °C for 24 hours. After cooling, 2 M HCl was added until the mixture was pH 5. The white precipitate was extracted with ethylacetate, and then dried to give the title compound (270 mg, 80%). ¹H NMR agreed with that described above.

2-(4-Benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064)

Method 1

4-Benzyl-1-cyanoacetylthiosemicarbazide (25) (500 mg, 2.01 mmol) was dissolved in 2 M NaOH (6.7 ml) and the reaction mixture was heated at reflux for 4 hours. After cooling, cold water was added to the solution and 2 M HCl was added until the mixture was pH 3 (pH paper). The acidified solution was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated to give the title compound as a white solid powder (76 mg, 15%) m.p. = 187 °C ; IR(cm⁻¹) 3283 (O-H), 2971, 2943, 2885, 2833, 2631, 1822, 1712 (C=O), 1578, 1492, 1455 (C=C), 1421, 1348 (C=S), 1158, 1121, 1075, 1028, 946, 868, 835, 715, 690, 657; δH (500 MHz; DMSO-d6) 13.88 (1H, s,
-COOH), 12.96 (1H, s, -NH), 7.33 (2H, br t, J 7.5 Hz, 3',5'-H), 7.29 (1H, d, J 7.5 Hz, 4'-H), 7.27 (2H, d, J 7.5 Hz, 2',6'-H), 5.23 (2H, s, -CH2-Ph), 3.68 (2H, -CH2-COOH); δc (126 MHz; DMSO-d6) 168.7 (5'-C), 167.7 (-COOH), 147.65 (3'-C), 135.71 (1'-C), 128.7 (3',5'-C), 127.9 (4'-C), 127.2 (2',6'-C), 46.1 (-CH2-Ph), 32.0 (-CH2-COOH); LCMS m/z (ESI-) 204 ([M-H]- (100%), 205 (75%), 460(32%), 431(28%), 460 (32%), 172 (25%), 113 (10%).

Method 2

4-Benzyl-1-cyanoacetylthiosemicarbazide (25) (200 mg, 0.81 mmol) was dissolved in aqueous NaHCO3 (1 M, 8.0 ml) and the reaction mixture was heated at reflux for 14 hours. After cooling, 2 M HCl was added until the mixture was pH 3. The acidified solution was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO4, filtered and concentrated to give the title compound as a white solid powder (160 mg, 86%); 1H NMR agreed with that described above.

2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)-N-(propan-2-yl)acetamide
(UNIS00067)

To dry DCM (7.3 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064) (100 mg, 0.401 mmol), isopropylamine (28 mg, 0.48 mmol), and EDC (130 mg, 0.677 mmol). The mixture was stirred for 26 h at RT. Dilute HCl (0.1 M) was added until the mixture was pH 5 (pH paper) and it was then extracted with DCM (3 × 7 mL). The combined organic layers were dried over MgSO4, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the
title compound as a white solid (33 mg, 28%); m.p. = 187 °C (sharp); Rf (DCM/ethylacetate [8:2] 0.6; IR (cm⁻¹) 3287 (N-H), 3091, 3038, 2929, 2851, 2762, 2429, 2359, 2224, 1627 (C=O), 1566 (C=N), 1504, 1439 (C=C), 1348(C=S), 1161, 1079, 941, 891, 769, 721; δH (500 MHz; CD3OD) 7.33 (2H, t, J 7.1 Hz, 3',5'-H), 7.31 (1H, t, J 7.1 Hz, 4'-H), 7.25 (2H, d, J 7.1 Hz, 2',6'-H), 5.34 (2H, s, -CH2Ph), 3.87 (1H, septet, J 6.7 Hz, -CHMe2), 3.46 (2H, s, -CH2CO-), 1.10 (6H, d, J 6.7 Hz, -CHMe2); δC (126 MHz; CD3OD) 169.6 (-CO-), 167.2 (-C=S), 149.5 (-C-CH2), 136.5 (1'-C), 129.9 (2',6'-C), 129.1(4'-C), 128.2(3',5'-C), 47.9 (-CH2Ph), 43.0 (-CHMe2), 34.1 (-CH2CO-), 22.4 (-CHMe2); m/z (ESI) 289 ([M-H]-, 37%), 204 (100%), 172 (11%), 112 (49%).

2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)-N-cyclopropylacetamide (UNIS00069)

To dry DCM (14.6 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064) (200 mg, 0.802 mmol), cyclopropylamine (55 mg, 0.962 mmol), and EDC (259 mg, 0.677 mmol). The mixture was stirred for 24 h at RT. Dilute HCl (0.1 M) was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 x 10 mL). The combined organic layers were dried over MgSO4, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound as a white powder solid (120 mg, 52%); m.p. = 170 °C ; Rf (DCM/ethylacetate [7:3] 0.3; IR (cm⁻¹) 3292 (N-H, stretch), 2929, 2906 (C-H, stretch), 1642 (C=O, stretch), 1537(C=C), 1350 (C=S) ; δH (500 MHz; CD3OD) 0.43 (2H,m, J 6.90Hz,2"-H), 0.677 (2H, m, J 6.90 Hz, 3"-H), 2.61 (1H,m, ,1"-H), 3.50
(2H, s, -CH$_2$-CO-), 5.34 (2H, s, -CH$_2$-Ph), 7.25 (2H, br d, J 7.6 Hz, 2',6'-H), 7.34 (2H, m, , 3',5'-H), 7.30 (1H,m, 4'-H); δC (126 MHz; CD$_3$OD) 6.34 (2"-C), 6.34 (3"-C), 23.52 (1"-C), 169.62 (-CO-), 33.90 (-CH$_2$-CO-), 149.33 (3-C), 169.78 (5-C), 136.48 (1'-C), 128.27 (2',6'-C), 129.93 (3',5'-C), 129.16 (4'-C); LCMS m/z (ESI-) 204 (100%), 287 [M-H] (56%), 597 (13%), 112 (49%). HRMS (ESI) found: [M+H]$^+$, 289.1115. C$_{14}$H$_{16}$N$_4$O requires [M+H]$^+$, 289.1045

**2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)-N-methylacetamide (UNIS00068)**

![Chemical Structure](image)

To dry DCM (14.60 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064)(200 mg, 0.802 mmol), methylvamine (31 mg, 0.96 mmol), and EDC (259 mg, 0.677 mmol). The mixture was stirred for 28 h at RT. Dilute HCl [0.1M] was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 x 10 mL). The combined organic layers were dried over MgSO$_4$, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound as a yellow powder (98 mg, 47%); m.p. = 155 °C; R$_f$ (DCM/ethylacetate [7:3] 0.26; IR (cm$^{-1}$) 1642 (C=O, stretch), 1484(C=C), 1363 (C=S), 2924, 2852 (C-H, stretch), 3266 (N-H, stretch);δ$_H$ (500 MHz; CD$_3$OD) 7.32(2H, m, 3',5'-H), 7.29 (1H, m, 4'-H), 7.25 (2H,d, J 7.5 Hz, 2',6'-H), 5.34 (2H, s, -CH$_2$-Ph), 3.49 (2H, s, -CH$_2$-CO-) 2.61 (3H, s, CH$_3$-NH-); , δC (126 MHz; CD$_3$OD) 169.7 (5-C), 168.7 (-CO-), 149.3 (3-C), 129.9 (3',5'-C), 129.1 (4'-C), 136.5 (1'-C), 128.3 (2',6'-C), 47.9 (-CH$_2$-Ph), 34.0 (-CH$_2$-CO-), 26.5 (CH$_3$-NH-); LCMS m/z (ESI-) (204 (100%), 261 [M-H]-(82%), 545 (6%), 112 (4%). HRMS (ESI) found: [M+H]$^+$, 263.0964. C$_{21}$H$_{14}$N$_4$OS requires [M+H]$^+$, 263.0888.
2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)-N-cyclohexyl-N-methylacetamide (UNIS00070)

To dry DCM (0.055 M, 14.60 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanyl-4H-1,2,4-triazol-3-yl)acetic acid (UNIS00064) (200 mg, 0.802 mmol), methyl cyclohexylamine (113 mg, 0.962 mmol), and EDC (259 mg, 0.677 mmol). The mixture was stirred for 29 h at RT. Dilute HCl (0.1 M) was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound as a ~1:1 mixture of atropisomers as a white powder (80 mg, 40%); m.p. = 183 °C; Rᵣ (DCM/ethylacetate [7:3] 0.3; IR (cm⁻¹) 1642 (C=O, stretch), 1453 (C=C), 1353 (C=S), 2922, 2853 (C-H, stretch); δH (500 MHz; CDCl₃) 7.27-7.20 (5H, m, Ar-H), 5.35 (1H, s, -CH₂-Ph), 5.34 (1H, s, -CH₂-Ph), 4.33 (0.5H, m, 1"-H), 3.68 (0.5H, -CH₂-CO-), 3.54 (0.5H, -CH₂-CO-), 3.33 (0.5H, m, 1"-H), 2.77 (1.5H, s, -CH₃), 2.68 (1.5H, s, -CH₃), 1.74 (2H, m, Cy), 1.57 (3H, m, Cy), 1.41 (1H, qd, J 12.5, 3.1 Hz, Cy), 1.31 (2H, m, Cy), 1.21-1.12 (1H, m, Cy), 1.00 (1H, m, Cy); δC (126 MHz; CD₃OD) 168.7 (5-C), 165.3 (-CO-), 148.6 (3-C), 148.5 (3-C), 134.96 (1'-C), 134.99 (1'-C), 129.00 (3',5'-C), 128.98 (3',5'-C), 128.29 (4'-C), 128.27 (4'-C), 127.61 (2',6'-C), 127.57 (2',6'-C), 57.2 (1"-C), 53.2 (1"-C), 47.5 (-CH₂Ph), 47.4 (-CH₂Ph), 46.15 (-CH₂-Ph), 33.1 (-CH₂-CO-), 32.5 (-CH₂-CO-), 30.8 (Cy), 29.9 (Cy), 29.9 (-CH₃), 29.8 (Cy), 27.7 (-CH₃), 25.1 (Cy), 25.47 (Cy), 25.51 (Cy), 25.55 (Cy); LCMS m/z (ESI-) 343 ([M-H]⁻, 100%), 252 (18%), 702 (18%). HRMS (ESI) found: [M+H]⁺, 345.1793. C₁₈H₂₄N₄OS requires [M+H]⁺, 345.1671.
To dry DCM (4.6 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064)(200 mg, 0.802 mmol), cyclohexylamine (95 mg, 0.96 mmol), and EDC (259 mg, 0.677 mmol). The mixture was stirred for 21 h at RT. Dilute HCl (0.1 M) was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 x 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound (40 mg, 20%); m.p. = 211 °C; Rf (DCM/ethylacetate) [7:3] 0.3; IR (cm⁻¹) 3280 (N-H, stretch), 2932, 2853 (C-H, stretch), 1643 (C=O, stretch), 1444 (C=C), 1349 (C=S); δH (500 MHz; CD3OD) 7.33(2H, t, J 7.2 Hz, 3',5'-H), 7.30 (1H, t, J 7.2 Hz, 4'-H), 7.24 (2H, d, J 7.2 Hz,2',6'-H), 7.30 (1H, t, J 7.2 Hz, 4'-H), 7.24 (2H, d, J 7.2 Hz,2',6'-H), 5.34 (2H, s, -CH₂-Ph), 3.54 (1H, tt, J 10.7, 3.8 Hz, 1''-H), 3.46 (2H,s, -CH₂-CO-), 1.72 (2H, m, Cy), 1.62 (1H, m,Cy), 1.37-1.28 (2H, m, Cy), 1.23-1.12 (3H, m, Cy), 1.18, 1.80 (2H, m, Cy), δc (126 MHz; CD3OD) 169.7 (5-C), 167.2 (-CO-), 149.6 (3-C), 136.5 (1'-C), 129.9 (3',5'-C), 129.1 (4'-C), 128.2 (2',6'-C), 50.26 (1''-C), 47.9 (-CH₂-Ph), 34.1 (-CH₂-CO-), 33.6 (2'',6''-C), 26.6 (4''-C), 26.0 (3'',5''-C); LCMS, m/z (ESI-) (204 (100%), 329 [M-H]⁻, 50%), 681(30%), 113 (4%). HRMS (ESI) found: [M+H]+, 331.1583. C₁₇H₂₂N₄OS requires [M+H]+, 331.1514.
2-(4-Benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)-N,N-dimethylacetamide (UNIS00071)

To dry DCM (10.9 ml) under nitrogen, was added 2-(4-benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00064)(150 mg, 0.60 mmol), dimethylamine (0.08 mg, 0.72 mmol), and EDC (194.38 mg, 1.01 mmol). The mixture was stirred for 21 h at RT and then heated at 42 °C for 3 h. Dilute HCl (0.1M) was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound as a white powder (39 mg, 26%); m.p. = 110 °C; Rf (DCM/ethylacetate [7:3] 0.26; IR (cm⁻¹) 2935, 2853 (C-H, stretch), 1627 (C=O, stretch), 1453(C=C), 1353 (C=S); δH (500 MHz; CD₃OD) 7.35 (2H, t, J 7.4 Hz, 3',5'-H), 7.31 (1H, t, J 7.4 Hz, 4'-H), 7.25 (2H, d, J 7.4 Hz, 2',6'-H), 5.30 (2H, s, -CH₂-Ph), 3.65 (2H, s, -CH₂-CO-), 2.91 (3H, s, -NCH₃), 2.86 (3H, s, -NCH₃); δC (126 MHz; CD₃OD) 169.6 (5-C), 168.2 (-CO-), 149.6 (3-C), 136.4 (1'-C), 129.9 (3',5'-C), 129.2 (4'-C), 128.5 (2',6'-C), 48.1 (-CH₂-Ph), 37.8 (-N-CH₃), 35.9 (-N-CH₃), 32.6 (-CH₂-CO-); LCMS m/z (ESI-) 275 ([M-H]⁻, 100%), 184 (31%), 243 (10%). HRMS (ESI) found: [M+H]⁺, 277.1121. C₁₃H₁₆N₄OS requires [M+H]⁺, 277.1045.
N,N-Diethylhydroxylamine (77 mg, 0.87 mmol) was added to a mixture of 2-(4-benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)acetonitrile (UNIS00073) (200 mg, 0.87 mmol) and water (2.6 ml). The reaction was heated at reflux for 2 h 45 minutes. Dilute HCl 0.1 M was added until the mixture was pH 3 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:2], [7:3], [1:1]) gave the title compound as a white powder (20 mg, 10%); m.p. = 150 °C; Rₜ (DCM/ethylacetate [7:3] 0.29; IR (cm⁻¹) 3329 (N-H), 2923, 2875 (C-H, stretch), 1680 (C=O, stretch), 1495 (C=C), 1342 (C=S); δ_H (500 MHz; CD₃OD) 7.35 (2H, t, J 7.4 Hz, 3',5'-H), 7.30 (1H, t, J 7.4 Hz, 4'-H), 7.28 (2H, d, J 7.4Hz, 2',6'-H), 5.75 (1H, d, 15.4 Hz, -CH₂-Ph), 5.05 (1H, d, 15.4 Hz, -CH₂-Ph), 4.44 (1H, s, -CH-CO-), 2.68-2.55 (4H, m, -CH₂-CH₃), 0.77 (6H, t, J 7.3 Hz, -CH₂-CH₃); δ_C (126 MHz; CD₃OD) 173.2 (5-C), 169.6 (-CO-), 151.0 (3-C), 136.9 (1'-C), 130.0 (3',5'-C), 129.2 (4'-C), 128.3 (2',6'-C), 62.5 (-CH-CO-), 47.9 (-CH2-Ph), 46.4 (-CH₂-CH₃), 13.8 (-CH₂-CH₃); LCMS m/z (ESI-) 318 (275 (100%), 246 (51%), 217 (21%), [M-H]⁻, 11%). HRMS (ESI) found: [M+H]⁺, 320.1534. C₁₅H₂₁N₅OS requires [M+H]⁺, 320.1467.
A solution of 2-(4-benzyl-5-sulfanyl-4H-1,2,4-triazol-3-yl)acetonitrile UNIS00073 (100 mg, 0.43 mmol) in a mixture of TFA (2 mL) and H₂SO₄ (1 mL) was stirred at RT for 2 h. The reaction mixture was then poured into ice-cold water purified using reverse-phase flash silica column chromatography, gradient elution (MeCN/water [5:95 %], [15:85%], [20:80%], [100:0%]) and then water was evaporated using a lyophiliser. This gave the title compound as a white powder (54 mg, 50%); Rf (DCM/ethylacetate [3:7] 0.8; IR (cm⁻¹) 3366 (N-H), 2950, 2927 (C-H, stretch), 1660 (C=O, stretch), 1452 (C=C), 1343 (C=S); δH (500 MHz; CD₃OD) 7.36 (2H, t, J 7.5 Hz, 3',5'-H), 7.32 (1H, t, J 7.5 Hz, 4'-H), 7.28 (2H, d, J 7.5 Hz, 2',6'-H), 5.35 (2H, s, -CH₂-Ph), 3.52 (2H, s, -CH₂-CO-); δC (126 MHz; CD₃OD) 170.9 (5-C), 169.6 (-CO-), 149.40 (3-C), 136.5 (1'-C), 130.0 (3',5'-C), 129.2 (4'-C), 128.4 (2',6'-C), 48.0 (-CH₂-Ph), 33.6 (-CH₂-CO-); LCMS, m/z (ESI) 204 (100%), 247 [M-H]⁺, 72%, 517 (55%), 113 (31%), 172 (19%).

β-Alanine Methyl Ester (46)

Thionyl chloride (9 g, 78.60 mmol) was added dropwise to a suspension of β-alanine (5 g, 56 mmol) in (42 ml) methanol at 0 °C. The reaction was stirred at 0 °C for 24 h and then concentrated using a rotary evaporator. The residual oil was triturated with cold ether and β-alanine methyl ester precipitated as a white powder (6.90 g, 88%); m. p. = RT; IR (cm⁻¹) 2940 (N-H₂), 1660 (C=O), 1597, 1523, 1392, 1344, 1213, 1001, 891, 797; δH (500 MHz; CD₃OD) 8.20 (2H, NH₂), 3.73 (3H, Me),
3.37 (2H, q, J 6.60 Hz, 3-H), 2.94 (2H, t, J 6.60 Hz, 2-H); δ (126 MHz; CD$_3$OD) 171.75 (-CO-), 52.39 (-OMe), 35.78 (3-C), 31.24 (2-C); m/z (GC-MS, El) 143 (10%), 129 (5%), 103 (M+, 15%), 85 (18%), 70 (15%).

$N$-(tert-Butoxycarbonyl)-β-alanine methyl ester (48)

![Structure of $N$-(tert-Butoxycarbonyl)-β-alanine methyl ester]

To a solution of β-alanine methyl ester hydrochloride (5 g, 35.8 mmol) in 108 ml methanol cooled in an ice bath was added triethylamine (15 ml) with vigorous stirring. Di-tert-butyl dicarbonate was then added to the mixture in portions (12 g, 53.7 mmol) and the reaction was stirred for 12 h. The mixture was concentrated to half its volume under reduced pressure, and triethylammonium hydrochloride was filtered from solution. The filtrate was diluted with chloroform (17 ml), and the mixture was washed with water (17 ml), and then with 10% w/w aqueous citric acid (17 ml). The organic layer was evaporated to give $N$-(tert-butoxycarbonyl)-β-alanine methyl ester as a transparent oil (4.80 g, 96%), IR (cm$^{-1}$): 3372 (N-H), 1700 (-C=O), 1693 (tBu-O-CO-), 1515, 1366, 1392, 1248, 1150 (-C-O-Me), 1076; δ (300 MHz; CDCl$_3$) 5.05 (1H, s, -NH), 3.71 (3H, s, -OMe), 3.42 (2H, q, J 6.30 Hz, 2-C), 2.54 (2H, t, J 6.35 Hz, 2-C), 1.45 (9H, s, -(Me)$_3$); δ (300 MHz; CDCl$_3$) 173.1 (1-C), 155.9 (-CO-NH-), 79.5 (-C-(Me)$_3$), 51.9 (-OMe), 36.2 (3-C), 34.6 (2-C), 28.5 (-(Me)$_3$); m/z (GC-MS, El) 147 ([M-tBu+H]$^+$, 50%), 130 ([M-tBuO]$^+$, 38%), 116 (46%), 98 (30%), 57 (tBu$^+$, 100%).

$N$-Boc-β-alanine hydrazide (50)

![Structure of $N$-Boc-β-alanine hydrazide]

To $N$-Boc-β-alanine methyl ester (2 g, 9.84 mmol) in 3 ml MeOH was added hydrazine hydrate (1.89 ml, 38.8 mmol) and the mixture was heated at reflux for 16 h. The mixture was evaporated and redissolved in chloroform (10 ml) and then
washed with water (10 ml). The mixture was dried over MgSO₄ and evaporated to dryness to give the title compound as a white solid (1.3 g, 67%); m. p. = 125 °C; IR(cm⁻¹) 3283 (N-H₂), 1688 (-CO-OMe), 1632 (tBu-O-CO-), 1520, 1364, 1280, 1247, 1163, 1013, 970, 939, 872; δH (500 MHz; DMSO) 8.90 (1H, s, NH-NH₂), 6.7 (1H, s, Boc-NH-), 4.20 (2H, s, -NH₂), 3.12 (2H, q, J 7.16 Hz, -NH-CH₂-), 2.15 (2H, t, J 7.16, -CH₂-CO-), 1.36 (9H, s, -(Me)₃); δC (126 MHz; DMSO-d₆) 170.2 (C=O), 155.9 (tBu-O-CO-), 152.0 (CO-NH), 1365, 1287, 1228 (S=C), 1162; δH (500 MHz; DMSO-d₆) 9.78 (1H, s, NH-CO-), 9.24 (1H, s, NH-NH-CS-), 8.40 (1H, s, -NH-CH₂Ph), 6.79 (1H, s, -NH-Boc), 7.29 (2H, m, 3',5'-H), 7.28 (2H, m, 2',6'-H), 7.22 (1H, t, J 7.2 Hz, 4'-H), 4.72 (2H, d, J 6.4 Hz, CH₂-Ph), 3.16 (2H, q, J 7.16 Hz, -NH-CH₂-), 2.30 (2H, t, J 7.16, -CH₂-CO-), 1.36 (9H, s, -(Me)₃); δC (126 MHz; DMSO-d₆) 182 (S=C), 170 (CO-NH-NH₂), 156 (tBu-O-CO-), 139.8 (1'-C), 128.5 (3',5'-C), 127.28 (2',6'-C), 127.22(4'-C), 78.9 -(C-(Me)₃), 47.09 (CH₂-Ph), 37.2 (Boc-NH-CH₂-), 34.4

**tert-Butyl N-(2-[(benzylcarbamothioyl)amino]carbamoyl)ethyl)carbamate (52)**

N-Boc-β-alanine hydrazide 50 (400 mg, 1.20 mmol) and benzyl isothiocyanate (353 mg, 2.36 mmol) were dissolved in ethanol (3.90 ml) and heated at reflux for 2 h. The reaction mixture was left to cool and then evaporated to give a white solid product. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:3], [1:1], [3:7]) gave the title compound as a white powder solid (642 mg, 92%); m.p. = 145 °C; IR (cm⁻¹) 3272 (N-H), 3182, 2993 (CH arom.), 1700 (-CO-OMe), 1548 (tBu-O-CO-), 1365, 1287, 1228 (S=C), 1162; δH (500 MHz; DMSO-d₆) 9.78 (1H, s, NH-CO-), 9.24 (1H, s, NH-NH-CS-), 8.40 (1H, s, -NH-CH₂Ph), 6.79 (1H, s, -NH-Boc), 7.29 (2H, m, 3',5'-H), 7.28 (2H, m, 2',6'-H), 7.22 (1H, t, J 7.2 Hz, 4'-H), 4.72 (2H, d, J 6.4 Hz, CH₂-Ph), 3.16 (2H, q, J 7.16 Hz, -NH-CH₂-), 2.30 (2H, t, J 7.16, -CH₂-CO-), 1.36 (9H, s, -(Me)₃); δC (126 MHz; DMSO-d₆) 182 (S=C), 170 (CO-NH-NH₂), 156 (tBu-O-CO-), 139.8 (1'-C), 128.5 (3',5'-C), 127.28 (2',6'-C), 127.22(4'-C), 78.9 -(C-(Me)₃), 47.09 (CH₂-Ph), 37.2 (Boc-NH-CH₂-), 34.4 (-CH₂-
CO\textsubscript{3}), 28.71 (-C-(Me)\textsubscript{3}); LCMS m/z (ESI-) 351 ([M-H]\textsuperscript{-} (30%), 277(100%), 243 (35%), 234 (60%), 128 (20%).

tert-Butyl-N-[2-(4-benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)ethyl]carbamate (UNIS00075)

![Chemical Structure Image]

tert-Butyl-N-(2-(((benzylcarbamothioyl)amino)carbamoyl)ethyl)carbamate (52) (200 mg, 0.57 mmol) was dissolved in 1 M NaHCO\textsubscript{3} (5.70 ml) and MeOH (2.5 ml) and the reaction mixture was heated at 60 °C for 24 h. After cooling, 2 M HCl was added until the mixture was pH 6. The white precipitate was extracted with ethylacetate, and then dried to give the title compound (150 mg, 79%); m.p. = 160 °C; IR (cm\textsuperscript{-1}) 3263 (-NH-Boc) 3116, 2970, 2925 (C-H arom.), 1666 (tBu-O-CO\textsubscript{-}), 1502, 1454, 1399 (CH aliph.),1356 (C=S), 1167, 1140, 721; δ\textsubscript{H} (500 MHz; (CDCl\textsubscript{3}) 11.88 (1H, s, 1H), 7.27 (2H, m, 3',5'-H), 7.23-7.25 (3H, m, 2', 4', 6'-H), 5.24 (2H, s, -CH\textsubscript{2}-Ph), 5.00 (1H, br s, Boc-NH\textsuperscript{-}), 3.39 (2H, q, 6.8 Hz, Boc-NH-CH\textsubscript{2}-), 2.61 (2H, t, 6.22 Hz, Boc-NH-CH\textsubscript{2}-CH\textsubscript{2})), 1.35 (9H, s, -(Me)\textsubscript{3}); δ\textsubscript{C} (126 MHz; (CDCl\textsubscript{3}) 168.1 (5-C), 155.8 (tBu-O-CO\textsubscript{-}), 151.3 (3-C), 134.5 (1'-C), 129.1 (3',5'-C), 128.4 (2',6'-C), 127.5 (4'-C), 79.9 (-C-(Me)\textsubscript{3}), 47.1 (-CH\textsubscript{2}-Ph), 36.6 (Boc-NH-CH\textsubscript{2}-), 28.3 -(Me)\textsubscript{3}), 26.8 (Boc-NH-CH\textsubscript{2}-CH\textsubscript{2})); LCMS m/z (ESI-) 333.3 ([M-H]\textsuperscript{-} (9%), 259.1 (100%), 168 (18%).
3-(2-Aminoethyl)-4-benzyl-4,5-dihydro-1H-1,2,4-triazole-5-thione (UNIS00076)

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\begin{align*}
\text{Tert-butyl-N-[2-(4-benzyl-5-sulfanyl-1H-1,2,4-triazol-3-yl)ethyl]carbamate} & \quad \text{(100 mg, 0.30 mmol)} \text{ were dissolved in 6 ml of 1M of (HCl/EtOAc) (1:1) and heated at reflux for 1 h. The mixture was evaporated to form a yellow solid product (70 mg, 86\%) m.p. = 220 °C ; IR(cm}^{-1} \text{) 3028 (-NH}_2\text{)}, 2930, 2753 (\text{C-H arom.}), 1578, 1473 (\text{C=C}, \text{ (CH aliph.)}, 1346 (\text{C=S}), 1250, 1173 (\text{CH aliph.}); \delta (500 MHz; (CD}_3\text{OD}) 7.30-7.38 (5H, m, 2',3',4',5',6'-H), 5.34 (2H, s, -CH}_2\text{-Ph), 3.25 (2H, t, 6.4 Hz, NH}_2\text{-CH}_2\text{-}), 2.91(2H, t, 6.44 Hz, NH}_2\text{-CH}_2\text{-CH}_2\text{-); } \delta (126 MHz; (CD}_3\text{OD}) 168.8 (5-C), 149.4(3-C), 135.1 (1'-C), 128.7 (3',5'-C), 127.9 (4'-C), 127.1 (2',6'-C), 46.2 (-CH}_2\text{-Ph), 35.3 (NH}_2\text{-CH}_2\text{-), 23.4 (NH}_2\text{-CH}_2\text{-CH}_2\text{-); LCMS m/z (ESI-) (233 ([M-H]}^-(100%), 203.9 (30%), 174.7 (28%).
\end{align*}
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4-Benzyl-3-[2-(cyclohexylamino)ethyl]-4,5-dihydro-1H-1,2,4-triazole-5-thione (UNIS00077)

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\begin{align*}
\text{Acetic acid (0.031 ml) and sodium triacetoxyborohydride (114 mg, 0.54 mmol) were added to a mixture of the 3-(2-aminomethyl)-4-benzyl-4,5-dihydro-1H-1,2,4-triazole-5-thione (100 mg, 0.37 mmol) and cyclohexanon (36.31 mg, 0.37 mmol) in DCM (2.89 ml). The mixture were stirred at RT for 24 h. The mixture were}
\end{align*}
\]
concentrated by evaporating most of the solvent and then saturated NaHCO₃ was added until the mixture was pH 7 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/MeOH [8:2], [1:1], [2:8]) gave the title compound (60 mg, 63%); m.p. = 147°C; Rᵢ (DCM/MeOH) [9:1] 0.3; IR (cm⁻¹) 3228 (N-H), 2865, 2851 (C-H, stretch), 1575, 1456 (C=C), 1364 (C=S), 1109, 903, 696; δ₇ (500 MHz; CD₃OD) 7.31 (2H, m, 3',5'-H), 7.32 (1H, m, 4'-H), 7.35 (2H, m,2',6'-H), 5.34 (2H, s, -CH₂-Ph), 3.08 (2H, t, 6.9 Hz, -NH-CH₂-), 2.83 (2H, t, 6.9 Hz, -NH-CH₂-CH₂-), 2.70 (1H, tt, J 10.7 Hz, 1''-H), 1.91 (2H, m, Cy), 1.78 (2H, m,Cy), 1.65 (1H, m, Cy), 1.32-1.24 (2H, m, Cy), 1.21-1.13 (3H, m, Cy), 1.65 (1H, m, Cy); δC (126 MHz; CD₃OD) 169.4 (5-C), 151.6 (3-C), 136.8 (1'-C), 130.0 (3',5'-C), 129.2 (4'-C), 128.4 (2',6'-C), 58.1 (1'' -C), 47.5 (-CH₂-Ph), 42.3 (-NH-CH₂-), 31.8 (2'',6''-C), 26.4 (4''-C), 25.6 (3'',5''-C), 25.30 (-NH-CH₂-CH₂-); LCMS m/z (ESI-) (653(5%), 315 [M-H] -, 100%), 265 (5%).

4-Benzyl-3-(2-[(propan-2-yl)amino]ethyl)-4,5-dihydro-1H-1,2,4-triazole-5-thione (UNIS00078)

Acetic acid (0.031 ml) and sodium triacetoxyborohydride (114.4 mg, 0.54 mmol) were added to a mixture of 3-(2-aminoethyl)-4-benzyl-4,5-dihydro-1H-1,2,4-triazole-5-thione (100 mg, 0.37 mmol) and acetone (21 mg, 0.37 mmol) in DCM (2.89 ml). The mixture were stirred at RT for 24 h. The mixture were concentrated by evaporating most of the solvent and then saturated NaHCO₃ was added until the mixture was pH 7 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/MeOH [8:2], [1:1], [2:8])
gave the title compound (60 mg, 59%); m.p. = 144 °C; Rf (DCM/MeOH) [9:1] 0.43 IR (cm⁻¹) 3233 (N-H), 2968, 2852, 2720, 2476 (C-H, stretch), 1624, 1575, 1421 (C=C), 1350 (C=S), 1286, 1013, 896, 722; δH (500 MHz; CD₃OD) 7.02 (2H, m, 2',6'-H), 7.32-7.30 (3H, m, 3',5',4'-H), 5.34 (2H, -CH₂Ph), 2.99 (2H, t, 6.9 Hz, -NH-CH₂-), 2.96 (1H, m, -CHMe₂), 2.79 (2H, t, 6.9 Hz, -NH-CH₂-CH₂-), 1.11 (6H, d, J 6.5 Hz, -CHMe₂); δC (126 MHz; CD₃OD) 169.4 (-C=S), 151.6 (3-C), 136.8 (1'-C), 130.0 (2',6'-C), 129.2 (4'-C), 128.3 (3',4'-C), 50.5 (-CH₂Me), 47.6 (-CH₂Ph), 42.9 (-NH-CH₂-), 25 (-NH-CH₂-CH₂-), 21.0 (-CHMe₂); LCMS m/z (ESI-) (573.5 (5%), 275 [M-H], 100%), 260.9 (2%).

4-Benzyl-3-[2-(methylamino)ethyl]-4,5-dihydro-1H-1,2,4-triazole-5-thione (UNIS00079)

Dimethyl sulphate (69 mg, 0.55 mmol) was added to a mixture of 3-(2-aminoethyl)-4-benzyl-4,5-dihydro-1H-1,2,4-triazole-5-thione (100 mg, 0.37 mmol) and 1 M NaHCO₃ (0.3 ml) and HFIP (0.4 ml). The mixture were stirred at RT for 5 h. 2M HCl was added into the mixture until the mixture was pH 2 and it was extracted with DCM three times and then 1M NaHCO₃ was added into the aqueous layer until the mixture was pH 8. The aqueous layer was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layer was then purified using Reverse-phase flash silica column chromatography, gradient elution (water in 0.1% formic acid/MeCN in 0.1% formic acid [5:95%], [15:85%], [20:80%], [100:0%]) which then gave the title compound as a transparent sticky solution (70 mg, 78%). m.p. = 80°C; IR (cm⁻¹) 3311 (N-H), 2932, 2868 (C-H, stretch), 3311 (N-H), 1360 (C=S), 1274, 1154, 959; δH (500 MHz; CD₃OD) 8.40(1H, 1'-H), 7.38 (2H, t, J 7.20 Hz, 3',5'-H), 7.33 (1H, J 7.20 Hz, 4'-H), 7.16 (2H, d, J 7.20 Hz, 2',6'-H), 5.23 (2H, -
CH$_2$Ph), 3.36 (2H, t, 6.4 Hz, -NH-CH$_2$-), 3.06 (2H, t, 6.4 Hz, -NH-CH$_2$-CH$_2$-), 2.64 (3H, -CH$_3$); $\delta$$_C$ (126 MHz; CD$_3$OD) 168.5 (-C=S), 154.6 (3'-C), 135.9 (1'-C), 130.2 (3',5'-C), 129.50 (4'-C), 127.8 (2',6'-C), 48.1 (-CH$_2$Ph), 37.4 (-NH-CH$_2$-), 24.1 (-NH-CH$_2$-CH$_2$-), 15.8 (-CH$_3$); LCMS $m/z$ (ESI+) (129.8 (15%), 219.9 (10%), 249 [M-H]$^+$, 100%), 271 (20%).

**N-(tert-Butoxycarbonyl)glycine hydrazide (47)**

![N-(tert-Butoxycarbonyl)glycine hydrazide](image)

To N-(tert-Butoxycarbonyl)glycine methyl ester (2 g, 10.60 mmol) in 3 ml MeOH was added hydrazine hydrate (2.0 ml, 41.65 mmol) and the mixture was refluxed for 16 h. The mixture was evaporated and redissolved in (10 ml) chloroform and then washed with (10 ml) water. The mixture was dried over magnesium sulphate and evaporated to dryness to give title compound as a white solid (1.3 g, 67%); m. p. = 99 °C; IR(cm$^{-1}$) 3288(N-H$_2$), 1701 (-CO-OMe), 1657 (tBu-O-CO-), 1542, 1366, 1251, 1248, 1053, 941, 675; $\delta$$_H$ (500 MHz; DMSO) 9.01 (1H, s, NH-NH$_2$), 6.84 (1H, t, J 6.35 Hz, Boc-NH$_2$), 4.19 (2H, s, -NH$_2$), 3.48 (2H, d, J 6.28 Hz, -NH-CH$_2$-), 1.33 (9H, s, -(Me)$_3$); $\delta$$_C$ (126 MHz; DMSO) 169.5(-CO-NH-NH$_2$), 156.3(tBu-O-CO-), 78.9 (-C-(Me)$_3$), 42.2 (-NH-CH$_2$-), 28.5 (-C-(Me)$_3$); m/z (GC-MS, EI) 133([M-tBu+H]$,^\circ$, 51%), 116 ([M-tBuO]$^\circ$, 48%), 89 ([M-Boc], 75%), 74 (25%), 57 (tBu $^\circ$, 100%)

**tert-Butyl N-(((benzylcarbamothioyl)amino)carbamoyl)methyl)carbamate (51)**

![tert-Butyl N-(((benzylcarbamothioyl)amino)carbamoyl)methyl)carbamate](image)

N-Boc-β-glycine hydrazide (1.200 g, 6.34 mmol) and benzyl isothiocyanate (114 g, 7.61 mmol) were dissolved in ethanol (12.68 ml) was heated at reflux for 2 hours.
The reaction mixture was left to cool and the mixture was evaporated to form a white solid product. Flash silica column chromatography, gradient elution (DCM/ethylacetate [8:3], [1:1], [3:7]) gave the title compound as a white powder solid (642 g, 92%); m. p.=90 °C; Rf (DCM/EtOAc) [6:4] 0.27 ;IR(cm\(^{-1}\)) 3385 (N-H), 3175, 3062, 2983 (CH arom.), 1700 (-CO-OMe), 1570(tBu-O-CO-) 1451, 1219 (S=C), 1168, 967; δ\(^1\)H (500 MHz; DMSO-d6) 9.93(1H, s, N\(-\)CO\(-\)), 9.42 (1H, s, NH-NH-CS-), 8.31 (1H, s, NH-CH\(_2\)-Ph), 7.29 (2H, m, 3',5'-H), 7.27 (2H, m, 2',6'-H), 7.21 (1H, m, 4'-H), 4.73 (2H, d, J 6.4 Hz, CH\(_2\)-Ph), 3.61 (2H, d, J 5.68 Hz, CH\(_2\)-CO-), 1.34 (9H, s, (Me)\(_3\)) ; δ\(^13\)C (126 MHz; DMSO-d6) 181 (C=S), 168 (-CO-NH-NH\(_2\)), 155.5 (tBu-O-CO-), 138.5 (1'-C), 127.5 (3',5'-C), 126.32 (2',6'-C), 126.32 (4'-C), 78.1 (-C-(Me)\(_3\)), 46.2 (-CH\(_2\)-Ph), 41.5 (-CH\(_2\)-CO-), 27.6 (-C-(Me)\(_3\)); LCMS m/z (ESI) 697.5 [M\(_{2}\)Na\(^+\)] (20%), 337 ([M-H\(^-\)] (29%), 263 (100%), 162 (4%), 114 (5%).

**tert-butylN-[(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl]carbamate (UNIS00080)**

*tert-butyl N-([(benzylcarbamothioyl)amino]carbamoyl)methyl)carbamate* (1.17 g, 3.46 mmol) were dissolved in 1M NaHCO\(_3\) (34.68 ml) and (15 ml) MeOH and the reaction mixture was heated at 60 °C for 24 hours. After cooling, (2 M) HCl was added until the mixture was pH 6. The white precipitate was extracted with ethylacetate, and then dried to give the titled compound (887 mg, 80%) m.p. = 198 °C; IR(cm\(^{-1}\)) 3291 (-NH-Boc) 3046, 2929, 2768 (C-H arom.), 1650 (tBu-O-CO-), 1537, 1442, 1428 (CH aliph.),1350 (C=S), 1289, 1031, 779; δ\(^1\)H (500 MHz; (DMSO) 13.73 (1H, s, 1H), 7.39 (1H, s, Boc-NH\(-\)), 7.34 (2H, t, J 7.6 Hz, 3',5'-H), 7.29 (1H, d, J 7.15 Hz, 4'-H), 7.25 (2H, d, J 7.15 Hz, 2',6'-H), 5.23 (2H, s, -CH\(_2\)-Ph), 4.1 (2H, d, 5.7 Hz, Boc-NH-CH\(_2\)-), 1.31 (9H, s, -(Me)\(_3\)); δ\(^13\)C (126 MHz; (DMSO) 167.7 (5-
C), 155.8 (tBu-O-CO), 150.2 (3-C), 135.6 (1'-C), 128.5 (3',5'-C), 127.5 (2',6'-C), 126.9 (4'-C), 78.43 (-C-(Me)₃), 45.6 (-CH₂-Ph), 35.4 (Boc-NH-CH₂), 28.0 (-{(Me)₃};

LCMS m/z (ESI-) (319.1, [M-H]⁻ (20%), 244.9 (100%), 232.8 (4%), 154.7 (5%).

[(4-Benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl](chloro)aminyl (UNIS00081)

\[
\begin{array}{c}
\text{ tert-butyl-N-[}{(4\text{-benzyl}-5\text{-sulfanylidene}-4,5\text{-dihydro}-1\text{-H}-1,2,4\text{-triazol}-3\text{-yl})}\text{methyl]carbamate (540 mg, 1.69 mmol) was dissolved in 34 ml of 1 M HCl in EtOAc (1:1) and heated at reflux for 1 h. The mixture was evaporated to form a yellow solid product (400 mg, 92%) m.p. = 200 °C ; IR(cm⁻¹) 3022 (-NH₂), 2910, 2751 (C-H arom.), 1574, 1470 (C=C), 1343(C=S), 1272, 1146, 983, 737; δH (500 MHz; (DMSO-d₆)) 14.14 ((1H, s, 1H)), 8.75 (3H, s, -NH₂.HCl), 7.38 (2H, t, J 7.6 Hz, 3',5'-H), 7.34 (1H, d, J 7.15 Hz, 4'-H), 7.29 (2H, d, J 7.15 Hz, 2',6'-H), 5.33 (2H, s, -CH₂-Ph), 4.1 (2H, s, -NH₂-CH₂); δC (126 MHz; (DMSO-d₆)) 168.1 (5-C), 146.8(3-C), 135.0 (1'-C), 128.8 (3',5'-C), 128.0 (4'-C), 127.1 (2',6'-C), 45.6 (-CH₂-Ph), 33.6 (NH₂-CH₂-); LCMS m/z (ESI-) (218.9 ([M-H]⁻) (100%), 186.9 (10%), 174.7 (8%), 127.7 (15%).
\end{array}
\]
Acetic acid (0.031 ml) and sodium triacetoxyborohydride (114 mg, 0.54 mmol) were added to a mixture of the [(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl](chloro)aminyl (100 mg, 0.37 mmol) and cyclohexanone (36.31 mg, 0.37 mmol) in DCM (2.89 ml). The mixture was stirred at RT for 24 h. The mixture were concentrated by evaporating most of the solvent and then saturated NaHCO₃ was added until the mixture was pH 7 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/MeOH [8:2], [1:1], [2:8]) gave the title compound (70 mg, 59%); m.p. = 166°C; Rf (DCM/MeOH) [9:1] 0.28; IR (cm⁻¹) 3190 (N-H), 2919, 2851 (C-H stretch), 1514, 1447 (C=C), 1366 (C=S), 1298, 1252, 1097, 932, 730; δH (500 MHz; CD₃OD) 7.36 (2H, m, 3',5'-H), 7.32-7.30 (3H, m, 2',6',4'-H), 5.41 (2H, s, -CH₂-Ph), 3.68 (2H, s, -NH-CH₂-), 2.27 (1H, tt, J 10.7 Hz,1''-H), 1.67 (4H, m, Cy), 1.15 (3H, m, Cy), 0.96 (2H, m, Cy); δC (126 MHz; CD₃OD) 169.7 (5-C), 152.6 (3-C), 137.1 (1'-C), 130.0 (3',5'-C), 129.2 (4'-C), 128.2 (2',6'-C), 56.9 (1'' -C), 47.4 (-CH₂-Ph), 41.8 (-NH-CH₂-), 33.5 (2'',6''-C), 27.0 (4''-C), 25.9 (3'',5''-C); LCMS m/z (ESI-) (625(20%), 301 [M-H]⁻, 100%), 269 (5%).
4-Benzyl-3-([(propan-2-yl)amino]methyl)-4,5-dihydro-1H-1,2,4-triazole-5-thione (UNIS00083)

Acetic acid (0.033 ml) and sodium triacetoxyborohydride (114.4 mg, 0.54 mmol) were added to a mixture of the [(4-benzyl-5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl][(chloro)aminy]l (100 mg, 0.37 mmol) and acetone (21 mg, 0.37 mmol) in DCM (2.89 ml). The mixture were stirred at RT for 24 h. The mixture was concentrated by evaporating most of the solvent and then saturated NaHCO₃ was added until the mixture was pH 7 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/MeOH [8:2], [1:1], [2:8]) gave the title compound (60 mg, 58%); m.p. =144 °C; Rf (DCM/MeOH) [9:1] 0.32 IR (cm⁻¹) 3195 (N-H), 2966, 2918, 2862 (C-H, stretch), 1582, 1512, 1447 (C=C), 1362 (C=S), 1302, 1298, 1255, 1160, 1012, 920, 728; δH (500 MHz; CD₃OD) 7.35 (2H, m, 2',6'-H), 7.31-7.29 (3H, m, 3',5',4'-H), 5.40 (2H, -CH₂Ph), 3.64 (2H, s, -NH-CH₂-), 2.65 (1H, m, -CHMe₂), 0.96 (6H, d, J 6.3 Hz, -CHMe₂); δc (126 MHz; CD₃OD) 169.7 (-C=S), 152.4 (3-C), 137.0 (1'-C), 130.0 (2',6'-C), 129.2 (4'-C), 128.3 (3',4'-C), 48.4 (-CHMe₂), 47.5 (-CH₂Ph), 42.3 (-NH-CH₂-), 22.2 (-CHMe₂); LCMS m/z (ESI⁺) (263 [M+H]⁺, 100%), 222 (5%), 203.9 (80%), 174.9 (5%).

Ethyl 3-ethoxy-3-iminopropanoate (117)
acetyl chloride (839 mmol, 59.7 ml) was added dropwise into dry ethanol (49.6 ml) at 0 °C. Ethyl cyanoacetate (88.40 mmol) was then added. The reaction was stirred at 22 °C for 20 h and then concentrated and triturated with diethyl ether to give a white powder salt (8.4 g, 70%). m.p. = 86°C; IR (cm⁻¹) 2865, 2851 (C-H, stretch), 1740 (C=NH, stretch), 1662 (C=O); δH (500 MHz; CDCl₃) 12.71 (1H, HCl), 11.98 (1H, NH), 4.70 (2H, q, J 7.13 Hz, 2-H), 4.21 (2H, q, J 7.13 Hz, 6-H), 3.87 (2H, s, 4-H), 1.48 (3H, t, J 7.13 Hz, 1-H), 1.27 (3H, t, J 7.13 Hz, 7-H); δC (126 MHz; CDCl₃) 172.2 (5-C), 164.4 (3-C), 71.8 (2-C), 62.7 (6-C), 39.3 (4-C), 14.1 (1-C), 13.6 (7-C); LCMS m/z (ESI⁺) (160 [M+H]⁺, 100%), 146 (80%), 115 (40%).

Methyl 3-[(Z)-(1,3-dimethoxy-3-oxopropylidene)amino]-3-iminopropanoate (115)

acetyl chloride (478 mmol, 34 ml) was added dropwise into dry methanol (28 ml) at 0 °C. Methyl cyanoacetate (50.5 mmol, 4 ml) was then added. The reaction was stirred at 0 °C for 20 h and then concentrated and triturated to give a white sticky powder (2.4 g, 28%). m.p. = 120 °C; IR (cm⁻¹) 3115 (N-H), 3000, 2804 (C-H, stretch), 1719 (C=NH, stretch), 1655 (C=O), 1395, 1205; δH (500 MHz; DMSO-d6) 3.63 (6H, s, -COOMe), 3.59 (3 H, s, -N=C-OMe), 3.51 (2H, s, -CH₂-C=NH), 3.20 (2H, s, -N=C-CH₂-COOMe); δC (126 MHz; DMSO-d6) 168 (-N=C-OMe), 167.2 (-C=NH), 167.0 (-CO-CH₂-C=NH), 167.0 (-CO-OMe), 52.2 (-COOMe), 51.75 (-N=C-OMe), 42.2 (-N=C-CH₂-COOMe), 41 (-CH₂-C=NH); LCMS m/z (ESI⁺) (208 (100%), 213 (50%), 231 [M+H]⁺, 5%), 260 (5%), 265 (30%).
**Methyl 3-methoxy-3-iminopropanoate (111)**

Acetyl chloride (240 mmol, 17 ml) was added dropwise to methanol (14 ml) at 0 °C. Methyl cyanoacetate (25 mmol, 2 ml) was then added. The reaction was stirred at 22 °C for 4 h and then concentrated and triturated with diethyl ether to give a white powder (3.2 g, 75%); m.p. = RT; IR (cm⁻¹) 2788, 2735 (C-H, stretch), 1732 (C=NH, stretch), 1664 (C=O); δH (500 MHz; CDCl₃) 12.82 (1H, HCl), 12.1 (1H, NH), 4.34 (3H, s, 1-H), 3.93 (2H, s, 3-H), 3.76 (3H, s, 5-H); δC (126 MHz; CDCl₃) 173.3 (2-C), 164.8 (4-C), 61.6 (5-C), 62.7 (6-C), 53.4 (1-C), 38.9 (3-C); LCMS m/z (ESI⁺) (132 [M-H]⁺, 100%), 167 (10%), 204 (15%), 263 (20%).

**Methyl 2-[N'-(3-methoxy-3-oxopropanoyl)hydrazinecarbonyl]acetate (104)**

Malonyl chloride (3.66 mmol, 0.4 ml) was added dropwise to a hydrazine (7.32 mmol) in water (19.3 ml). The reaction was stirred at 0 °C for 2 h and then extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography, gradient elution (DCM/MeOH [9:1]) gave the title compound as white crystal (200 mg, 41%); m.p. = 118 °C; IR (cm⁻¹) 3186 (NH), 1738, 1583 (C=O), (C=O-NH) δH (500 MHz; CD₃OD) 3.71 (6H, s, MeO-), 3.38 (4H, s, -CH₂-COOMe); δC (126 MHz; CD₃OD) 169.1(-CO-NH-), 166.8 (-COOMe), 52.8 (MeO-); LCMS m/z (ESI⁺) (101(5%), 233 [M-H]⁺, 3%), 255 (100%).
**Ethoxycarbonylacetoxydrazide (106)**

![Chemical structure](image)

Hydrazine hydrate (7.48 mmol) was added at RT to a solution of diethyl malonate (2 g, 12.48 mmol) in ethanol (0.9 ml). The mixture was stored overnight, and malonohydrazide (240 mg) was separated by filtration. The filtrate was triturated with diethylether. Evaporation of the filtrate left a partly white crystalline residue (410 mg, 40%), m.p. = 59 °C , IR (cm⁻¹) 3296, 3048 (NH₂, NH), 1725, 1637 (C=O), δH (500 MHz;DMSO-d6) 9.18 (1H, s, NH), 4.06 (2H, q, J = 7.1, -CH₂-CH₃), 3.14 (2H, s, -CH₂), 1.17 (3H, t, J = 7.1, -CH₃); δC (126 MHz; DMSO-d6) 168.2 (-NH-CO), 165 (-COOEt), 41.1 (-CH₂-CH₃), 14.5 (CH₃).

**Malonohydrazide (107)**

![Chemical structure](image)

White powder (240 mg), IR (cm⁻¹) 3295, 3100 (NH₂, NH), 1640 (C=O), δH (500 MHz;DMSO-d6) 9.05 (2H, s, NH), 4.22 (4H, s(broad), -NH₂), 2.39 (2H, s, -CH₂-); δC (126 MHz;DMSO-d6) 166 (-CO-), 40 (-CH₂-)

**Methyl 2-(benzylcarbamoyl)acetate (123)**

![Chemical structure](image)

Methyl malonyl chloride (2 g, 14.64 mmol) was added drop wise over 30 minutes into a mixture of benzyl amine (1.57 g, 14.64 mmol), triethylamine (1.63 g,16 mmol), DCM (49 ml). The reaction mixture was stirred for 4 h at rt. The triethylamine salt was then filtered and DCM (40 ml) was added into the filtrate. The filtrate was washed with saturated ammonium chloride and brine. The mixture
then was concentrated. Flash silica column chromatography, gradient elution (DCM/EtOAc (8/2) gave the title compound as a white solid powder (1.20 g, 34%). m.p. = 65°C; IR (cm\(^{-1}\)) 3285.7 (N-H, stretch), 1742.8 (NH-C=O, stretch), 1633 (CH\(_2\)-C=O); \(\delta_H\) (500 MHz; CDCl\(_3\)) 7.30-7.28 (3H, d, J 7.57 Hz, 3',4',5'-H), 7.35-7.32 (2H, t, J 7.57 Hz, 2',6'-H), 4.49 (2H, d, J 5.55 Hz, -CH\(_2\)-Ph), 3.74 (3H, s, CH\(_3\)), 3.38 (2H, s, -CO-CH\(_2\)); \(\delta_C\) (126 MHz; CDCl\(_3\)) 170 (NH-CO\(-\)), 164.6 (1-C), 137.8 (1'-C), 128.8 (2',6'-C), 127.7-127.5 (3',4',5'-C), 43.62 (-CH\(_2\)-Ph), 52.5 (CH\(_3\)) , 40.8 (2-C); LCMS \(m/z\) (ESI\(^+\)) (230(95%), 227 (25%), 208 (5%, [M+H]\(^{+}\)).

**Ethyl 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetate (UNIS00085)**

Oxalyl chloride (2.60 g, 20.5 mmol) was added to a solution of N-benzylacetamide (3 g, 20.5 mmol) and 2,6-lutidine (4.40 g, 41 mmol) in DCM (102 ml) at 0 °C which caused heavy gas [CAUTION: carbon monoxide] evolution. The mixture was stirred for 40 min, and ethoxycarbonylacetoxydrazide (3 g, 20.5 mmol) was added. The reaction mixture was stirred for 3 h at RT. The volatiles were then removed under reduced pressure. The residue was dissolved in toluene/MeCN (100/50 ml) and refluxed for 24 h. After cooling to 0 °C, the precipitate was filtered and the filtrate was collected. The solvent was evaporated from the filtrate to give an oily sticky crude product. Flash silica column chromatography, gradient elution (DCM/EtOAc/MeOH (8/1.5/0.5)) gave the title compound as a white solid powder (2.64 g, 50%). m.p. = 60 °C ; IR (cm\(^{-1}\)) 3370 (C-H arom.), 1733.4 (C=O), 1534, 1440, 1162 (CH aliph.); \(\delta_H\) (500 MHz; DMSO-d6) 7.36 (2H, t, J 7.0 Hz, 3',5'-H), 7.30 (1H, t, J 7.0 Hz, 4'-H), 7.0 (2H, d, J 7.0 Hz, 2',6'-H), 5.21 (2H, s, -CH\(_2\)-Ph), 3.96 (2H, q, J 7.0 Hz, -CH\(_2\)-CH\(_3\)) 3.89 (2H, s, -CH\(_2\)-COOEt), 2.23 (3H, s, CH\(_3\)-C=N-), 1.10 (3H, .
t, J 7.0 Hz, -CH₂CH₃); δC (126 MHz; DMSO-d6) 168.6 (−CO), 152.1 (5−C), 149 (3−C), 136.1 (1′−C), 129.2 (3′,5′−C), 128.2 (4′−C), 127 (2′,6′−C), 61.4 (−CH₂−CH₃), 46.6 (−CH₂−Ph), 31.5 (−CH₂−COOEt), 14.3 (−CH₂−CH₃), 11.1 (CH₃−C=N−); LCMS m/z (ESI+) 282 (20%), 260 ([M+H]+ 100%, 208 (18%), 143 (100%); Elemental analysis; calculated: C, 64.85; H, 6.61; N, 16.20; found: C, 60.27; H, 6.80; N, 16.01.

2-(4-Benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetic acid (103)

Method 1

LiOH (146 mg, 3.47 mmol) was added to a mixture of ethyl 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetate (300 mg, 1.15 mmol), THF (7 ml) and water (3 ml) and the reaction mixture was stirred for 2 h 30 at RT. 2 M HCl was added to the solution until the mixture was pH 2 (pH paper). The acidified solution was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated to give the title compound as an oil (55 mg, 18%), IR (cm⁻¹) 1703 (C=O), 1533, 1334, 712 (CH aliph.); δH (500 MHz; DMSO-d6) 7.36 (2H, t, J 7.0 Hz, 3′,5′−H), 7.30 (1H, t, J 7.0 Hz, 4′−H), 7.0 (2H, d, J 7.0 Hz, 2′,6′−H), 5.21 (2H, s, −CH₂−Ph), 3.85 (2H, s, −CH₂−COOH), 2.25 (3H, s, CH₃−C=N−), δC (126 MHz; DMSO-d6) 169.7 (−CO), 151.4 (5−C), 149.2 (3−C), 135.6 (1′−C), 128.7 (3′,5′−C), 127.6 (4′−C), 126.5 (2′,6′−C), 46.1 (−CH₂−Ph), 31.3 (−CH₂−COOEt), 10.6 (CH₃−C=N−); LCMS m/z (ESI+) 276 (70%), 250 (25%), 232 ([M+H]+ 22%), 186 (15%), 106 (8%).

Method 2

LiOH (48.6 mg, 1.16 mmol) was added to a mixture of ethyl 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetate (100 mg, 0.39 mmol), THF (2 ml) and water (1 ml) and
the reaction mixture was heated at 90 °C for 22 h. 1 M NaHCO₃ was added to the solution until the mixture was pH 2 (pH paper). The solution was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated to give the title compound as an oil (55 mg, 38%).

**Method 3**

LiOH (146 mg, 3.47 mmol) was added to a mixture of ethyl 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetate (300 mg, 1.16 mmol), THF (7.2 ml) and water (3 ml) and the reaction mixture was stirred for 2 and a half hours at RT. The mixture was purified using a resin (Dowex 1 x 2 chloride form) column that was prepared to have hydroxide ions. The column was eluted first with (formic acid in methanol pH~3) followed by (0.1 M HCl in MeOH). The collected fraction was evaporated using a lyophiliser to give the title compound as an oil (267 mg, 100%).

**4-Benzyl-3,5-dimethyl-4H-1,2,4-triazole (UNIS00086)**

![Chemical Structure](image)

LiOH (859 mg, 20.5 mmol) was added to a mixture of ethyl 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetate (1.70 g, 6.55 mmol), THF (42.6 ml) and water (17.7 ml) and the reaction mixture was stirred for 24 h at rt. (2 M) HCl was added to the solution until the mixture was pH 2 (pH paper). Saturated NaCl was added to the acidified solution and was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated to give an oil (acid 103). Leaving this oily product at RT for around 22 h gave the title compound as an oil (437 mg, 34%). IR (cm⁻¹) 3336 (C-H strech.), 1534, 1440, 1162 (CH aliph.); δH (500 MHz; DMSO-d6) 7.37 (2H, t, J 7.0 Hz, 3',5'-H), 7.31 (1H, t, J 7.0 Hz, 4'-H),
To dry DCM (15 ml) under nitrogen, was added 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetic acid (200 mg, 0.86 mmol), isopropylamine (61 mg, 1.03 mmol), and EDC (78 mg, 1.45 mmol). The mixture was stirred for 22 h at RT. 1 M NaHCO₃ was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Preparative silica TLC, (DCM/EtOAc/MeOH [6:3.5:0.5]) gave the title compound as an oil (30 mg, 15%); IR (cm⁻¹) 3368 (C-H arom.), 1700 (C=O), 1530, 1435, 1173 (CH aliph.); δH (500 MHz; (CDCl₃) 7.35-7.28 (3H, m, 3',4',5'-H), 6.96 (2H, d, J 6.79 Hz,2',6'-H), 5.11 (2H, s, -CH₂-Ph), 3.76 (2H, s, -CH₂-CO-), 3.62 (3H, -CO-CH₃), 2.23 (3H, s, CH₃-C=N-); δC (126 MHz; (CDCl₃) 168.49 (-CO-), 152.70 (5-C), 148.72 (3-C), 134.70 (1'-C), 129.34 (3',5'-C), 128.52 (4'-C), 126.14 (2',6'-C), 52.67 (COO-CH₃), 47.18 (-CH₂-Ph), 31.76 (-CH₂-COOMe), 11.19 (CH₃-C=N-); LCMS m/z (ESI+) 106 (30%), 132 (20%), 246 ([M+H]+,15%, 268 (100%), 295 (30%).
2-(4-Benzyl-5-methyl-4H-1,2,4-triazol-3-yl)-N-cyclohexylacetamide (UNIS00088)

To dry DCM (4.30 ml) under nitrogen, was added 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetic acid (55 mg, 0.237 mmol), cyclohexylamine (28 mg, 0.033 mmol), and EDC (78 mg, 0.4 mmol). The mixture was stirred for 24 h at RT. 1 M NaHCO₃ was added until the mixture was pH 5 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Preparative silica TLC (DCM/EtOAc/MeOH [6:3.5:0.5]) gave the title compound as white powder (37 mg, 50%); m.p. = 230 °C; Rₜ (DCM/EtOAc/MeOH [6:3.5:0.5] 0.8 ; IR (cm⁻¹) 3385, 3241 (N-H, stretch), 2934 (CH₃), 1650 (C=O, stretch), 1444 (C=C), 1352 (C=S); δₓ (500 MHz; CDCl₃) 7.36-7.30 (3H, m, 3',4',5'-H), 7.13 (1H, b, -CO-NH-), 6.96 (2H, d, J 6.79 Hz,2',6'-H), 5.17 (2H, s, -CH₂-Ph), 3.70 (1H, tt, J 8.31, 3.90 Hz,1''-H), 3.60 (2H, s, -CH₂-CO-), 2.39 (3H, s, CH₃-C=N-), 1.8 (2H, m, Cy), 1.62 (5H, m, Cy), 1.58 (1H, m, Cy), 1.36-1.29 (2H, m, Cy), 1.20, 1.13 (3H, m, Cy); δₜ (126 MHz; CDCl₃) 165.2 (-CO-), 152.4 (5-C), 150.3 (3-C), 134.3 (1'-C), 129.4 (3',5'-C), 128.5 (4'-C), 126.1 (2',6'-C), 48.8 (1''-C), 47.0 (-CH₂-Ph), 33.6 (-CH₂-CO-), 32.8 (2'',6''-C), 25.5 (4''-C), 24.8 (3'',5''-C), 11.1 (CH₃-C=N-); LCMS m/z (ESI-) (313 [M-H]⁻, (100%)), 231(80%), 214 (70%), 186 (60%).
2-(4-Benzyl-5-methyl-4H-1,2,4-triazol-3-yl)-isopropylacetamide (UNIS00089)

Method 1

To dry DCM (14.62 ml) under nitrogen, was added 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetic acid (186 mg, 0.80 mmol) and isopropylamine (57 mg, 0.96 mmol), followed by EDC (260 mg, 1.35 mmol). The mixture was stirred for 24 h at RT. 1 M NaHCO₃ was added until the mixture was pH 7 and it was extracted with DCM/MeOH [9:1] (3 × 10 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated. Flash silica column chromatography (DCM/EtOAc/MeOH (6/3/1)) gave the title compound as a white powder (4 mg, 2%); m.p. =174 °C; IR (cm⁻¹) 3244 (N-H, stretch), 2972 (CH₃), 1643 (C=O, stretch), 1532, 1443, 1173; δH (500 MHz; DMSO-d6) 8.09 (1H, d, J 7.31 Hz, -CO-NH-), 7.36-7.27 (3H, m, 3',4',5'-H), 7.05 (2H, d, J 7.3 Hz, 2',6'-H), 5.23 (2H, s, -CH₂-Ph), 3.76 (1H, sextet, J 6.8 Hz, -CH₂Me₂), 3.57 (2H, s, -CH₂-CO-), 2.17 (3H, s, CH₃-C=NH-), 1.01 (6H, d, J 6.6 Hz, -Me₂); δC (126 MHz; DMSO-d6) 166.2 (-CO-), 151.8 (5-C), 150.5 (3-C), 136.3 (1'-C), 129.2 (3',5'-C), 128.12 (4'-C), 127 (2',6'-C), 46.5 (-CH₂-Ph), 41.2 (-CH₂Me₂), 32.7 (-CH₂-CO-), 22.6 (-Me₂), 11.1 (CH₃-C=N-); LCMS m/z (ESI⁺) (123 (10%), 186 (65%), 214 (80%), 273 [M-H]⁺, (100%)), 295 (90%).

Method 2

To isopropyl amine (25 mg, 0.42 mmol) under nitrogen, was added a solution of 2-(4-benzyl-5-methyl-4H-1,2,4-triazol-3-yl)acetic acid (80 mg, 0.35 mmol) in DMF (0.58 ml) followed by a solution of a mixture of EDCI (106 mg, 0.55 mmol), HOBT (51 mg, 0.38 mmol) in DMF (0.61 ml). Finally DIPEA (89 mg, 0.69 mmol) was added dropwise to the mixture. The mixture was stirred for 35 h at RT. The DMF
was evaporated and the mixture was purified using reverse-phase flash column chromatography, gradient elution (MeCN (1% formic acid)/water (1% formic acid) [5:95 %], [15:85%], [20:80%], [100:0%]) and then water was evaporated using a lyophiliser to gave the title compound as a white powder (56 mg, 59%).

**Ethyl 2-[(carbamothioylamino)carbamoyl]acetate (133)**

Method 1

Thiosemicarbazide (121 mg, 1.33 mmol) was dissolved in dry MeCN (2 ml) under nitrogen. Ethyl-3-chloro-3-oxopropanoate (200 mg, 1.33 mmol) was added dropwise at 0 °C to the mixture. The mixture was then heated to reflux for 2 h. It was then filtered and the precipitate was collected and recrystallized using ethanol to give white crystals (63 mg, 23%), m.p. = 165 °C, IR (cm⁻¹) 3327, 3129 (NH₂, NH), 1695, 1652 (C=O), δH (500 MHz; DMSO-d6) 10.1 (1H, s, -CO-NH-), 9.36 (1H, s, -CO-NH-NH-), 7.97 (1H, s, -CS-NH₂), 7.35 (1H, s, -CS-NH₂), 4.09 (2H, q, J= 7.1, -CH₂-CH₃), 3.28 (2H, s, -CO-CH₂), 1.19 (3H, t, J= 7.1, -CH₃); δC (126 MHz;DMSO-d6) 182.3 (-CS-), 168.1 (-CO-NH-), 165.2 (-COOEt), 61.2(-CH₂-CH₃), 14.5 (CH₃), LCMS Rₜ 0.7, no corresponding molecular ion could be detected.

Method 2

Thiosemicarbazide (605 mg, 6.64 mmol) was dissolved in dry MeCN (11 ml) and pyridine (525 mg, 6.64 mmol) was added. Ethyl-3-chloro-3-oxopropanoate (1 g, 6.64 mmol) was added dropwise to the mixture at 0 °C. The mixture was then heated at reflux for 2 h. The mixture was filtered and the solid was collected and recrystallized using ethanol to give white crystals (184 mg, 13%).
Ethyl 2-(5-sulfanylidene-1,4-dihydro-1,2,4-triazol-3-yl)acetate (UNIS00090)

Dry EtOH (4 ml) was added into ethyl 3-ethoxy-3-iminopropanoate (200 mg, 1.02 mmol) under nitrogen followed by the addition of Et₃N (103 mg, 0.4 mmol, 1.02 mmol). Thiosemicarbazide (93 mg, 1.02 mmol) was added to the mixture at 0 °C and the mixture was stirred at RT for 2 h. The mixture was then heated at reflux for 24 h. After cooling to 0 °C, the precipitate was filtered and the filtrate was collected. The solvent was evaporated from the filtrate to give a yellow powder. Flash silica column chromatography (DCM / EtOAc (7:3) gave the title compound as a yellow powder (112 mg, 60%). m.p. = 179-180 °C; IR (cm⁻¹) 1738 (C=O), 1593, 1469; δ_H (500 MHz; DMSO-d6) 13.38 (1H, s, 1-H), 13.21 (1H, s, 4-H), 4.27 (2H, q, J 7.14 Hz, -CH₂-CH₃), 3.74 (2H, s, -CH₂-COOEt), 4.27 (2H, q, J 7.14 Hz, -CH₂-CH₃); δ_C (126 MHz; DMSO-d6) 168.6 (5-C), 166.8 (-CO-), 146.7 (3-C), 61.6 (-CH₂-CH₃), 31.9 (-CH₂-COOEt), 14.4 (-CH₂-CH₃); LCMS m/z (ESI+) 262 (40%), 234 (20%), 188 ([M+H]^+, 35%), 156 (20%), 142 (40%), 132 (80%), 114 (100%). Elemental analysis; calculated: C, 38.49; H, 4.85; N, 22.45; found: C, 38.53; H, 4.99; N, 15.98.

2-(5-Sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetic acid (UNIS00091)

Method 1

LiOH (66.7 mg, 1.59 mmol) was added to a mixture of ethyl 2-(5-sulfanylidene-1,4-dihydro-1,2,4-triazol-3-yl)acetate (100 mg, 0.53 mmol), THF (3.31 ml) and water (1 ml) and the reaction mixture was stirred for 24 h at RT. 2 M HCl was added to the solution until the mixture was pH 2 (pH paper). The acidified solution was then
extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated to give the title compound as a yellow powder (10 mg, 11%), m.p. = 178 °C; IR (cm⁻¹) 3119 (N-H, stretch), 1743 (C=O), 1473, 1429 (C=S), 1227, 989; δH (500 MHz; DMSO-d6) 13.38 (1H, s, 1-H), 13.21 (1H, s, 4-H), 12.92 (1H, s, -COOH), 3.63 (2H, s, -CH₂-COOH); δC (126 MHz; DMSO-d6) 169.6 (5-C), 166.8 (-CO-), 147.2 (3-C), 32.2 (-CH₂-COOH); LCMS m/z (ESI⁻) 114 (100%), 158 ([M+H]^−, 5%), 180 (5%), 317 (5%), 339 (7%).

Method 2

LiOH (67 mg, 1.59 mmol) was added to a mixture of ethyl 2-(5-sulfanylidene-1,4-dihydro-1,2,4-triazol-3-yl)acetate (100 mg, 0.53 mmol), THF (3.31 ml) and water (1 ml) and the reaction mixture was stirred for 1 hour at RT. The mixture was purified using a resin (Dowex 1 × 2 chloride form) column that was prepared to have hydroxide ions. The column was eluted first with formic acid in methanol pH~ 3 followed by 0.1 M HCl in MeOH. The collected fraction was evaporated using a lyophiliser to give the title compound as a yellow powder (90 mg, 100%).

**N-Cyclohexyl-2-(5-sulfanylidene-4,5-dihydro-1H-1,2,4-triazol-3-yl)acetamide (UNIS00092)**

![Chemical Structure](image)

Ethyl 2-(5-sulfanylidene-1,4-dihydro-1,2,4-triazol-3-yl)acetate (100 mg) was added into a mixture of NaOMe (57.70 mg, 1.06 mmol), cyclohexylamine (137 mg, 1.38 mmol) in THF (2 ml). The reaction mixture was stirred for 24 h at 50 °C. Saturated NH₄Cl was added to the solution, followed by 2 M HCl until the mixture was pH 2 (pH paper). The acidified solution was then extracted with DCM/MeOH [9:1] (3 × 7 mL). The organic layer was dried over MgSO₄, filtered and evaporated. Flash silica
column chromatography (DCM /EtOAc (6:4)) gave the title compound as a white powder (5 mg, 3%). m.p. = 230 °C ; IR (cm⁻¹) 3304 (N-H, stretch), 2929, 2852 (C-H, stretch), 1652 (C=O, stretch), 1548, 1350 (C=S), 1334, 1038, 810; δH (500 MHz; CD₃OD ) 5.48 (2H, s, -CH₂-CO-), 3.64 (1H, tt, J 10.7, 3.8 Hz, 1-H), 1.87 (2H, m, Cy), 1.75 (2H, m, Cy), 1.63 (1H, m, Cy), 1.39-1.16 (5H, m, Cy); δC (126 MHz; CD₃OD ) 167.87 (5-C), 167.68 (-CO-), 149.37 (3-C), 54.80 (1 -C), 50.14 (-CH₂-CO-), 33.64 (2,6-C), 26.61 (4-C), 26 (3,5-C); LCMS m/z (ESI-) 125 (78%), 174 (45%), 207 (60%), 239 [M-H]- (20%).

8.3 AAG Inhibitor biochemical assay

General

All surface-bound substrate DNA oligomer bioassays for testing the potency of inhibitors against AAG were carried out in 96-well Thermo Scientific Nunc Immobilizer Amino plates (Thermo Fisher Scientific, Loughborough, UK). Each plate used for testing inhibitors included a simple standard curve assay, where the AAG enzyme was tested in the absence of inhibitors, at varying concentrations, for its natural activity and to verify the A concentration of AAG was being used with inhibitors that was on a linear point of the standard curve and thus would provide a discernable readout.

Materials and buffers

Oligonucleotides were purchased from Integrated DNA Technolgies, Leuven, Belgium and were supplied lyophilized and they were resuspended in ultrapure (milliQ) water to a concentration of 1 mM then diluted to 10 μM solutions in the appropriate buffer. T4 DNA Ligase was purchased from Promega (Southampton, UK). It was supplied in the following storage buffer: 10mM Tris-HCl (pH 7.4), 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% glycerol. AAG enzyme (10,000 U/mL), in which U represents unit of the enzyme and is defined as the amount of enzyme
that catalyses the conversion of 1 μmol of substrate per minute, was supplied by New England Biolabs, Hitchin, UK. This enzyme was supplied in the following storage buffer: 10 mM Tris-HCl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.5% Tween® 20, 0.5% NP-40. The goat anti-fluorescein horseradish peroxidase conjugate was from Abcam, Cambridge, UK. It was supplied at a concentration of 1 mg/ml in a buffer consisting of 0.42% Potassium phosphate, 0.87% Sodium chloride, 1% BSA (pH 7.2) containing 0.1% Gentamicin sulphate. It was diluted 10-fold into PBST containing 1% w/v BSA and stored as single use aliquots at -20°C. 3,3’,5,5’-Tetramethylbenzidine (TMB) peroxidase substrate and peroxidase substrate solution B from were purchased from Insight Biotechnology Ltd (Wembley, UK).

Bicarbonate buffer was prepared from 0.18 g of NaHCO₃ and 0.04 g of Na₂CO₃ in 50 ml milliQ water (pH= 9.6). Phosphate buffered saline with 0.1% v/v Tween® 20 (PBST) was prepared from 1 ml of Tween® 20 (Sigma-Aldrich) in 1 L of milliQ water that had 10 tablets of PBS (Sigma-Aldrich) dissolved in it. AAG glycosylase buffer was made from 4 ml of 1 M Tris (Sigma-Aldrich) (pH 7.8), 20 ml of 1 M of KCl, 4 ml of 0.25 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) (pH 8), 400 μl of 500 mM of egtaacidic acid (EGTA) (Sigma-Aldrich) and 69.6 μl of β-mercaptoethanol (Sigma-Aldrich) in 171.5 ml of milliQ water. T₄ ligase buffer comprised 6 ml of 1 M Tris (pH 7.8), 6 ml of 1 M NaCl, and 2 ml of 1 M of MgCl₂ in 186 ml of milliQ water. T₄ DNA Ligase solution was prepared by dissolving 4 ul of 3U/ul T₄ DNA ligase in 12 ml buffer freshly prepared by adding 120 μl of 1 M DTT (Sigma-Aldrich) and 24 μl of 0.5 M ATP (Sigma-Aldrich) to hybridisation buffer (30 ml of 20 X SSC buffer (Sigma-Aldrich), 1 ml of 500 mM EDTA, and 100 ul of Tween® 20 in 68.9 ml milliQ water). Alkaline denaturation buffer comprised 10 ml of 5 M of NaOH and 2.5 ml of 20 X SSC buffer in 487.5 ml milliQ water, AAG buffer for LCMS comprised 8 ml of 0.5 M HEPES (Sigma-Aldrich) (pH 7.8), 20 ml of 1 M of KCl, 4 ml of 0.25 M EDTA (Sigma-Aldrich) (pH 8), and 250 mg of TCEP (Sigma-Aldrich) in 168 ml milliQ water.
Representative procedure of AAG surface-bound fluorescein-linked duplex DNA oligonucleotide substrate built from three smaller oligonucleotides: INC01, HX01 and REP04

The oligonucleotide complex used for the assay consisted of three oligonucleotides referred to as INC01 (sequence from 5’ to 3’: [OH] CTA CAC CAC ATA CAC-NH₂), HX01 ([P] AAC ACH GTC CAC TCA CCT ACT CG [Fluorescein]) and REP04 (lower case letters represent phosphorothioate-linked nucleotides: ga TGT GGT GTA TGT GTT GTG TCA GGT GAG TGG ATG A gc). 2.5 pmol/100 µl of INC01 was prepared in Bicarbonate buffer (pH 8.5) and added to the surface-activated plate and incubated at RT for 2 h or at 4 °C overnight. The liquid was decanted from the wells and the plate was washed (3 x 100 µl/well) with PBST.

The oligonucleotide HX01 and REP04 were hybridised previously to form the duplex and prepared at a concentration of 10 uM. For the hybridisation and ligation of this duplex with INC01, 30 µl of 10 uM HX01/REP04 duplex was diluted into 12 ml of T4 DNA Ligase solution, to give a 2.5 pmol/100 ul of pre-hybridized complex. 100 µl of this was added to each well. The plate was sealed and incubated at 30 °C for 2 h. The liquid was decanted from the wells and the wells were washed with PBST (3 x 100 µl/well). Increasing concentrations of AAG enzyme from 0 U/well to 0.1 U/well were prepared by diluting the stock solution of the AAG enzyme into 2% DMSO in glycosylase buffer or just glycosylase buffer (depending on the experiment) and all dilutions were kept in ice or at RT (depending on the experiment). These solutions were then added into the wells (100 µl/well) and the plate was incubated at 37 °C for 1-2 h. The liquid was decanted from the wells and the wells were then washed with PBST (3 x 100 µl/well). 125 µl of alkaline denaturation buffer was added to each well and the plates were sealed and incubated at 75 °C for 30 minutes. 100 ul of the solution in each well was transferred to the corresponding well on a standard black plate and a further 100 µl of NaOH solution was added to each well which was then also transferred into
the black plate to give 225 µl in each black plate well. The eluted fluorescence signals were detected using plate reader.

**Representative procedure of AAG surface-bound fluorescein-linked hairpin loop DNA oligonucleotide substrate built from oligonucleotides HX02 and Loop01**

The synthetic oligonucleotide used in this assay bore a 3’-NH₂ group, a 5’-fluorescein moiety, a hairpin loop, and an internal hypoxanthine as the ‘damaged base’ AAG substrate. It was prepared *in situ* by hybridisation between the two oligonucleotides named HX02 and Loop01 whose sequences (from 5’ to 3’) are as follows:

HX02: [P] CAC GAA HCA ACT CAG CAA CTC C tt [Amc7T]-NH₂

Loop01: [Fluorescein] tt GGA GTT GCT GAG TTG ATT CGT GAG CAC CAA CCG GTG CT [OH]

A 1 nM solution of oligonucleotide HX02 was prepared in bicarbonate buffer (pH~9.6) and was added to the Nunc Immobilizer Amino plate (0.1 pmol, 100 µl/well) which was incubated 2 h at RT or at 4 °C overnight. The liquid was then decanted from the wells and the plate was washed with PBST (3 × 100 µl/well). A solution of oligonucleotide Loop01 was prepared by diluting 10 uM stock solution into the hybridisation buffer to give 1 nM. This solution was then added into the wells (100 µl/well) and the plate was heated to 95 °C and then slowly cooled to RT to promote annealing of the DNA strands. The liquid was decanted from the wells and the plate was then washed with PBST (3 × 100 µl/well). T4 DNA ligase (3 U/µl, 2.6 µl) solution was then added into the wells (100 µl/well). The plate was incubated at 37 °C for 1 h. The liquid was decanted from the wells and the plate was then washed with PBST (3 × 100 µl/well). Increasing concentrations of AAG enzyme from 0 U/well to 0.1 U/well were prepared by diluting the stock solution of the AAG enzyme into 0 or 2% DMSO in glycosylase buffer (depending on
experiment) and all dilutions were kept in ice or RT (depending on experiment). These solutions were then added to the wells (100 µl/well) and the plate was incubated at 37 °C after adding the enzyme for 1 h. The liquid was decanted from the wells and the wells were then washed with PBST (3 × 100 µl/well). 125 µl of alkaline denaturation buffer was added to each well and the plates were sealed and incubated at 75 °C for 30 minutes. The liquid was decanted from the wells and the plate was then washed with PBST (3 × 100 µl/well). 1% v/v BSA in PBST was prepared by dissolving 100 mg BSA in 10 ml PBST. To this was added 10 ul of 1:10 diluted goat anti-fluorescein HRP conjugate. This solution was then added into the wells (100 µl/well) and the plate was incubated at RT for 1 h. The liquid was decanted and the plate was then washed with PBST (3 × 100 µl/well). To each well was added a mixture of an equal volume of (TMB) peroxidase substrate and peroxidase substrate solution B (100 µl/well). After the development of sufficient colour (as judged by gradual decrease in blue colour with increasing AAG enzyme concentration in the well) the reaction was stopped by adding phosphoric acid (concentration, volume) to give a yellow colour. Absorbance at 450 nm was then read in the plate reader.
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