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Solid-Phase Synthesis of $s$-Tetrazines: Method Development and Applications in Chemical Biology

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Doctorate of Philosophy
University of Edinburgh
2024
Lay Abstract

In the realm of modern chemistry, a field known as "bioorthogonal chemistry" has sparked much intrigue (as well as a Nobel Prize). This offers ways to tag molecules within living organisms, all without disrupting the delicate balance of natural biological function. At the forefront of this lies tetrazine, inverse demand Diels-Alder chemistry, renowned for its rapid and biocompatible nature. To date, this has been used for diverse applications ranging from medical imaging to drug delivery.

Despite their potential, the routine synthesis of s-tetrazines can pose significant challenges. Traditional solution-phase routes often suffer from laborious synthesis, yielding mixtures of products and necessitating multiple purification steps. Herein, a new approach, based on solid-phase methods, was developed to efficiently produce s-tetrazines in high yields without needing expensive metals or harsh conditions. This method opens doors for the rapid and versatile production of s-tetrazines, facilitating their broader use in bioorthogonal chemistry.

The application of chemical modification of biomolecules for imaging purposes is crucial for advancing our understanding of biological processes and diseases. Here, a small library of peptide-based probes was developed for the labelling of mycobacteria, including Mtb. These were labelled with fluorophores spanning the wavelength range from green to NIR-I, as targeted imaging agents, offering valuable insights into mycobacterial infections and holding promising implications for future research.
Abstract

Since the emergence of bioorthogonal chemistry in the 20th century, s-tetrazines stand out from the bioorthogonal toolbox due to their superior reaction kinetics over azide/alkyne chemistries, and high chemical selectivities. Specifically, s-tetrazines selectively undergo inverse electron-demand Diels-Alder reactions with dienophiles within complex biological systems, which has allowed for their use in various biological scenarios such as in sensing, imaging and drug delivery. However, despite the growing applications of tetrazine bioorthogonal chemistry, their development has been hampered due to a lack of practical approaches that facilitate the emergence of new applications.

In this thesis, an efficient and high-yielding solid-phase route to s-tetrazines was successfully developed without the need for metal catalysts or harsh reaction conditions. This method provides a versatile route to the synthesis, under mild conditions, of unsymmetrical aryl and alkyl tetrazines, as well as more reactive monosubstituted tetrazines, with possible applications in bioorthogonal chemistry.

To explore the bioorthogonal activation of tetrazines and of mycobacteria labelling, a small library of fluorescent probes was initially developed based on the mycobacterial membrane-associated disruption peptide (MAD1), with evaluation of both enantiomers of the MAD1 peptides, decorated with four different fluorophores (ranging from green to far-red), including solvato-fluorogenic dyes.
Fluorescent labelling on *M. smegmatis*, showed robust and stable labelling. Two of the D-probes (5-CF-D-MAD1 and NBD-D-MAD1) successfully labelled *Mtb*. A norbornene-labelled MAD1 peptide was then synthesised, which was used to bind *M. smegmatis*, and trigger the activation of the quenched fluorophore (BODIPY-Tz). This process facilitated a 61-fold amplification of the fluorescent signal upon bioorthogonal activation, allowing efficient free-wash labelling.
Declaration of Authorship

The research detailed within this thesis has been performed by the author in the duration of the PhD studentship between the dates of May 2019 and February 2024 under the supervision of Professor Mark Bradley and Dr. Annamaria Lilienkampf, School of Chemistry, University of Edinburgh. The work, data, and interpretation presented here are those of the author unless there was significant collaborative made, in which case it has been clearly recognised. References have been provided to all supporting literature and resources, including the published work of others. This work has not been submitted for any other degrees or professional qualifications.

Parts of the work presented herein have been published or submitted as:


Signed:

Zainab Saeed A. Alghamdi
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BODIPY</td>
<td>Boron-dipyrromethene</td>
</tr>
<tr>
<td>CF</td>
<td>Carboxy Fluorescin</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Cu(I)-Catalysed Azide–Alkyne Cycloaddition</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N-Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDG</td>
<td>Electron donating group</td>
</tr>
<tr>
<td>ELSD</td>
<td>Evaporative light scattering detector</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EWG</td>
<td>Electron withdrawing group</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
</tr>
<tr>
<td>gem</td>
<td>Geminal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest Occupied Molecular Orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HSPyU</td>
<td>Dipyrrolidino(N-succinimidyl)carbenium hexafluorophosphate</td>
</tr>
<tr>
<td>IEDDA</td>
<td>Inverse-electron-demand Diels–Alder reaction</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Second order rate constant</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest Occupied Molecular Orbital</td>
</tr>
<tr>
<td>Mero</td>
<td>Merocyanine</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>µW</td>
<td>Microwaves</td>
</tr>
<tr>
<td>NBD</td>
<td>Nitrobenzoxadiazole</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared Region</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>Oxyma</td>
<td>Ethyl (hydroxyimino)cyanoacetate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced Electron Transfer</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>iPrOH</td>
<td>iso-Propanol</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescent unit</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse-phase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SPAAC</td>
<td>Strain-Promoted Alkyne–Azide Cycloaddition</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid-Phase Peptide Synthesis</td>
</tr>
<tr>
<td>Sulf</td>
<td>Sulfonated</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBET</td>
<td>Through Bond Energy Transfer</td>
</tr>
<tr>
<td>TCO</td>
<td>trans-Cyclooctene</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>t&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Retention time</td>
</tr>
<tr>
<td>Tz</td>
<td>Tetrazine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible spectroscopy</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1 Tetrazines

Tetrazines are small aromatic heterocycles with a benzene-like structure but with the highest permissible nitrogen-to-carbon proportion within a single ring. There are three isomeric tetrazine forms based on the locations of the four nitrogen atoms (Figure 1), namely 1,2,3,4-tetrazine, 1,2,3,5-tetrazine and 1,2,4,5-tetrazine (the so-called symmetric or s-tetrazines).\(^1\)

![Figure 1.1. Isomeric forms of tetrazines.](image)

Among these isomers, the symmetrical s-tetrazines (1,2,4,5-tetrazines) are the most extensively studied, owing to their higher stability, rich chemistry, and peculiar reactivities stemming from their reduced aromaticity stabilisation (E ≈ 14-15 kcal/mol) compared to benzene (33 kcal/mol).\(^1^2\) The established structure for s-tetrazine, with two degenerate Kekulé structures, was discerned by Hantzsch in 1900 (Figure 1.2).\(^3\)

![Figure 1.2. Kekulé structures for s-tetrazines.](image)
1.2 Inverse-electron-demand Diels–Alder reactions of s-tetrazines

The development of s-tetrazine chemistry has seen significant progress since its discovery by Pinner in the late 19th century. However, it wasn't until the 1950s that a fuller understanding of their chemical and physical properties was obtained, with Carboni showing their outstanding reactivity as dienes with strained alkenes and alkynes, offering an attractive route to the synthesis of pyridazines.

The Carboni reaction was further developed by Sauer and Boger to encompass a wide range of s-tetrazines and strained ring systems, ultimately earning the designation of the inverse-electron-demand Diels–Alder reactions, or so-called IEDDA reactions. Since then, the IEDDA chemistry of s-tetrazines has been widely utilised across various domains, including organic synthesis, natural products, high-energy materials, sensors, and coordination chemistry. However, it was not until approximately three decades later that Fox and Hilderbrand independently discovered the potential of this chemistry as a new bioorthogonal reaction. The rapid reactivity of s-tetrazines, their nontoxic by-products (N₂), inertness to biological media, and high reaction rate (10⁷ M⁻¹ s⁻¹) compared to other bioorthogonal reactions make the s-tetrazines the perfect tool for in vivo applications. A decade ago, Robillard uncovered the ability of s-tetrazines to facilitate "click-to-release" strategies, with superior efficacy compared to traditional methods. Notably, a bioorthogonally triggered drug release based on an IEDDA reaction, prompted the initiation of phase I clinical studies in 2020.
The IEDDA reaction is a [4+2] cycloaddition between an electron-poor diene “tetrazine” and an electron-rich dienophile, typically unsaturated compounds (alkene or alkyne), resulting in the formation of a highly strained bicyclic intermediate. Subsequently, this undergoes a retro [4+2] cycloaddition, eliminating nitrogen to yield a 4,5-dihydro pyridazine, that can isomerise to a 1,4-dihydropyridazine and then oxidise to a pyridazine (Scheme 1.1). The kinetics of this final, irreversible reaction, are influenced by various factors, including electronics, ring strain, stereochemistry, and solvent effects.

Scheme 1.1. IEDDA reaction between a s-tetrazine and an alkene.

Based on frontier molecular orbital theory, the overall reactivity of IEDDA cycloaddition is determined by the interaction between the highest occupied molecular orbital of the dienophile (HOMO$_{dienophile}$) and the lowest unoccupied molecular orbital of the diene (LUMO$_{diene}$). In an IEDDA reaction, the LUMO$_{diene}$ and the HOMO$_{dienophile}$ are closer in...
energy than the traditional $\text{HOMO}_{\text{dienec}}$ and $\text{LUMO}_{\text{dienophile}}$ pair of a conventional Diels-Alder reaction, resulting in a strong interaction and rapid bond formation.\textsuperscript{23}

The electronic and steric effects of substituents on both the diene and the dienophile in the IEDDA were extensively studied by Boger\textsuperscript{9,24} and Sauer\textsuperscript{7,10} through the comparative analysis of various 3,6-disubstituted $s$-tetrazines. The investigations revealed the introduction of electron-withdrawing groups on the diene to lower the $\text{LUMO}_{\text{dienec}}$ energy (\textit{e.g.}, carboxyl, pyrimidyl, and pyridinyl) and electron-donating groups on the dienophile to increase the $\text{HOMO}_{\text{dienophile}}$ energy (\textit{e.g.}, methyl and methoxy), leading to a faster reaction rate by reducing the $\text{HOMO}_{\text{dienophile}}$–$\text{LUMO}_{\text{dienec}}$ energy gap (Figure 1.3A).\textsuperscript{22,23} However, it is important to bear in mind that the potential enhancement of reaction rates by electron-withdrawing groups on $s$-tetrazines is accompanied by the need to maintain a delicate balance between reactivity and stability, considering that several $s$-tetrazines bearing strong electron-withdrawing groups are unstable under aqueous conditions (Figure 1.3B).\textsuperscript{15,18-25}

Recently, it has also been found that the lone-pair repulsion between the core nitrogen atoms ($s$-tetrazine) and heteroatoms in substituents results in a repulsive force influencing tetrazine reactivity.\textsuperscript{26} Closer proximity of heteroatoms to nitrogen increases tetrazine reactivity inductively without suffering from stability limitations with this effect. (Figure 1.3C).
Figure 1.3. A. Electronic effects of substituents on the Diels–Alder reaction. (EDG: electron-donating group, EWG: electron-withdrawing group, HOMO: highest occupied molecular orbital, LUMO: lowest unoccupied molecular orbital energy, ΔE: energy gap). A tetrazine is an extreme example of an electron deficient diene (low LUMO), while a strained alkene has a higher energy HOMO than a similar unstrained alkene. B. Stability to aqueous environment and reactivity in IEDDA chemistry of some common substituted s-tetrazines. C. Intramolecular repulsion effects on IEDDA reactivity of s-tetrazine, with 2-pyridyl and vinyl ether substituents.¹⁵,¹⁸,²⁶
Ring strain exerts another significant influence on accelerating the IEDDA reaction. Sauer and his co-workers found that strained rings of dienophiles showed higher reactivity.\textsuperscript{27} Among them, cyclopropene exhibited the highest reaction rate, followed by cyclobutene, cyclopentene, cyclohexene, and cis-cyclooctene (cis-CO). The highly strained \textit{trans}-cyclooctene (TCO) demonstrated remarkable reactivity with several \textit{s}-tetrazines having second order rate constants of up to $10^6$ M$^{-1}$ s$^{-1}$. This is attributed to its 'crown' conformation,\textsuperscript{28,29} wherein the double bond is twisted, generating a significantly strained system with a high-energy HOMO,\textsuperscript{30} and increasing its reactivity some 7 orders of magnitude higher than the 'half-chair' conformation of the \textit{cis}-isomer (Figure 1.4).\textsuperscript{15} Despite the high reactivity of TOC, it is less stable, exhibiting rapid isomerisation towards the much less reactive \textit{cis}-isomer in biological environments such as serum-containing media with a half-life of approximately 1 hour.\textsuperscript{31} The stereochemistry of strained dienophiles also impacts the rate of the IEDDA reaction, for instance, the axial isomer of functionalised TCO (TCO-OR) can react with \textit{s}-tetrazines ($k_2 = 8.0 \times 10^4$ M$^{-1}$ s$^{-1}$) approximately four times faster than the equatorial isomer ($k_2 = 2.3 \times 10^4$ M$^{-1}$ s$^{-1}$).\textsuperscript{31,32} Similarly, \textit{exo}-norbornene reacts with \textit{s}-tetrazines roughly three times faster than the \textit{endo}-isomer (Figure 1.4).\textsuperscript{33}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_4.png}
\caption{Examples of stereoisomeric influence on dienophiles reactivities in IEDDA chemistry.\textsuperscript{15,32,33}}
\end{figure}
Sterics on the s-tetrazine have also been found to influence the cycloaddition rate, with monosubstituted s-tetrazines generally exhibiting faster reactivity compared to disubstituted s-tetrazines, even those with electron-withdrawing groups.\textsuperscript{34-37}

Protic solvents have also been found to accelerate IEDDA reactions.\textsuperscript{38,39} and it is believed that water can enhance the hydrophobic–hydrophobic interactions between the s-tetrazine and the dienophile during the reaction leading to locally enhanced effective concentrations accelerating the rate of the reaction ($k_2 = 2000 \text{ in } 10\% \text{ H}_2\text{O/MeOH} \text{ and } k_2 = 1400 \text{ M}^{-1} \text{ s}^{-1} \text{ in MeOH}$).\textsuperscript{32} Additionally, protic solvents can facilitate hydrogen bonding with tetrazines, reducing the LUMO energy, similar to the effect of EWGs and thereby increasing the reaction rate.\textsuperscript{39}

### 1.3 Synthesis of s-tetrazines

Pinner was the first to synthesise s-tetrazines in 1890 based on the reaction between ethyl benzimidate hydrochloride "imidoester" with hydrazine attacking the imidoester, forming the amidrazone, which subsequently reacts with another equivalent of the imidoester to yield the 1,2- or 1,4-dihydrotetrazines. These were then oxidised to give the s-tetrazine (Scheme 1.2A).\textsuperscript{4} The synthetic route was improved by using aryl nitriles as precursors instead of the imidoester (Scheme 1.2B).\textsuperscript{40} To date, the Pinner synthesis stands as the most common approach for obtaining symmetrical s-tetrazines, albeit with some limitations. The reported yields vary between 5\% and 85\%, underscoring the substantial impact of core substituents on reaction outcomes. Indeed, this synthetic
method is effective with aryl nitriles but fails with alkyl nitriles. Additionally, the method is inherently unsuitable for synthesising unsymmetrical s-tetrazines, often requiring the use of a significant excess of one nitrile, resulting in low yields and complex product mixtures.

Scheme 1.2. The first synthetic route of s-tetrazines, originating from imidoesters (A) or nitriles (B) with hydrazine.\textsuperscript{440}
In 1900, Hantzsch unwittingly generated 3,6-tetrazinedicarboxylic acid and the unsubstituted s-tetrazine via the base mediated dimerization of ethyl diazoacetate to give the dihydro-s-tetrazine that was then neutralised, oxidised, and subsequently decarboxylated to give s-tetrazine with very low yield ≥ 2% yield (Scheme 1.3). Subsequent studies by Curtius, Wood, Spencer, and others improved the yield to almost 17%.

![Scheme 1.3. The low yielding Hantzsch pathway to s-tetrazines.](image)

Stollé introduced a substituent-independent method, further expanded by others, for synthesising both symmetrical and unsymmetrical alkyl s-tetrazines (Scheme 1.4). This method also enabled the production of s-tetrazines bearing strong electron-withdrawing groups ‘for more reactive tetrazines’ or bulky groups, which are usually difficult to obtain using the classical Pinner method via acyl chloride precursors. The method entailed synthesising acyl hydrazides, followed by their activation with phosphorus pentachloride (PCl₅) and condensation with hydrazine to afford dihydro-s-tetrazines, which were oxidised to give s-tetrazines in good to moderate yields (45–75%).
Microwave irradiation significantly enhanced the yield of the condensation step by up to 20% and drastically reduced the reaction time to just 30 minutes.\textsuperscript{47}

\begin{align*}
\text{R}^1\text{OCl} + \text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O} \rightarrow \text{R}^1\text{N} \cdot \text{O} \cdot \text{R}^2
\end{align*}

**Scheme 1.4.** Stollé’s method for the synthesis of \textit{s}-tetrazines via acyl chlorides\textsuperscript{45-47}

An alternative approach to prepare \textit{s}-tetrazines using \textit{gem}-difluoroalkenes as the starting material was originally reported by Carboni\textsuperscript{,48} but with low yields and further improved by others to access both symmetrical and unsymmetrical \textit{s}-tetrazines in high yields (60–90\%).\textsuperscript{49} The reaction between \textit{gem}-difluoroalkenes and hydrazine generated very reactive fluorohydrazone species that self-condensed in air to form the disubstituted \textit{s}-tetrazines (Scheme 1.5).
Scheme 1.5. Synthesis of s-tetrazines starting from gem-difluoroalkenes.\textsuperscript{49}

Over the years, modifications to the Pinner reaction have been explored in an effort to improve its efficiency and applicability. These investigations have led to the development of several Pinner-like reactions.\textsuperscript{50-52} Wiley, carried out the reaction under anhydrous conditions and obtained some 3,6-diaryl-s-tetrazines in 50–70% yields.\textsuperscript{53} In 1968, a significant modification was reported by Tolba with the incorporation of sulphur as a catalyst to give both symmetric and asymmetric s-tetrazines (Scheme 1.6).\textsuperscript{54} It was found that the presence of sulphur in the reaction improved the yields up to 80%. Sulphur (S\textsubscript{8}) has been proposed to undergo a reaction with hydrazine, forming the active nucleophile H\textsubscript{2}N-NH-SH, which then reacts with a nitrile to yield the corresponding amidrazone. Upon condensation with a second nitrile, a reactive intermediate is generated, leading to the elimination of S\textsubscript{8} and subsequent transformation into the dihydro-s-tetrazine through electrocyclic rearrangement and oxidation to give the desired 3,6-disubstituted s-tetrazines.
tetrazine. However, this improved method still did not give alkyl tetrazines in good yields (< 20%) and remained inadequate for more sterically hindered nitriles.

\[
\begin{align*}
\text{NH}_2\text{NH}_2 + S_8 & \xrightarrow{\text{EtOH, 75 °C}} \text{NH}_2\text{NHSH} \\
\text{NC-R}^1 & \xrightarrow{\text{NC-R}^2} \text{HN-NH} \\
\text{N-N} & \xrightarrow{\text{Oxidation, NaNO}_2, 1 \text{ M HCl}} \text{N-N} \\
\text{N-N} & \xrightarrow{} \text{N-N} \\
\end{align*}
\]

**Substrate Scope:**

An additional strategy involving the substitution of nitriles with aldehydes was reported by Skorianetz.\textsuperscript{55,56} The reaction between alkyl aldehydes and hydrazine gave the hexahydro-\(s\)-tetrazine which, was then oxidised to \(s\)-tetrazine in two steps, albeit in a low yield (Scheme 1.7). Although the method was designed to offer branched substituents and mixed alkyl \(s\)-tetrazines, the compounds were highly volatile and prone to sublimation at room temperature, posing challenges in purification.\textsuperscript{56}
Scheme 1.7. Preparation of 3,6-dialky1 s-tetrazines using aldehydes and hydrazine.\textsuperscript{55,56}

Later, Johnson\textsuperscript{57} and Erickson\textsuperscript{58} developed an alternative approach for synthesising unsymmetrical alkyl and aryl s-tetrazines using the S-methylisothiocarbonohydrazide salt (Scheme 1.8). The salt was reacted with several carboxylic acid derivatives (e.g., orthoesters, dithiobenzoate esters, and amide acetal) to form the 6-alkyl-3-(methylthio)-s-tetrazines, which were converted through aromatic nucleophilic substitution (SNAr) chemistry with several N- and O-nucleophiles to 6-alkyl-3-aminotetrazines in high yields (70–90%).

Scheme 1.8. Synthesis of unsymmetrical s-tetrazines using S-methylisothiocarbonohydrazide salt as starting material.\textsuperscript{57,58}

In the early 1990s, Hiskey\textsuperscript{59} developed a synthetic pathway based on the initial work reported by Scott,\textsuperscript{60} facilitating the preparation of a variety of novel symmetric and unsymmetric s-tetrazines with 80–85\% yield (Scheme 1.9).
The bis(dimethyl-pyrazolyl)-s-tetrazine was generated from inexpensive and readily available starting materials (e.g., guanidine, hydrazine, and 2,4-pentanedione). The pyrazolyl groups then serve as soft leaving groups, enabling the introduction of a wide range of heterocyclic substituents through $S_N$Ar onto the tetrazine ring.

Since then, the synthesis of both unsymmetrical and symmetrical s-tetrazines through $S_N$Ar reaction of suitable tetrazine precursors has garnered significant interest and found widespread application. Indeed, the electron-deficient nature of tetrazines bearing appropriate leaving groups renders them highly suitable substrates for nucleophilic attack. To this end, Hiskey\cite{61} and others later expanded the synthetic method to include more efficient leaving group by converting bis(hydrazino)-s-tetrazine to dichloro-s-tetrazine using chlorine gas or trichloroisocyanuric acid.\cite{61-63} Due to its stronger electron affinity, dichloro-s-tetrazine more readily undergoes $S_N$Ar reactions as a precursor with a variety of nucleophiles, such as $NH_3$, morpholine, pyrrolidine, hydrazine, alcohols, mercaptans and carbanions, offering a wide range of s-tetrazines (Figure 1.5).
Figure 1.5. Examples of disubstituted s-tetrazines reported via S$_{N}$Ar chemistry using 3,6-dichloro-s-tetrazine as a precursor.61-63

In 2012, Devaraj reported a metal-catalysed approach to symmetrical and unsymmetrical s-tetrazines directly from nitriles and hydrazine (Table 1.1).64 It was proposed that the metal acts as a Lewis acid and coordinates to the nitrile promoting the nucleophilic addition by hydrazine. The investigation commenced by exploring the reaction of benzyl cyanide with neat hydrazine to screen a range of 24 Lewis acid catalysts at a 5 mol% loading. It was found that the addition of 5 mol% Ni(OTf)$_2$ led to a near-quantitative yield (95%) of 3,6-dibenzyl-s-tetrazine, and also Zn(OTf)$_2$ gave good yields (70%). These two catalysts were then tested for the synthesis of several asymmetric and symmetric s-tetrazines.
Table 1.1. Screening of 24 Lewis acids for the synthesis of 6-dibenzyl-s-tetrazine.\(^a\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst</th>
<th>Yield</th>
<th>Entry</th>
<th>Catalyst</th>
<th>Yield</th>
<th>Entry</th>
<th>Catalyst</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>without</td>
<td>0%</td>
<td>9</td>
<td>Sc(OTf)(_3)</td>
<td>26%</td>
<td>17</td>
<td>Cu(OTf)</td>
<td>57%</td>
</tr>
<tr>
<td>2</td>
<td>Ni(acac)(_2)</td>
<td>10%</td>
<td>10</td>
<td>Yt(OTf)(_3)</td>
<td>31%</td>
<td>18</td>
<td>Cu(OAc)(_2)</td>
<td>59%</td>
</tr>
<tr>
<td>3</td>
<td>Cu(OTf)(_2)</td>
<td>11%</td>
<td>11</td>
<td>Zn(OAc)(_2)</td>
<td>38%</td>
<td>19</td>
<td>MgCl(_2)</td>
<td>63%</td>
</tr>
<tr>
<td>4</td>
<td>ZnCl(_2)</td>
<td>11%</td>
<td>12</td>
<td>CuBr</td>
<td>42%</td>
<td>20</td>
<td>ZnI(_2)</td>
<td>68%</td>
</tr>
<tr>
<td>5</td>
<td>CuCl</td>
<td>12%</td>
<td>13</td>
<td>ZnBr(_2)</td>
<td>46%</td>
<td>21</td>
<td>Zn(OTf)(_2)</td>
<td>70%</td>
</tr>
<tr>
<td>6</td>
<td>CoCl(_2)· 6 H(_2)O</td>
<td>13%</td>
<td>14</td>
<td>Cul</td>
<td>50%</td>
<td>22</td>
<td>NiCl(_2)</td>
<td>73%</td>
</tr>
<tr>
<td>7</td>
<td>MgBr(_2)</td>
<td>15%</td>
<td>15</td>
<td>CuOAc</td>
<td>53%</td>
<td>23</td>
<td>Nil(_2)</td>
<td>93%</td>
</tr>
<tr>
<td>8</td>
<td>CuBr(_2)</td>
<td>23%</td>
<td>16</td>
<td>MnBr(_2)</td>
<td>55%</td>
<td>24</td>
<td>Ni(OTf)(_2)</td>
<td>95%</td>
</tr>
</tbody>
</table>

\(\text{a. With } \cong 50 \text{ equiv. of } \text{NH}_2\text{NH}_2\)

Indeed, the method also enabled the synthesis of both mono- and disubstituted alkyl s-tetrazines, addressing a challenge posed by the original Pinner reaction. Zinc triflate exhibited higher efficacy with aliphatic and sterically hindered nitriles (\(~20\text{–}95\%\)). Conversely, nickel triflate demonstrated better efficiency in generating various monosubstituted s-tetrazines, particularly from aromatic nitriles and formamidine salts, resulting in significantly higher yields (\(~70\%) (Figure 1.6). While several s-tetrazines have been successfully synthesised using this methodology, its substrate scope remains limited due to the requirement of hazardous anhydrous hydrazine (\(~ 50 \text{ equivalents to} \)
the nitrile precursors), high temperature, metal catalysts and excess formamidine salts (~ 10 equivalents).

\[
\text{NC-}R^1 + \text{NC-}R^2 \xrightarrow{\text{i. NH}_2\text{NH}_2, 60 ^\circ\text{C, 24 h}} \xrightarrow{\text{ii. NaNO}_2, 1 \text{ M HCl}} R^1, R^2\text{ alkyl or aryl}
\]

\[
\text{NC-}R + \text{NH}_2\text{NH}_2 \xrightarrow{\text{i. NH}_2\text{NH}_2, 60 ^\circ\text{C, 24 h}} \xrightarrow{\text{ii. NaNO}_2, 1 \text{ M HCl}} R\text{ aryl}
\]

**Substrate Scope:**

![Substrate Scope Image]

Figure 1.6. Synthesis of mono- and disubstituted s-tetrazines using a metal-catalysed approach.\(^{64}\)

In 2016, a non-metal-catalysed based Pinner synthesis was developed by Melguizo to access dialkyl s-tetrazines derivatives (Scheme 1.10).\(^{65}\) In their approach, \(N\)-acetylcysteine was used as the catalyst with hydrazine hydrate. Dialkyl s-tetrazines were successfully obtained in 35–40% yields, although with limited number of examples. It was proposed that \(N\)-acetylcysteine could act as a catalyst promoting the formation of an amidrazone intermediate from the nitrile precursor and hydrazine, which after
dimerization leads to the generation of dihydro-s-tetrazine which oxidised to the alkyl s-tetrazine.

**Scheme 1.10.** Synthesis of dialkyl s-tetrazines using N-acetylcysteine (NAC) catalyst.\(^{65}\)

Wu reported an efficient synthesis of s-tetrazines using thiol-containing catalysts,\(^ {66}\) inspired by the reversible interactions observed between nitriles and thiols (Scheme 1.11).\(^ {67}\) It was proposed that thiols promoted the reaction by forming a thioimidate ester, which subsequently undergoes attack by hydrazine, leading to the regeneration of the thiol and the formation of an amidrazone. This compound then reacts with another equivalent of the thioimidate ester to yield, after oxidation, s-tetrazines bearing various functional groups (e.g., carboxylic acid, amino, and alkyl) in 34–75% yield. (Figure 1.7).

**Scheme 1.11.** Synthesis unsymmetrical s-tetrazines using thiol catalysts.\(^ {66}\)
CHAPTER 1

The best results were achieved using 3-mercaptopropionic acid, N-Acetyl-L-cysteine or thioglycolic acid (~70%) as a catalyst with hydrazine hydrate and ethanol as co-solvent (Table 1.2).

![Chemical Structures]

**Figure 1.7.** Examples of unsymmetrical s-tetrazines reported by using thiol promoters."66

**Table 1.2.** Screening of thiol-containing catalysts for the synthesis of s-tetrazines."66

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst (R-SH)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cysteine</td>
<td>69%</td>
</tr>
<tr>
<td>2</td>
<td>1,3-Propanedithiol</td>
<td>49%</td>
</tr>
<tr>
<td>3</td>
<td>2-Aminoethanethiol</td>
<td>46%</td>
</tr>
<tr>
<td>4</td>
<td>3-Mercaptotropionic Acid</td>
<td>77%</td>
</tr>
<tr>
<td>5</td>
<td>Thioglycolic acid</td>
<td>70%</td>
</tr>
<tr>
<td>6</td>
<td>N-Acetyl-L-Cysteine</td>
<td>71%</td>
</tr>
<tr>
<td>7</td>
<td>Glutathione</td>
<td>66%</td>
</tr>
</tbody>
</table>
Interestingly, Audebert\textsuperscript{68} observed that dichloromethane can undergo sulphur-catalysed reactions\textsuperscript{54,69} with nitriles and hydrazine hydrate to form the monosubstituted 5-tetrazines, typically generated from formamidine (Scheme 1.12). The authors conducted experiments using 13C-labeled dichloromethane to confirm its role in 5-tetrazine ring formation and found that neither dichloroethane nor dibromomethane were effective in generating tetrazines. This metal-free approach was achieved using 2 equivalents of sulphur to the aromatic nitrile precursors, and only 1 equivalent of dichloromethane under microwave irradiation to obtain several monosubstituted 5-tetrazines with para-aryl substituents (e.g., carboxylic acid, halide, alcohol, protected amine, and pyridyl) in 40–70\% yield.

\[
\text{Ar-CN} + \text{CH}_{2}\text{Cl}_2 \xrightarrow{\text{S}_8, \text{N}_2\text{H}_4\cdot\text{H}_2\text{O}, \text{EtOH}} \xrightarrow{\mu\text{W}, 50^\circ\text{C}, 24\text{h}} \xrightarrow{\text{NaNO}_2, \text{HCl}} \text{R} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \\
\text{R= alkyl,aryl}
\]

**Substrate Scope:**

\[
\begin{array}{c}
\text{BocHN} \\
\text{40\%} \\
\text{Cl} \\
\text{54\%} \\
\text{CN} \\
\text{61\%} \\
\text{OH} \\
\text{70\%} \\
\text{43\%} \\
\text{56\%} \\
\text{64\%}
\end{array}
\]

**Scheme 1.12.** Sulphur-catalysed synthesis of the of monosubstituted 5-tetrazine using DCM as a starting material.\textsuperscript{68}
Herth expanded the Audebert approach, to synthesise another array of monosubstituted aryl s-tetrazines, containing meta-substituents on the phenyl ring, from bulkier and double substituted aryl nitriles.\textsuperscript{70,71} Despite the low isolated yields (14–40%), the method exhibited tolerance towards various functional groups, including ethers, esters, amides, sulfonamides, and halides (Figure 1.8).

![Figure 1.8. Example of monosubstituted aryl tetrazes with meta-substituents obtained through the sulfur-catalysed approach.\textsuperscript{70,71}]

In 2020, Fox introduced a novel one-pot strategy for converting a broad range of aliphatic and aromatic esters into unsymmetrical 3-thiomethyl-s-tetrazines (Scheme 1.13). The oxabicyclo[2.2.2]octyl (OBO) orthoester intermediates were synthesised by treating the esters with boron trifluoride diethyl etherate.\textsuperscript{72} Subsequently, 3-thiomethyl-s-tetrazines were obtained through the condensation of the orthoester intermediates with S-methylisothiocarbonohydrazidium iodide, followed by oxidation with phenyl iodine(III) diacetate (PIDA). The reaction exhibits remarkable substrate versatility, accommodating diverse ester substrates such as alkyl groups, substituted phenyls, N-heterocycles, steroidal structures, and biotin, forming thiomethyl-s-tetrazines in 50–79%
yield. Indeed, the prospect of converting almost any carboxylic acid into a tetrazine with good yields and without using hydrazine presents promising avenues for future research. Furthermore, the methyl thioether substituent was identified as a versatile functional handle that can be replaced with ethers, amines or aryl groups via Pd-catalysed cross-coupling reactions.

Scheme 1.13. One-pot synthesis of s-tetrazine from carboxylic ester precursors.

Finally, it is important to point out that s-tetrazines are commonly synthesised via oxidation of dihydro-s-tetrazines. Although atmospheric oxygen can be sufficient as an eco-friendly alternative for oxidation, most substrates and methods have used oxidising...
agents to push full conversion. Sodium or isopentyl nitrite, typically with either hydrochloric acid or acetic acid, are commonly used.\textsuperscript{73} However, early synthetic routes often employed nitrous gases,\textsuperscript{50} while more contemporary approaches use 2,3-dichloro-5,6-dicyano-1,4-benzoquinone,\textsuperscript{15} or hydrogen peroxide.\textsuperscript{74}

Despite the huge efforts toward enhancing tetrazine synthesis methods, the quest for more efficient, simple, rapid, and direct approaches remains ongoing. The goal is to develop methods with a wide scope that can cater to various applications, particularly in bioorthogonal chemistry. While significant progress has been made, there is still room for innovation and refinement. Further advancements in \textit{s}-tetrazine synthesis hold the promise of unlocking new possibilities and expanding its role in bioorthogonal chemistry and other applications.
Chapter 2. Aims and Objectives

Solid-phase synthesis and bioorthogonal chemistry represent two cutting-edge fields, offering unique opportunities for molecule assembly and selective labelling within biological systems. Solid-phase synthesis, in many cases, can streamline synthesis processes and facilitate the generation of compounds that are difficult to obtain by traditional solution-phase methods. Bioorthogonal chemistry focuses on chemical reactions that can occur within living organisms without interfering with native biological processes.

The overarching aims of this thesis were 1) to develop a first ever synthesis of s-tetrazines via solid-phase methods; 2) to investigate a library of fluorescent probes based on the mycobacteria membrane-associated disruption peptide (MAD1); and 3) the application of an intracellular bioorthogonal labelling approach to detect *Mycobacterium*.

Efficient solid-phase synthetic methods for s-tetrazines were developed to allow versatile synthesis under mild conditions, involving the optimisation of reaction conditions to achieve high yields and purities, and enhancing access to reactive s-tetrazines for bioorthogonal chemistry applications.

For the rapid and selective detection of mycobacteria, a series of fluorescent peptide-based probes containing either L- or D-amino acids were developed and their fluorescent properties as well as their performance in labelling clinically relevant bacterial strains, including *Mtb*, were evaluated. Additionally, a bioorthogonal labelling approach was developed using a tagged MAD1 peptide and a tetrazine-quenched fluorophore, with conditions optimised for the efficient and specific detection of mycobacteria.
Chapter 3. Solid-Phase Synthesis of s-Tetrazines


3.1 Introduction

Merrifield pioneered solid-phase synthesis in 1963, and initially the area focused on the synthesis of peptides and later oligonucleotides. Since then, the application of solid-phase based strategies has proven to be a highly effective and valuable technique for the efficient construction of diverse libraries of molecules, ranging from small compounds to biopolymers such as carbohydrates and other natural products. In the 1980’s and 1990’s, the use of solid supports marked a transformative era in the multiple parallel synthesis of peptides, exemplified by Geysen’s multipin technology and Houghten’s “tea-bag” approach, setting the stage for the development of combinatorial chemistry in the subsequent decades. In 1992, Ellman reported the first example of biologically-active small-molecules, based on 1,4-benzodiazepines, via a combinatorial approach, using a polystyrene resin functionalised with a hydroxymethylphenoxy-acetic acid-based linker (HMP), starting the era of the solid-phase synthesis of libraries of small molecules (Scheme 3.1).
Scheme 3.1. Solid-phase synthesis of benzodiazepines on a solid support.\textsuperscript{83}

Indeed, the synthesis of small organic molecules using solid-phase techniques is now well-established with a vast number of examples.\textsuperscript{84-86} Solid-phase synthesis methodologies have allowed the generation of combinatorial libraries of pharmacologically active scaffolds including indoles,\textsuperscript{87} coumarins,\textsuperscript{88} oxazoles,\textsuperscript{89} triazoles,\textsuperscript{90} piperazines,\textsuperscript{91} pyrimidines,\textsuperscript{92} thiophenes\textsuperscript{93} and thiazoles\textsuperscript{94} to name but a few (Figure 3.1).

Figure 3.1. Structures of some small organic molecule scaffolds that have been constructed by solid-phase methods.\textsuperscript{92-94}
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Solid-phase methodologies can, in many cases, streamline classical solution-phase methodologies that have traditionally been harnessed to access small molecules, while allowing library synthesis.\textsuperscript{95-98} The potential advantages of solid-phase techniques include:

- Immobilisation of starting materials: Immobilising reactants onto the solid support can effectively reduce the likelihood of undesirable side reactions.

- High concentrations of reagents can be used to drive reactions to completion, with excess of reagents simply washed away between reaction steps – something that is very problematic in solution-phase chemistry, with purification typically needed after each step. This expedites reaction workup and can help to increase the purity of the product, allowing multistep reactions to be completed within a few days in a reasonable yield.

- The ease of swapping solvents, allowing employment of a wide spectrum of organic solvents to accommodate various chemical processes, and allowing the performance of reaction workups in parallel.

- Automation: A notable strength of solid-phase synthesis lies in its capacity for parallel synthesis (in well plates) and possible automation, enabling the efficient synthesis of a diverse range of compounds.

- Site-to-site isolation on a solid-phase is a valuable strategy for separating reactive sites or functional groups on a solid support. Indeed, the ability to separate molecules from one another by attaching them to an inert, rigid matrix can offer an alternative to the high-dilution principle for the suppression of undesirable bimolecular side reactions.
- Stabilisation of reactive groups attached to a solid support with an extended life-time when compared to their counterparts in solution. This stability primarily arises from the protective shield offered by the solid support from environmental factors such as moisture, air. This is especially important when handling compounds that are prone to degradation or those possessing high reactivity, such as tetrazines.

- Detachment from the solid support: Upon completion of synthesis, the final product is detached from the solid support by conditions that can add a specific element of diversity to the compound library (e.g., amines can be used to cleave an ester).

Although solid-phase techniques have several advantages, there are also some limitations that must be considered. Solid-phase syntheses often entail extensive solvent usage for repeated washing steps to remove excess reagents and byproducts, which poses environmental concerns and higher costs. Also, reactions on solid-phase are often driven by high reactant concentrations. Moreover, scalability presents a significant challenge as gram quantities of resin are typically required to generate only modest quantities of the target compound (a few hundred milligrams). This limitation stems from the restricted loading capacity of the resins, making larger-scale production impractical. In addition, the resin materials are not always compatible with all reagents and reaction conditions, and reaction monitoring is more challenging compared to classical solution synthesis.
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Inspired by this flexible and robust technique, here an efficient solid-phase synthesis of s-tetrazines was developed, exploiting:

(i). The ability to use high concentrations or neat reagents;

(ii). Linkers to add diversity at the cleavage step;

(iii). The ability to employ a combination of solvents and/or reactants;

(iv). In situ oxidation, leading to the formation of the s-tetrazine ring on a solid support;

(v). Site-Site isolation.

3.2 Synthesis of s-tetrazines on-solid-supports

Herein, an expedient solid-phase synthesis route to both monosubstituted and unsymmetrical disubstituted s-tetrazines was developed, bearing different functional groups. Based on thiol-promoted chemistry, monosubstituted s-tetrazines were synthesised using dichloromethane as a carbon source, while disubstituted unsymmetrical aryl or alkyl s-tetrazines were synthesised using readily available nitriles (Scheme 3.2). This resin-supported approach to s-tetrazines used mild conditions and was investigated with a variety of resins and linkers routinely used in solid-phase synthesis. A variety of s-tetrazines including the most reactive and challenging monofunctionalised s-tetrazines as well unsymmetrical aryl and alkyl s-tetrazines were obtained in high yields (70–94%) after a single purification step.
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Thiol-promoted strategy\(^{(66)}\)

\[
R^1\text{CN} + R^2\text{CN} \xrightarrow{\text{N}_2\text{H}_4\text{H}_2\text{O}, \text{EtOH}} \xrightarrow{\text{NaNO}_2, \text{HCl}} R^1\text{C} - N - N - R^2
\]

\(R^1, R^2 = \text{alkyl, aryl}\)

First example of using DCM as a reagent\(^{(66)}\)

\[
\text{RCN} + \text{CH}_2\text{Cl}_2 \xrightarrow{\mu\text{W}, 50 ^\circ\text{C}, 24 \text{ h}} \xrightarrow{\text{NaNO}_2, \text{HCl}} N - N - R
\]

\(R = \text{alkyl, aryl}\)

This work:

\[
\text{NC} \xrightarrow{X = \text{CH, N}} \xrightarrow{\text{CH}_2\text{Cl}_2 \text{ or } \text{RCN}} \xrightarrow{\text{HS} \xrightarrow{\text{N}_2\text{H}_4\text{H}_2\text{O}, 40 ^\circ\text{C}, 24 \text{ h}}} \xrightarrow{\text{NaNO}_2, \text{HCl} \text{ Cleavage}} R - N - N - \text{X} \xrightarrow{Y = \text{OH, NH}_2} \]

\(R = \text{H, alkyl, aryl}\)

Scheme 3.2. Synthetic strategies used for the synthesis of \(s\)-tetrazines.

3.2.1 Functionalisation of the Rink amide linker for \(s\)-tetrazine generation

Aminomethyl ChemMatrix (an amino-functionalised PEG-based resin 0.5–0.7 mmol/g, 100–200 mesh) was initially selected as a solid support owing to its high loading capacity, broad chemical compatibility, and water-compatibility (Figure 3.2).\(^{99}\)

![Figure 3.2. Structure of aminomethyl ChemMatrix resin.\(^{99}\)](image-url)
The resin was functionalised with an Fmoc-Rink amide linker (Knorr linker). The 9-fluorenlymethoxycarbonyl (Fmoc) group is one of the most popular orthogonal protecting groups used in solid-phase synthesis to temporarily mask amino groups. The Fmoc group can be effectively removed under mildly basic conditions, while exhibiting robust resistance to acidic treatments (Scheme 3.3).  

![Scheme 3.3. Deprotection mechanism of the Fmoc group by piperidine.](image)

After removal of the Fmoc group with 20% piperidine, 4-cyanobenzoic acid (3 equiv.) was coupled as a nitrile precursor for the subsequent preparation of tetrazine, using DIC and Oxyma as the coupling combination (3 equiv. each) to form the nitrile-benzoic acid functionalised resin 1 (Scheme 3.4).

![Scheme 3.4. Functionalisation of the Rink linker to give the resin-bound nitrile 1.](image)

An investigation was undertaken into the synthesis of monosubstituted s-tetrazine 4 using thiol-promoted chemistry (Scheme 3.5).
Scheme 3.5. Solid-phase synthesis of monosubstituted s-tetrazine 4 using thiol-promoted chemistry.

Initially, the nitrile functionalised resin 1 (100 mg, loading 0.5–0.7 mmol/g, 100–200 mesh) was degassed and treated with 50–60% hydrazine hydrate (2 mL), 3-mercaptopropionic acid (3 equiv.), and dichloromethane (0.5 mL) with EtOH (0.5 mL) as a solvent. Dichloromethane here was utilised as a reagent in the synthesis of tetrazines, forming the terminal carbon of the monosubstituted s-tetrazine ring as previously reported. The subsequent in situ oxidation to the dihydro-s-tetrazine was carried out, on-resin, using an aqueous solution of NaNO₂ (0.1 M), followed by the dropwise addition of HCl (2 M) until pH ~3 was reached. The resin beads turned pink-orange within a few minutes indicating the formation of the monosubstituted, resin-bound tetrazine 3 (Scheme 3.6).

Scheme 3.6. Synthesis of the monosubstituted, resin-bound tetrazine 3 starting from the terminal nitrile benzoic acid functionalised Rink linker 1 using a thiol-catalyst and CH₂Cl₂ as the 1-C source.
Reaction mechanism

To get better insight into the reaction pathways leading to the monosubstituted resin-bound s-tetrazine 3, the following reaction mechanism was proposed (Scheme 3.7). First, a thiolate reacts with the aromatic nitrile 1, forming the reactive thioimidate ester 2 in an irreversible step. The intermediate 2 then undergoes a nucleophilic attack by hydrazine, leading to the formation of the corresponding amidrazone and the regeneration of the thiol organocatalyst. The terminal nitrogen on the amidrazone then displaces chloride from CH$_2$Cl$_2$, which is followed by a second substitution step by another hydrazine molecule. This sets up the scene for an intramolecular 1,2-addition, which closes the ring to give 1,2,4,5-tetrahydro-s-tetrazine. Next, hydrazine-mediated elimination of NH$_3$ occurs, followed by rapid in situ autoxidation generating the diazene intermediate as previously reported, before the final oxidation of the resulting dihydro-s-tetrazine to form the monosubstituted, resin-bound s-tetrazine 3.
Scheme 3.7. Proposed mechanism for the synthesis of monosubstituted, resin-bound s-tetrazine.
CHAPTER 3

poor conversion and irreproducible results were obtained after cleavage of monosubstituted tetrazine 4 from the resin with 90% TFA (Scheme 3.8).

![Scheme 3.8. Liberation of the monosubstituted s-tetrazine 4 from the Rink linker.]

$^1$H NMR analysis showed that only a small amount of s-tetrazine 4 was obtained (3–5% yield) with the crude product mainly composed of the unreacted nitrile 1, which was probably due to the poor swelling of resin beads in an organic solution enriched with 50–60% hydrazine hydrate (Figure 3.3).

![Figure 3.3. $^1$H NMR analysis showing the cleaved nitrile starting material 1 and the resulting tetrazine product 4 confirmed a low conversion rate of 3–5% yield. The spectra are zoomed into the “aromatic region”, showing the resonances corresponding to the s-tetrazine and phenyl ring protons.]

35
The ability of resin beads to swell in organic solvents is a key for successful solid-phase chemistry,\textsuperscript{102} as only when the beads are solvated do functional sites within the resin beads become chemically accessible. This is important as > 99% of the reaction sites are situated within the interior of a polymer bead.\textsuperscript{99,103} To improve the conversion of starting material 1 to s-tetrazine product 3, the reaction conditions were optimised (Scheme 3.9). This involved a 50% reduction of the volume of hydrazine hydrate (1 mL), while increasing the volume of DCM by four-fold (2 mL) to promote the resin swelling (Figure 3.4). Furthermore, ethanol was removed from the procedure, as DCM was entirely adequate in fulfilling the dual roles of both solvent and reactant. These alterations lead to a visually noticeable improvement as the resin beads (before cleavage) exhibited a more intense pink colour after the reaction sequence (Scheme 3.9). The isolated yield of the monosubstituted s-tetrazine 4, post cleavage and purification, was 12%, compared to 3–5% with the original method, showing slight improvement. However, the prominent visual colour of the resin strongly indicated significant tetrazine formation and thus, suggesting possible decomposition upon cleavage from the linker with 90% TFA. Thus, investigations were conducted to enhance the yield by tuning the cleavage conditions.

Scheme 3.9. Revised synthetic procedure for the synthesis of monosubstituted s-tetrazine 3.
Figure 4.5. Comparison of the resin beads’ swelling in the reaction mixture of the Initial method (right) and the optimised method (left), demonstrating the positive effect of increasing DCM volume by four-fold (from 0.5 to 2 mL) and reducing hydrazine hydrate volume by 50% (from 2 to 1 mL).

Optimisation of cleavage conditions

Cleavage off the solid support proved to be a crucial step in the synthesis, with the $s$-tetrazines prone to degradation under the strongly acidic conditions typically used to cleave acid-labile linkers in solid-phase synthesis (90% TFA is often used for cleaving the Rink linker – although this is known to be somewhat excessive!). The acid-mediated cleavage of $s$-tetrazine 3 from the Rink linker was investigated looking at different reaction times and concentrations of TFA (see Table 3.1). 90% TFA/10% H$_2$O (entries 1–3) gave the monosubstituted $s$-tetrazine in low yields (12–23%) with high levels of
degradation and decreased yields upon prolonged exposure to TFA, shown visually by the change in colour from pink to orange-yellow and the low isolated yield after the workup and purification. It should be noted that after cleavage, the combined filtrates were diluted with toluene to avoid degradation of the tetrazine by TFA when the solvents were evaporated. Decreasing the acid concentration to 70% (entry 4) increased the yield significantly (to 45%), which increased to 71% (entry 5) upon 3 × 1 h treatments without noticeable decomposition occurring. Similar yields (75%) were observed after 3 × 1 h treatments with 50% TFA. When water, which is traditionally used as a scavenger in the cleavage of the Rink linker, was removed from the “cleavage cocktail” and replaced with DCM (entries 7–9), the monosubstituted s-tetrazine 4 was isolated in 86% yield and the full conversion was confirmed by $^1$H NMR spectroscopy (Figure 3.5, no nitrile starting material was observed).

![Figure 3.5](image)

**Figure 3.5.** $^1$H NMR analysis of both the cleaved nitrile starting material 1 and the resulting s-tetrazine product 4 from the optimised procedure. The spectra are zoomed into the “aromatic region”, showing the resonances corresponding to the s-tetrazine and phenyl ring protons.
Table 3.1. Optimisation of resin cleavage conditions for the efficient liberation of the tetrazine from the Rink linker.\(^a\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cleavage Cocktail</th>
<th>Time</th>
<th>Yield(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90% TFA/H(_2)O</td>
<td>3 h</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>90% TFA/H(_2)O</td>
<td>2 h</td>
<td>15%</td>
</tr>
<tr>
<td>3</td>
<td>90% TFA/H(_2)O</td>
<td>1 h</td>
<td>23%</td>
</tr>
<tr>
<td>4</td>
<td>70% TFA/H(_2)O</td>
<td>1 h</td>
<td>45%</td>
</tr>
<tr>
<td>5</td>
<td>70% TFA/H(_2)O</td>
<td>3 x 1 h</td>
<td>71%</td>
</tr>
<tr>
<td>6</td>
<td>50% TFA/H(_2)O</td>
<td>3 x 1 h</td>
<td>75%</td>
</tr>
<tr>
<td>7</td>
<td>50% TFA/DCM</td>
<td>1 h</td>
<td>49%</td>
</tr>
<tr>
<td>8</td>
<td>50% TFA/DCM</td>
<td>2 x 1 h</td>
<td>80%</td>
</tr>
<tr>
<td>9</td>
<td>50% TFA/DCM</td>
<td>3 x 1 h</td>
<td>86%</td>
</tr>
</tbody>
</table>

\(^a\) Cleavage was performed at room temperature using 100 mg of ChemMatrix resin that had been pre-swollen in DCM. \(^b\) Isolated yield after purification by column chromatography.

Evaluation of Resins and Catalysts

The efficiency of the solid-phase synthesis of monosubstituted s-tetrazine 4 was examined with different resin type and loadings. Scaling up the reaction using 1 g of aminomethyl ChemMatrix resin (with loadings of 0.6 mmol/g or 1 mmol/g, 100–200 mesh) did not affect the yield of the reaction, giving 4 in 88% and 90% yield, respectively. Switching to aminomethyl polystyrene resin (PS, 0.745 mmol/g or 1 mmol/g, 100–200 mesh) maintained robust efficiency, yielding 4 in 79% and 82%, respectively, and
indicating the efficiency of the developed method (Table 3.2). The effect of using other catalysts commonly used in a solution-phase chemistry with the revised method, where 3-mercaptopropionic acid was replaced with sulfur (3 equiv.) or zinc triflate (3 equiv.) was investigated; but both gave 4 in lower yields 45% and 58%, respectively (Table 3.2).

Table 3.2. The isolated yields obtained with the optimised method used in the synthesis of the monosubstituted s-tetrazine 4 and its scale up.
**CHAPTER 3**

**Synthesis of disubstituted unsymmetrical s-tetrazines 5–10**

This efficient solid-phase method was further expanded to the generation of disubstituted unsymmetrical s-tetrazines 5–10, with a variety of aliphatic and aromatic nitriles (Scheme 3.10, Figure 3.6). Thus, the nitrile functionalised resin 1 was treated with hydrazine hydrate (1 mL), degassed 3-mercaptopropionic acid (3 equiv.) and the selected nitrile (3 equiv.), either neat or dissolved in 1,4-dioxane (2 mL). Note, it was crucial to eliminate any traces of DCM (typically used to swell the resin in solid-phase synthesis) in order to prevent preferential formation of monosubstituted s-tetrazine 4. This synthetic approach was compatible with nitriles bearing both electron-donating (*e.g.*, methoxy) and electron-withdrawing groups (*e.g.*, nitro and fluorine). The potential formation of the undesired symmetrical disubstituted s-tetrazine side product(s), typically found in solution-phase synthesis was totally avoided, due to site isolation on the solid-phase and the fact that any side products formed in solution are simply washed away.

![Scheme 3.10](image)

**Scheme 3.10.** Solid-phase synthetic route to the disubstituted unsymmetrical s-tetrazines 5–10, starting from the terminal nitrile benzoic acid functionalised Rink linker bound ChemMatrix 1 using commercially available alkyl or aryl nitriles.
Figure 3.6. Substrate scope – exploring the solid-phase synthesis of amide s-tetrazines 5–10.

tert-Butylcyanoacetate allowed access to carboxy-functionalised s-tetrazine 6 with the tert-butyl group removed during the acidic cleavage from the resin. However, due to the very poor solubility of compound 6 in NMR solvents tried (polar and non-polar), an NMR spectrum could not be recorded (Table 3.3), with the structure just confirmed by high resolution mass spectrometry (Figure 3.7).
Table 3.3. Solubility tests conducted on carboxy-functionalised tetrazine 6.

![Chemical Structure](image)

1. N$_2$H$_4$ · H$_2$O, 40 °C, 24 h
2. NaNO$_2$, 2 M HCl, r.t., 2–4 min
3. 50% TFA/DCM, 3 x 1 h

<table>
<thead>
<tr>
<th>Entry</th>
<th>NMR Solvents</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Deuterium oxide</td>
<td>Insoluble</td>
</tr>
<tr>
<td>2</td>
<td>Acetic acid-$d_4$</td>
<td>Poorly soluble</td>
</tr>
<tr>
<td>3</td>
<td>Dimethyl sulfoxide-$d_6$</td>
<td>Insoluble</td>
</tr>
<tr>
<td>4</td>
<td>$N,N$-Dimethylformamide-$d_7$</td>
<td>Poorly soluble</td>
</tr>
<tr>
<td>5</td>
<td>Methanol-$d_4$</td>
<td>Insoluble</td>
</tr>
<tr>
<td>6</td>
<td>Acetonitrile-$d_3$</td>
<td>Insoluble</td>
</tr>
<tr>
<td>7</td>
<td>Dichloromethane-$d_2$</td>
<td>Poorly soluble</td>
</tr>
<tr>
<td>8</td>
<td>Chloroform-$d_2$</td>
<td>Insoluble</td>
</tr>
<tr>
<td>9</td>
<td>Toluene-$d_8$</td>
<td>Insoluble</td>
</tr>
<tr>
<td>10</td>
<td>Benzene-$d_6$</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>
3.2.2 Functionalisation of the 2-chloro-trityl linker for s-tetrazine generation

The Rink linker is widely used in solid-phase synthesis leaving behind a primary amide group upon cleavage. However, since s-tetrazines are commonly used in bioconjugation reactions, the methodology was expanded to a linker that would provide a “conjugation handle”, namely a carboxylic acid, upon cleavage. Thus, a 2-chlorotrityl chloride linker (CLTR-Cl) attached to a polystyrene resin was explored (Scheme 3.11).
Scheme 3.11. Solid-phase synthetic route to carboxyl s-tetrazines 13–21, starting from the terminal nitrile benzoic acid or nitrile nicotinic acid functionalised 2-chlorotritylchloride linker bound polystyrene resin 11 using commercially available alkyl or aryl nitriles.

The scope of aryl nitriles attached to the support was then expanded, using either 4-cyanobenzoic acid or 6-cyanonicotinic acid, which were attached to the trityl linker (loading 0.95 mmol/g) by esterification. Indeed, pyridyl-substituted s-tetrazines have emerged as highly reactive s-tetrazines\textsuperscript{104-105} originating from the electron-withdrawing influence of the heteroaryl substituent resulting in a lowered orbital energy of the tetrazine (LUMO) thereby expediting IEDDA cycloadditions.\textsuperscript{106,107} To this end, monosubstituted and disubstituted carboxy-functionalised phenyl s-tetrazines 13–15, pyridyl s-tetrazines 16–18, and dipyrindyl s-tetrazines 19–21 were synthesised using the same synthetic steps described above but with cleavage from the 2-chlorotrityl linker.
enabled by 20% hexafluoroisopropanol (HFIP) in DCM, giving the carboxyl \(s\)-tetrazines under mild conditions and in excellent 78–94% yields (Figure 3.8).

\[ \text{Figure 3.8. Substrate scope – exploring the solid-phase synthesis of carboxyl } s\text{-tetrazines 13–21.} \]

To explore the efficiency of the solid-phase method in accessing \emph{ortho} and \emph{meta} substituted-aryl \(s\)-tetrazines, 2-cyanobenzaldehyde (\(o\)-aryl nitrile) 4-bromo-3-cyano pyridine (\(o\)-aryl nitrile) and 5-bromo-3-cyanopyridine (\(m\)-aryl nitrile) were reacted via hydrazine condensation with the nitrile functionalised CLTR linker bound resin 11. The \emph{ortho}-functionalised-aryl \(s\)-tetrazines were not accessible presumably owing to steric and
electronic limitations,\textsuperscript{108} while the \textit{meta}-functionalised-dipyridyl \textit{s}-tetrazine 19 was successfully synthesised in 85% yield (Scheme 3.12).

![Scheme 3.12. Solid-phase synthetic route to access \textit{ortho} and \textit{meta} substituted-aryl \textit{s}-tetrazines.](image)

The limited stability of \textit{s}-tetrazines is a known obstacle to their use. However, the tetrazines 4–21 reported here were observed to be robust for over 1–3 months on the solid support (storage in dark at \(-20 ^\circ\text{C}\)), presumably due to site-site isolation and the passive nature of the resin matrix.
3.3 Solid-phase tetrazine synthesis with a cyano-fluorophore

“Turn-on” fluorophores, that exhibit fluorescence enhancement upon undergoing specific reactions have many biological applications due to their ability to enhance imaging of cellular components by minimizing background fluorescence. Upon exposure to light or specific chemical treatments, turn-on fluorophores have their fluorescence restored upon activation. This capability grants precise control over when and where the fluorescence is restored, contingent upon the activation treatment. Tetrazines linked to fluorophores have garnered significant attention due to their dual roles as bioorthogonal reactive groups and fluorescence quenchers. This quenching can be achieved through various mechanisms, including through bond energy transfer (TBET), photoinduced electron transfer (PET), or Förster resonance energy transfer (FRET), owing to their high electron affinities and absorbance spectra (\( \lambda = 500–560 \) nm), which make them perfect as quenchers of several fluorophores.

Previous work has shown that the quenching efficiency of \( s \)-tetrazines in tetrazine-fluorophore pairs is strongly dependent on distance between the \( s \)-tetrazine and the fluorophore and its relative orientation. Weissleder first reported \( s \)-tetrazine fluorogenic probes, featuring a series of FRET tetrazine–quenched boron dipyrromethenes (BODIPYs) in which fluorescence could be restored upon IEDDA reaction in PtK2 cells with a trans-cyclooctene (TCO)–modified taxol, with a fluorescence turn-on ratio of up to 15 (Scheme 3.13 A). By having an \( s \)-tetrazine moiety close to the BODIPY scaffold, the fluorescence increase was reported to be 900–1600-fold upon an IEDDA reaction with the trans-cyclooctene (Scheme 3.13 B).
Scheme 3.13. A. BODIPY-tetrazine conjugate in which the s-tetrazine acts as a FRET quencher with BODIPY fluorescence restored upon an IEDDA reaction with a TCO-modified taxol. B. BODIPY-tetrazine conjugates with a minimal distance between the s-tetrazine core and BODIPY scaffold. Fold increase (F.I.) refers to an increase in fluorescence.\textsuperscript{115,117}
The influence of s-tetrazines on fluorescence relies on the precise structure of both the fluorophore and the tetrazine, and several fluorophores have been conjugated to the tetrazine moiety. Indeed, coumarins, fluorescein, oregon-green, rhodamines, phenoxazines and cyanines have all shown fluorescence quenching across the wavelength range from blue to NIR-I (Figure 3.9).

![Figure 3.9](image)

**Figure 3.9.** Examples of s-tetrazines attached to different fluorophores, where the tetrazines effectively quenches their fluorescence.

### 3.3.1 In Situ formation of s-tetrazine on Peptide

Herein, the synthetic approach to the solid-phase synthesis of s-tetrazines was extended to allow conjugation onto a peptide and incorporation of a cyano-fluorophore. The
cyanophenylboronic acid-coumarin conjugate (CN–CM) 24 was synthesised in two steps starting from the commercially available 7-diethylamino-4-methylcoumarin 22 (Scheme 3.14). Halogenation of the coumarin was achieved using iodine, and the iodo–coumarin derivative 23 was then subjected to a Suzuki coupling reaction with 3-cyanophenyl boronic acid, and bis(triphenyl phosphine)palladium(II) diacetate as catalyst to provide the CN–CM 24 in 91% yield.\textsuperscript{125}

![Scheme 3.14. Synthesis of the cyanophenylboronic acid-coumarin conjugate 24.\textsuperscript{124,125}]

To incorporate the nitrile precursor into a peptide, the antimicrobial peptide MAD1\textsuperscript{126} was selected (see Chapter 4). The 6-cyanonicotinic acid-L-MAD1 peptide (CN–MAD1) 27 was synthesised using standard solid-phase peptide chemistry with a Rink linker functionalised polystyrene resin (100 mg, loading 0.745 mmol/g, 100-200 mesh). 6-Cyanonicotinic acid was firstly activated using the activating agent dipyrrolidino-(N-succinimidyl)carbenium hexafluorophosphate (HSPyU) to form the corresponding NHS ester \textit{in situ}, which then coupled to alpha-N-terminus of the L-MAD1 31 peptide with couplings monitored by a ninhydrin test (Kaiser test) (Scheme 3.15).\textsuperscript{127} Synthesis was confirmed by HPLC and HRMS, with the formation of CN-MAD1 27 in 88% yield (Figure 3.10).
Scheme 3.15. Synthesis of 6-cyanonicotinic acid-L-MAD1 peptide conjugate 27.

Figure 3.10. HPLC and HRMS traces of the purified 6-cyanonicotinic acid-L-MAD1 conjugate 27.

To form the coumarin-tetrazine probe (CM–Tz–MAD1) 30, the coumarin nitrile 24 and the resin-bound nitrile 26 were reacted with hydrazine hydrate, and 3-mercaptopropionic acid (3 equiv.) as described in the developed solid-phase method,
with subsequent *in situ* oxidation again carried out, on-resin, using an aqueous solution of NaNO₂ (0.1 M), followed by the dropwise addition of HCl (2 M) until pH ≈ 3 was reached. The resin beads turned red/orange within a few minutes confirming the formation of the s-tetrazine 29 (Scheme 3.16). After cleavage and deprotection with 70% TFA, the formation of the coumarin-tetrazine on the L-MAD1 peptide 30 was confirmed after purification and analysis by HPLC and HRMS (Figure 3.11).

Figure 3.11. RP-HPLC trace of the purified CN–CM 24, CN–MAD1 27, and CM–TZ–MAD1 30 with detection at 350 nm and HRMS of probe 30.
The successful synthesis of the tetrazine-functionalised peptide 30 with 69% yield indicated that the on resin hydrazine treatments and oxidation processes had no discernible impact on the amino acids in the L-MAD1 peptide. However, it is important to consider that certain amino acids can be susceptible to oxidation. Particularly, methionine can undergo oxidation to form methionine sulfoxide, while cysteine residues can form disulfide bonds under oxidative conditions. Thus, these potential oxidative side reactions must be considered if this methodology is to be extended to other peptide sequences, and further experiments under tetrazine formation conditions with different peptides with varying compositions and sequences would be beneficial.
3.3.2 Tetrazine triggered fluorescence “switch-off”

Analysis showed a significant decrease in fluorescence upon s-tetrazine conjugation (24-30), due to proximal quenching and demonstrated that (CM–Tz–MAD1) 30 was a “silent probe” with a 160-fold decrease in fluorescence (Figure 3.12).

![Chemical structures](image1)

**Figure 3.12.** A. Normalized absorption and emission spectra of CN–CM 24 and CM–Tz–MAD1 30 (both at 10 μM in ACN). B. Images of cuvettes containing CN–CM 24 and CM–Tz–MAD1 30 demonstrating the “switch-off” of fluorescence by the s-tetrazine (illuminated with a 254 nm handheld UV light).
3.4 Conclusions and Future Work

In conclusion, a practical method for the solid-phase synthesis of s-tetrazines has been developed, with the thiol-promoted pathway yielding mono- or disubstituted s-tetrazines. The methodology was compatible with different resin supported aryl nitriles, and aliphatic and aromatic acceptor nitriles with either electron-withdrawing or electron-donating groups. The method was versatile, using either DCM as the carbon source for monosubstituted s-tetrazines, or nitriles (either as solvent or as a reactant) for the disubstituted derivatives. All s-tetrazines (16 compounds) were synthesised in excellent yields (70–94%) without the need for metal catalysts or high temperatures, and notably required only a single purification step after cleavage. This solid-phase approach naturally overcomes the problems typically associated with disubstituted s-tetrazine synthesis in solution, namely the formation and separation of the undesired symmetrical, disubstituted adducts. The method was compatible with different types of resins and linkers typically used in solid-phase synthesis. This route paves the way for applications in chemical biology where s-tetrazines can be synthesised in situ attached to peptides, thus, providing a range of chemical handles that can be exploited in bioorthogonal chemistries, and also opens up routes to “on-resin” cyclisation reactions. As a proof of concept, a tetrazine-coumarin probe was synthesised in which the s-tetrazine core was directly synthesised on a peptide using a nitrile containing coumarin scaffold. The resulting coumarin-tetrazine conjugate was highly quenched with a 160-fold drop in the fluorescence signal compared to the unconjugated coumarin-nitrile.
Chapter 4. Labelling *Mycobacteria*

Parts of this chapter have been submitted as: Alghamdi, Z.S.; Sharma, R.; Kiruthiga, N.; Ucuncu, M.; Klausen, M.; Santra, M.; Devi, U.; Venkateswaran, S.; Lilienkampf, A.; Bradley, M. Lighting up Mycobacteria with fluorescent membrane associated disruption targeting peptides.

4.1 Introduction

Tuberculosis (TB) affects with its high infectiousness an estimated one-fourth of the world's population and remains a prominent cause of death.\textsuperscript{128} The recent COVID-19 pandemic had huge detrimental consequence on TB infection rates by hampering access to diagnosis and treatment, as well as increasing TB's prevalence due to “lock-downs” promoting transmission.\textsuperscript{129} Indeed, much of the rapid progress made in the five years prior to 2020 has been undone, with many people undiagnosed/untreated during the pandemic, and with 2–3 times as many deaths from TB every decade than of COVID to date.\textsuperscript{130,131}

4.1.1 *Mycobacterium* cell envelope

*Mycobacterium tuberculosis* (*Mtb*), the causative pathogen of TB, is a member of the mycobacteria genus that has thick hydrophobic/waxy cell envelope which is notably different from other bacteria (Figure 4.1).\textsuperscript{132} This protective cell envelope of mycobacteria generally consists of the following components: the outer membrane (OM), the cell wall, and a cytoplasmic or inner membrane (CM). The mycobacteria outer
membrane is approximately 60% lipid, as compared to some 20% for the lipid-rich cell walls of gram-negative bacteria. The major lipid components of the mycobacteria outer membrane are long-chain fatty acids (up to C₉₀) known as mycolic acids.¹³₂,¹³³

![Diagram of cell envelopes](image)

**Figure 4.1.** Representation of the main components of the cell envelopes of gram-negative bacteria, gram-positive bacteria, and mycobacteria the generic structure of mycolic acids. Reproduced from reference 132, licensed under (CC BY-NC-ND 4.0), copyright © 2022.¹³²

The outer membrane of *Mtb* also contains inert waxes, protein channels (porins), and glycolipids that are covalently linked with mycolates, forming its lipid bilayer. Mycolic
acids are also connected to the cell wall polysaccharide arabinogalactan within the complex peptidoglycan layers, extending into the periplasm, which separates the cell wall from the inner membrane. Each bacterial envelope features peptidoglycan outside the cytoplasmic membrane, providing essential shape and integrity to the bacterial cell wall (Figure 4.1). Gram-positive bacteria feature thick multilayers of peptidoglycan, whereas mycobacteria and gram-negative bacteria have thinner layers.132,134

### 4.2 Strategies for *Mycobacterium tuberculosis* diagnosis

A key need for a successful TB treatment and prevention is early diagnosis, which prevents the spread of infection and permits early therapeutic intervention. A fundamental aspect of any diagnosis is accurate detection and identification of the pathogenic bacteria and this entails assessing factors such as sensitivity, specificity, cost-effectiveness (for resource-limited settings) and the time required for detection of bacteria from clinical samples.124,135

Key methods include:

- Traditional smear sputum microscopy relies on the direct acid-fast staining of *Mtb* in sputum, such as the Ziehl–Neelsen (colour–based) or Auramine–Rhodamine (fluorescence–based) staining.136,137 However, key drawbacks of sputum smears are their lack of sensitivity and specificity. Factors such as sample collection and preparation can drastically affect the sensitivity of Ziehl–Neelsen staining.138 Auramine–Rhodamine staining provides high sensitivity but there is a lack of
selectivity against other bacteria because of non-specific dye incorporation resulting in false positives. However, the high demand for microscopy-based tests for sputum samples is driven by their simplicity and cost-effectiveness. Therefore, there is still a need to develop stains that are able to selectively and sensitively detect \textit{Mtb}, enabling rapid and accurate early-stage identification.

- Microbial culture-based methods (the gold standard for TB diagnosis) involve the isolation of the \textit{Mtb} bacteria in a clinical specimen, growth and subsequent identification through biochemical analysis, phenotypic observations, and drug sensitivity tests. However, the process is time-consuming, often taking weeks due to the slow growing nature of \textit{Mtb} which hinders the diagnostic and timely initiation of the treatment.

- Genetic methods are largely based on the polymerase chain reaction (PCR) and provide rapid and generally robust results. PCR methods are often used to amplify \textit{Mtb} specific DNA sequences to determine antibiotic resistance profiles (e.g., GeneXpert). However, the accessibility of PCR methods is hampered by their cost and the requirement for cold chains. Moreover, they require specific laboratory facilities and trained personnel, thereby constraining their use in regions with limited resources.

**Fluorescent probes for \textit{Mtb} dedication**

Continuous advancements have established fluorescence imaging as an indispensable method for visualising biological processes at a molecular level and monitoring changes within living systems. The Jablonski diagram is widely used to illustrate how photons
interact with molecules (Figure 4.2). In fluorescence imaging, external light of an appropriate wavelength is used to excite an electron in a fluorophore from a ground state molecular orbital ($S_0$) to a higher energy molecular orbital ($S_1$). This is followed by rapid ($10^{-12}$-$10^{-10}$ s) relaxation to the lowest vibrational energy level of $S_1$, then followed by slower ($10^{-10}$-$10^{-7}$ s) relaxation back down to the ground state $S_0$ (so-called radiative decay) with emission of a photon into various vibrationally excited $S_0$ levels. This emission of light is known as fluorescence, and because the energy of the emitted photons is always less than that of the exciting photons (energy is lost due to the vibrational levels within the system), it always corresponds to a longer wavelength. 

![Jablonski diagram illustrating the energy transitions leading to fluorescence within a molecule after irradiation.](image)

Fluorescent probes that selectively target components of the mycobacterial cell envelope have emerged as invaluable tools in bioimaging applications. For example, fluorescent β-lactam probes have been designed for the recognition and covalent modification of target enzymes involved in peptidoglycan synthesis, such as Penicillin Binding Proteins (PBPs) and transpeptidases. This covalent modification can alter the
fluorescence properties of the probe, allowing the enzyme to be visualised and tracked. (Figure 4.3A).\textsuperscript{145-148}

**Figure 4.3.** Examples of fluorescent probes targeting the mycobacterial envelope\textsuperscript{148-152}

Fluorescent trehalose probes have also been used to label glycolipids in mycobacteria, primarily via the trehalose metabolic pathways (Figure 4.3B).\textsuperscript{149-153} Trehalose is a
precursor for glycolipids like trehalose dimycolate (TDM) and monomycolate (TMM), essential constituents of the outer membrane. These probes are incorporated as trehalose analogues, undergo processing by the enzyme antigen 85 (Ag85), leading to their incorporation into the mycolyl arabinogalactan (mAG) layer, thereby metabolically forming TDM. Alternatively, trehalose can be transported into the cytoplasm of the bacteria by the SugABC-LpqY protein complex. Inside the cytoplasm, trehalose is converted into TMM and subsequently transported to the outer membrane via the MmpL3 system (Figure 4.4).  

Figure 4.4. Illustration of mycobacterial trehalose metabolism.  

4.3 Lighting up Mycobacteria with fluorescent membrane associated disruption targeting peptides

Natural host defence peptides have gained attention as potential therapeutics, often acting via membrane-specific, bactericidal mechanisms that are capable of remarkably
selective actions, making them distinct from traditional antibiotics.\textsuperscript{154,155} Although natural host defence peptides offer a pool of molecules, rationally designed \textit{de novo} sequences can also give peptides possessing novel bioactivity.\textsuperscript{156} Medina reported a biomimetic, \textit{de novo} designed \(\alpha\)-helical “defence peptide” (H-KRWHWWRRHWVVW-NH\(_2\)),\textsuperscript{126} referred to as a mycobacteria membrane-associated disruption peptide (MAD1), which was inspired by the unique features of the mycobacteria-specific transmembrane protein porin A (MspA) that differs from porins of non-mycobacterial microbes.\textsuperscript{126,157} The MAD1 peptide was designed and optimised \textit{in silico} to self-assemble within the mycolic-acid rich outer membrane of \textit{Mtb}, with MAD1 found to selectively kill \textit{Mtb} with an MIC of 2.5–5 \(\mu\)M by induction of supramolecular membrane defects, without notable activity against Gram-positive or Gram-negative bacteria or mammalian cells.\textsuperscript{158,159} The other enantiomer of MAD1, prepared from non-natural D-amino acids, was found to be equally active against mycobacteria as its natural L-analogue, suggesting that the antimycobacterial activity of this peptide occurs through a physical mechanism, presumably by the mycolic acid-rich cell envelope being disrupted.\textsuperscript{126}

MAD1 undergoes supramolecular assembly, forming tryptophan-zippered structures that mimic the \(\beta\)-sheet-rich features of the MspA porin. This biomimetic behaviour allows MAD1 to selectively target the mycolic-acid-rich surface of \textit{Mtb}.\textsuperscript{126} Circular dichroism spectroscopy revealed a combination of \(\alpha\)-helical (at 206 nm and 219 nm) and \(\beta\)-sheet (at 212 nm) structures in MAD1 at pH 7.4. Additionally, an exciton band observed at 228 nm indicated interactions between tryptophan indole chromophores. However, at pH 4.5, MAD1 formed monomorphic amyloid-like fibres (300–700 nm in length and 5.5 nm in width), observed by transmission electron microscopy (TEM), suggesting the presence
of a tryptophan-zippered structure; whereas at pH 7.4, MAD1 organised into short fibrils (100–200 nm in length and 6 nm in width). This indicated that the solubility and structural organisation of MAD1 are highly pH-dependent, with more extensive fibre formation occurring under acidic conditions.

Here, in pursuit of the specific recognition and fluorescent labelling of *Mtb*, the MAD1 peptide was identified as a suitable targeting ligand. To this end, a small library of peptide-based probes for the labelling of mycobacteria, spanning the wavelength range from green to NIR-I, was developed as targeted imaging agents (Figure 4.5). The ability of the probes to label microorganisms were investigated with the “L”- and “D”-enantiomers of MAD1, as D-amino acid containing peptides are known to be much more robust to proteolysis. Here, both enantiomers of the peptides were decorated with four different fluorophores, two that were solvato-fluorogenic nitrobenzoxadiazole (NBD, green) and merocyanine (MeroCy, orange) to allow “wash-free” labelling, as well as more traditional “always-on” 5-carboxy-fluorescein (5-CF, green) and sulfonated Cy5 (sulf-Cy5, red) fluorophores.
4.3.1 Synthesis of MAD1 peptides

The L- and D-enantiomers of the MAD1 peptides were synthesised on a gram scale of resin using an SPPS approach on Fmoc-Rink-linker functionalised aminomethyl polystyrene resin (0.745 mmol/g, 100-200 mesh). Fmoc-protected amino acids were coupled to the linker with Oxyma/DIC (3 equiv., each) as the coupling combination until the 13-residue sequence was constructed (Scheme 4.1). The N-Fmoc protecting groups were removed using 20% piperidine between each coupling step. The deprotection and coupling steps were monitored by a Kaiser test\textsuperscript{127} and small-scale cleavage tests were performed after every three couplings to allow the intermediate peptides to be analysed.
by HPLC and LC-MS and ascertain problematic couplings. The MAD1 peptides L-32 and D-32 were cleaved from the resin using a mixture of TFA:TIS:DCM (90:5:5) and the crude products were purified by semi-preparative HPLC and characterised by HPLC and HRMS (Table 4.1).

Scheme 4.1. Synthesis of the MAD1 peptides (L-32, D-32) using a standard Fmoc solid-phase approach on a Rink amide linker functionalised polystyrene resin.
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Table 4.1. HPLC and HRMS data of the MAD1 peptides (L-32, D-32).

<table>
<thead>
<tr>
<th>Probe 32</th>
<th>HPLC*</th>
<th>HRMS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_t$ (min)</td>
<td>Detection (nm)</td>
</tr>
<tr>
<td>L-MAD1</td>
<td>2.29</td>
<td>254</td>
</tr>
<tr>
<td>D-MAD1</td>
<td>2.28</td>
<td>254</td>
</tr>
</tbody>
</table>

*HPLC and HRMS traces are in the appendices.

4.3.2 Synthesis and photophysical properties of MAD1-based "Green" fluorescent probes

Synthesis of the 5-CF-MAD1 probes

5-Carboxyfluorescein (5-CF), a traditional “always-on” fluorophore was selected as a proof of concept to provide a signal upon interaction with the target regardless of its localisation. To this end, the 5-carboxyfluorescein diacetate $N$-hydroxysuccinimide (NHS) ester 33 was synthesised† following the previously reported procedure by our group$^{161}$ and incorporated following Fmoc deprotection onto the alpha-amino terminus of the Lysine residue of both L- and D-MAD1 peptides. Deprotection of the acetyl groups was achieved with 20% piperidine and the acid labile sidechain protecting groups and cleavage from the resin were achieved under acidic conditions (Scheme 4.2). The peptides were subsequently purified by semi-preparative reverse phase HPLC to give 5-CF-MAD1 L-35 and D-35 as orange lyophilised powders in high yield < 92% (Table 4.2).

†Synthesised by Dr Muhammed Üçüncü.
Table 4.2. HPLC and HRMS data of the 5-CF-MAD1 peptides (L-35, D-35).

<table>
<thead>
<tr>
<th>Probe 35</th>
<th>HPLC*</th>
<th>HRMS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R_t) (min)</td>
<td>Detection (nm)</td>
</tr>
<tr>
<td>5-CF-L-MAD1</td>
<td>2.71</td>
<td>495</td>
</tr>
<tr>
<td>5-CF-D-MAD1</td>
<td>2.71</td>
<td>495</td>
</tr>
</tbody>
</table>

* HPLC and HRMS traces are in the appendices.

Synthesis of the NBD-MAD1 probes

Nitrobenzoxadiazole (NBD-Cl) is one of the most frequently used fluorescent membrane labels, recognised for its characteristics as a solvato-fluorogenic fluorophore, allowing for high signal-to-noise ratios to be generated.\(^{162}\) NBD demonstrates sensitivity to environmental polarity, manifesting fluorescence activation during the transition from aqueous to hydrophobic environments (here upon interaction with the bacterial hydrophobic lipid membrane).

The commercially available dye NBD-Cl 36 (3 equiv.) was coupled to the alpha-\(N\)-terminus of the resin-bound MAD1 peptide 31 through an aromatic substitution reaction (\(S_{n}\text{Ar}\)) with a displacement of the chlorine from NBD-Cl in the presence of DIPEA as a base (3 equiv.). (Scheme 4.3). After acidic cleavage off the resin and global deprotection, NBD fluorogenic probes were purified by preparative HPLC to give NBD-MAD1 L-38 and D-38 as yellow lyophilised powders in high yield \(\leq 91\) (Table 4.3).
Scheme 4.3. Synthesis of the NBD-MAD1 peptides (L-38, D-38). The NBD was linked to the alpha-N-terminus lysine residue of the enantiomeric peptides via an aromatic substitution reaction (SnAr).
Table 4.3. HPLC and HRMS data of the NBD-MAD1 peptides (L-38, D-38).

<table>
<thead>
<tr>
<th>Probe 38</th>
<th>HPLC*</th>
<th>HRMS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_t$ (min)</td>
<td>Detection (nm)</td>
</tr>
<tr>
<td>NBD-L-MAD1</td>
<td>2.67</td>
<td>495</td>
</tr>
<tr>
<td>NBD-D-MAD1</td>
<td>2.68</td>
<td>495</td>
</tr>
</tbody>
</table>

* HPLC and HRMS traces in the appendices.

Photophysical Characterisation

All photophysical studies were performed with freshly prepared air-equilibrated solutions at room temperature (298 K). Fluorescence quantum yields of the fluorophores were measured according to literature procedures.$^{163,164}$ The emission quantum yield values $\Phi_f$ of the sample ($s$) and reference ($ref$) were calculated using the following equation,$^{165}$ taking into account the refractive index ($n$), the absorbance ($A$), and the integral of the fluorescence plot $[I_f(\lambda_{exc}, \lambda_f)]$:

$$\Phi_f = \Phi_{f,ref} \times \left( \frac{n^n}{n^{ref}} \right)^2 \times \frac{1 - 10^{-A^{ref}(\lambda_{exc})}}{1 - 10^{-A^n(\lambda_{exc})}} \times \frac{\int_0^\infty I_f^{ref}(\lambda_{exc}, \lambda_f) \, d\lambda_f}{\int_0^\infty I_f(\lambda_{exc}, \lambda_f) \, d\lambda_f}$$

The absorption and fluorescence properties of the 5-CF-L-MAD1 and NBD-L-MAD1 probes were investigated in different mixtures of water and DMSO (Table 4.4).
Table 4.4. Photophysical properties of the “always-on” 5-CF-L-MAD1 and the “environmentally sensitive” NBD-L-MAD1 probes. For NBD, DMSO was used as a more hydrophobic environment to promote the probes’ maximal fluorescence.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent</th>
<th>( \lambda_{\text{abs}}^{\text{max}} ) (nm)</th>
<th>( \varepsilon_{\text{max}}^{\text{max}} ) (M(^{-1}) cm(^{-1}))</th>
<th>( \lambda_{\text{em}}^{\text{max}} ) (nm)</th>
<th>Stokes Shift (nm)</th>
<th>( \Phi_f )</th>
<th>( \varepsilon_{\text{max}}^{\text{max}}\Phi_f ) (M(^{-1}) cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CF-L-MAD1</td>
<td>H(_2)O/DMSO (9/1, v/v)</td>
<td>494</td>
<td>5.9 \times 10^4</td>
<td>522</td>
<td>28</td>
<td>0.17(^a)</td>
<td>10.0 \times 10^3</td>
</tr>
<tr>
<td>NBD-L-MAD1</td>
<td>DMSO</td>
<td>464</td>
<td>1.2 \times 10^4</td>
<td>545</td>
<td>81</td>
<td>0.06(^b)</td>
<td>0.72 \times 10^3</td>
</tr>
</tbody>
</table>

Fluorescence quantum yield standards: \( a \). Fluorescein in 0.1 M NaOH (\( \Phi_f = 0.90 \)); \( b \). Rhodamine 6G in EtOH (\( \Phi_f = 0.94 \)).

The photophysical properties of the “always-on” 5-CF-L-MAD1 probe in aqueous DMSO were comparable to the well-described optical properties of the parent dyes. As expected, 5-CF-L-MAD1 presents strong absorption in the green with an emission band at 522 nm (Figure 4.6). The environmentally sensitive NBD-L-MAD1 probe was first investigated in pure DMSO and showed similar behaviour to reference dye (Fluorescein) with absorbance at 464 nm and fluorescence emission at 545 nm (Figure 4.6). Interestingly, the absorption and emission bands of the green environmental probe NBD-L-MAD1 were much broader than the 5-CF counterpart and had the largest Stokes shift in the library (81 nm).
Figure 4.6. Normalised absorption (solid line) and emission (dashed line) spectra of 5-CF-L-MAD1 in H$_2$O/DMSO (9/1, v/v) (top) and NBD-L-MAD1 in DMSO (lower).

The solvato-fluorogenic property of NBD provides a significant advantage as it allows for labelling without the necessity of washing away excess probe from the sample, with the fluorescence remaining “off” in aqueous media and reducing background signal.\textsuperscript{166} To this end, the environmental sensitivity of NBD-MAD1 was further explored by measuring the fluorescence spectra in PBS solutions with gradually increasing amounts of DMSO. As expected, NBD-L-MAD1 showed a significant increase in fluorescence with an
increasingly hydrophobic environment (Figure 4.7). A 9-fold increase in fluorescence signal was observed for the NBD-L-MAD1 probe (10 µM) in a 9:1 (v/v) mixture of DMSO/PBS compared to a 1:9 mixture.

**Figure 4.7.** The evolution of the fluorescence intensity with increasing amounts of DMSO in PBS for solutions of NBD-L-MAD1 (λ_{exc} = 464 nm).

4.3.3 Synthesis and photophysical properties of MAD1-based "Red" fluorescent probes

Fluorescence imaging *in vivo* encounters limitations when using wavelengths in the visible spectrum (400–700 nm). The decline in resolution and contrast at increased tissue depths is attributed to various factors, including photon absorption, photon scattering, and high levels of tissue autofluorescence from endogenous fluorophores.\textsuperscript{167,168} Fluorescence exhibits less scattering at longer wavelengths (≈ 1/λ\textsuperscript{4}), resulting in superior tissue penetration and reduced fluorescent background, for near infrared dyes compared
to shorter wavelengths. This characteristic enables imaging at deeper anatomical sites with enhanced precision (Figure 4.8).^{169}

**Figure 4.8.** Absorption and scattering coefficients of *in vivo* chromophores, tissues, and water across (200–2000 nm) wavelengths with corresponding tissue penetration. Reproduced from reference 169, licensed under (CC BY 4.0), copyright © 2022.^{169}

Over the past few decades, there has been a significant effort dedicated to advancing Near-Infrared Region-I (NIR-I, ~650–900 nm) labelling with cyanine (Cy) fluorophores due to their high fluorescence and extinction coefficients, favourable solubility (particularly when sulfonated), and their synthetic and commercial availability.^{170-173} Cyanine dyes are composed of two heterocyclic “head groups” linked by an odd number of methine groups (Figure 4.9).
The nomenclature of cyanine dyes is based on the number of carbon atoms in the polymethine chain, denoted by \( n = 1, 3, 5, \) or \( 7 \), which correspond to mono-, tri-, (cyanine 3), penta- (cyanine 5), or heptamethine (cyanine 7) cyanines, respectively. Merocyanine is a cyanine-like polymethine chain but with only one indole group and an additional electron-donating or electron-withdrawing group, leading to a modified conjugated system (Figure 4.9). Merocyanines are also environmentally sensitive dyes exhibiting enhanced fluorescence intensity in hydrophobic environments, comprising an indoline electron-donating (D) and an accepting (A) carbonyl component, linked together by a polyethylene chain. Their emission properties can be fine-tuned across a broad range of the spectrum (500–900 nm) by extending the polymethine chain through the incorporation of vinylene groups or aromatic rings. The introduction of functional groups, such as carboxylic acids within the structure, facilitates conjugation to biomolecules.

![Figure 4.9. Generic structure polymethine dyes.](image)

**Synthesis of the merocyanine fluorophore**

The synthesis of the carboxylic acid functionalised merocyanine dye (MeroCy-CO\(_2\)H) followed previously reported procedures (Scheme 4.4).
The acceptor formation involved treating benzo[b]thiophene-3-(2H)-1,1-dioxide 39 with malonaldehyde bis(dimethyl) acetal 40 (5 equiv.) to produce the methyl enol ether 41 through an acid-catalysed process. Meanwhile, the donor component 44 was achieved through N-alkylation of 2,3,3-trimethyl-3H-indole 42 with 6-bromohexanoic acid 43 (2 equiv.). Subsequently, a condensation reaction between the acceptor 41 and donor 44 in the presence of sodium acetate (1 equiv., each) gave the desired product. The crude product was purified by flash chromatography to afford the MeroCy-CO$_2$H 45 as a blue solid in 90% yield.

Scheme 4.4. Synthesis of merocyanine functionalised with a carboxylic acid (45).

Synthesis of a sulfonated cyanine 5 fluorophore

The sulfonated cyanine fluorophore that has two sulfonate groups and emits in the deeper red region (λ$_{ex/em}$ = 640/670 nm) compared to MeroCy was also selected due to its high-water solubility resulting from the sulfonate and pyridine groups as previously
reported by our group (Scheme 4.5).\textsuperscript{177} The synthesis of sulfonated cyanine functionalised with a carboxylic acid (sulf-Cy5-CO\textsubscript{2}H) \textit{51} began by forming and activating the potassium 2,3,3-trimethyl-3\textsubscript{H}-indole-5-sulfonate \textit{48} for conjugation through quaternization with iodomethane (5 equiv.) to form 1,2,3,3-tetramethyl-3\textsubscript{H}-indolium-5-sulfonate \textit{49}.\textsuperscript{176} Subsequently, the condensation reaction between \textit{49} (2.2 equiv.) and the commercially available 2-(3-hydroxycarbonyl-6-pyridyl)malondialdehyde \textit{50} (1 equiv.) in the presence of sodium acetate, resulted in the formation of the desired product. The crude product was collected following precipitation and washed thoroughly with diethyl ether and purified by flash chromatography to afford the sulf-Cy5-CO\textsubscript{2}H \textit{51} as a blue solid in 90\% yield.

\begin{align*}
\textbf{Scheme 4.5.} \text{ Synthesis of sulfonated Cyanine 5 functionalised with a carboxylic acid (51).}\textsuperscript{176,177}
\end{align*}
Synthesis of the Cyanine-MAD1 probes

The carboxylic acid group of both the cyanine and merocyanine fluorophores was activated in situ using the activating agent dipyrrolidino(N-succinimidylcarboxyl) carbenium hexafluorophosphate (HSPyU, 3 equiv.) in the presence of base (DIPEA, 3 equiv.). The mechanism involves the conversion of the carboxylic acid into an NHS-ester that reacts readily with the free amino group of the resin-supported peptide (Scheme 4.6).

Scheme 4.6. The activation mechanism of MeroCy-CO₂H 45 and sulf-Cy5-CO₂H 51 through NHS ester formation using HSPyU as activating agent.

The activated MeroCy 45 and sulf-Cy5 51 fluorophores (3 equiv., each) were thus coupled to the deprotected alpha-N-terminus lysine on the MAD1 peptide-bound resins in the presence DIPEA (3 equiv.) (Scheme 4.7). After the cleavage and deprotection the red probes 53 and 54 were purified by preparative HPLC and characterised by HPLC and HRMS (Table 4.5).
Scheme 4.7. Synthesis of red probes 53 and 54 through the coupling of activated MeroCy or sulf-Cy5 fluorophores onto the alpha-N-terminus lysine residue of the resin-bound MAD1 peptides.
Table 4.5. Characterisation data for the synthesised red probes L-53, D-53, L-54 and D-54.

<table>
<thead>
<tr>
<th>Probe 53,54</th>
<th>HPLC*</th>
<th>HRMS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_t$ (min)</td>
<td>Detection (nm)</td>
</tr>
<tr>
<td>MeroCy-L-MAD1</td>
<td>3.40</td>
<td>600</td>
</tr>
<tr>
<td>MeroCy-D-MAD1</td>
<td>3.44</td>
<td>600</td>
</tr>
<tr>
<td>sulf-Cy5-L-MAD1</td>
<td>3.07</td>
<td>650</td>
</tr>
<tr>
<td>sulf-Cy5-D-MAD1</td>
<td>3.08</td>
<td>650</td>
</tr>
</tbody>
</table>

* HPLC and HRMS traces are in the appendices.

Photophysical Characterisation

The absorption and fluorescence properties of the L-MAD1 based red fluorescent probes were also investigated in different mixtures of water and DMSO (Table 4.6). All probes showed similar behaviours to the parent or reference dyes and the fluorescence quantum yields were measured according to literature procedures as previously described.$^{163-165}$ The absorption and emission profiles of the sulf-Cy5-L-MAD1 conjugate in aqueous DMSO were significantly red-shifted in comparison with MeroCy-L-MAD1 and green MAD1 probes, with narrow bands at 641 and 659 nm respectively (Figure 4.10). In comparison with the “always-on” sulf-Cy5-L-MAD1 probe, the environmental derivative
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MeroCy-L-MAD1 showed a slight hypsochromic shift in absorption and emission at 600 and 627 nm, respectively, with a lower extinction (Table 4.6).

**Table 4.6.** Photophysical properties of the “environmentally sensitive” MeroCy-L-MAD1 and the “always-on” sulfo-Cy5-L-MAD1 probes. For MeroCy, DMSO was used as a more hydrophobic environment to switch-on the probes’ fluorescence.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent</th>
<th>λ_{abs}^{max} (nm)</th>
<th>ε_{max}^{max} (M⁻¹ cm⁻¹)</th>
<th>λ_{em}^{max} (nm)</th>
<th>Stokes Shift</th>
<th>Φ_f</th>
<th>ε_{max}Φ_f (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeroCy-L-MAD1</td>
<td>DMSO</td>
<td>600</td>
<td>2.4 × 10⁴</td>
<td>627</td>
<td>27</td>
<td>0.02⁻</td>
<td>0.48 × 10³</td>
</tr>
<tr>
<td>sulf-Cy5-L-MAD1</td>
<td>H₂O/DMSO (9/1, v/v)</td>
<td>641</td>
<td>4.3 × 10⁴</td>
<td>660</td>
<td>18</td>
<td>0.08⁻</td>
<td>3.44 × 10³</td>
</tr>
</tbody>
</table>

Fluorescence quantum yield standards: a. Rhodamine 6G in EtOH (Φ_f = 0.94), b. Cresyl Violet in MeOH (Φ_f = 0.54).

The optical properties of merocyanine dyes are known to be highly sensitive to parameters such as polarity and hydrogen-bonding.¹⁷⁸ The presence of a shoulder peak in the absorption spectrum of the MeroCy-L-MAD1 probe at 570 nm could also be a sign of self-orientation of the dyes.¹⁷⁹ The sensitivity to the solvation environment was explored by measuring the fluorescence spectra in PBS containing gradually increased amount of DMSO content. As expected, MeroCy-L-MAD1 (10 µM) showed a 43-fold fluorescence increase with increasingly hydrophobic environments in a 9:1 (v/v) mixture of DMSO/PBS compared to a 1:9 (v/v) mixture (Figure 4.10).
Figure 4.10. Normalised absorption (solid line) and emission spectra (dashed line) of sulf-Cy5-L-MAD1 in H2O/DMSO (9/1, v/v) (top) and MeroCy-L-MAD1 in DMSO (middle). The evolution of the fluorescence intensity with increasing amounts of DMSO in PBS for solutions of MeroCy-L-MAD1 ($\lambda_{exc} = 592$ nm, lower).
4.3.4 Biological evaluation of the MAD1 probes

The choice of fluorophores was made considering various factors, including solubility, overall charge, and membrane-binding affinity. Solvato-fluorogenic fluorophores like NBD and merocyanine may exhibit distinct solubility properties, compared to traditional fluorophores like 5-CF and sulf-Cy5; however, all the MAD1 probes demonstrated good solubility in water when inspected visually. The overall charge of the MAD1 conjugates may also be influenced by the fluorophore. C5-CF and sulf-Cy5 contribute negative charges, potentially impacting electrostatic interactions with the cell membrane, whereas NBD and merocyanine are hydrophobic and may possess neutral or variable charges, influencing the peptides’ membrane affinity and subsequent self-assembly. Additionally, fluorophore can influence the peptide’s membrane binding affinity.

The biological evaluation of the fluorescently labelled MAD1-based probes was initially performed using the non-infectious and fast-growing species Mycobacterium smegmatis (M. smegmatis), which is a well-established model in the TB-related research commonly used due to the high biosafety restrictions (level 3) mandated for handling M. tuberculosis. The change in the intensity of labelling with concentration of the probes was studied with 1, 5 and 10 µM of the probes showing rapid, concentration-dependent, labelling (Figure 4.11). The labelling efficiency of all eight probes showed robust and stable labelling of M. smegmatis by confocal microscopy (Figure 4.12). Notably, the probes synthesised with the D-amino acids showed almost no pronounced difference to the L-enantiomer. The labelling experiments were performed by Dr Richa Sharma.
Figure 4.11. Concentration dependent labelling of *M. smegmatis* incubated with the L and D forms of the MAD1 probes. The bacteria were incubated with the probes (white = 1 μM, grey = 5 μM and black = 10 μM) for 1 h, washed and imaged, and the fluorescence intensity calculated by ImageJ, Fiji with standard errors (n = 25). Filters used for fluorescence imaging: Cy5 for sulf-Cy5-MAD1, Alexa 594 for MeroCy-MAD1, Alexa 488 for 5-CF-MAD1 and NBD-MAD1. Data from Dr Richa Sharma.
Figure 4.12. Labelling of *M. smegmatis* with different concentrations of the “L” and “D” enantiomers of the MAD1-based probes. The bacteria were incubated with the probes (1, 5 or 10 μM) for 1 h, washed and imaged. Filters used for fluorescence imaging (left panel): Cy5 for sulf-Cy5-MAD1, Alexa 594 for MeroCy-MAD1, Alexa 488 for 5-CF-MAD1 and NBD-MAD1. Right panels of all the fluorescent images are the brightfield images (Scale bar = 50 μm). Data from Dr Richa Sharma.
5-CF-MAD1 showed the strongest labelling, even at 1 µM, which was perhaps not surprising as 5-carboxyfluorescein conjugates are known to have high emissions at physiological pH, due to their high extinction coefficient of $5.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Table 4.4)\textsuperscript{181} For 5-CF-MAD1 and sulf-Cy5-MAD1 better imaging was achieved using a washing step to remove the background from the “always-on” probes; however, such background issues were avoided with NBD-MAD1 and MercoCy-MAD1 which were decorated with environmentally sensitive fluorophores (Figure 4.13).

![Figure 4.13. The M. smegmatis were incubated with the probes at 10 µM for 1 h, imaged and washed twice with PBS before being imaged again. The bacteria showed lower background fluorescence for the solvato-fluorogenic probes NBD-D-MAD1 and MercoCy-D-MAD1 without a washing step. Filters used for fluorescence imaging (left panel): Cy5 for sulf-Cy5-MAD1, Alexa 594 for MercoCy-MAD1, Alexa 488 for 5-CF-MAD1 and NBD-MAD1. Right panels of all the fluorescent images are the brightfield images (Scale bar = 50 µm). Data from Dr Richa Sharma.](image-url)
The labelling intensity of both the L and D-MAD1 probes remained intense even after 24 hours after initial labelling (Figure 4.14).

**Figure 4.14.** The fluorescence signal remains stable for ≥ 24 h post-labelling. *M. smegmatis* stained with the probes (1 μM) for 1h, followed by removing the probes and imaging after 24 h. L and D correspond to the enantiomers of the probes. Filters used for fluorescence imaging (left panel): Cy5 for sulf-Cy5-MAD1, Alexa 594 for MeroCy-MAD1, Alexa 488 for 5-CF-MAD1 and NBD-MAD1. Right panels of all the fluorescent images are the brightfield images (Scale bar = 50 μm). Data from Dr Richa Sharma.

In order to evaluate the specificity of the probes, the labelling of two common pathogenic bacteria *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) were examined with 5-CF-D-MAD1 and NBD-D-MAD1 by confocal microscopy (Figure 4.15A). Neither 5-CF-D-
**MAD1** nor **NBD-D-MAD1** showed off-target labelling at 2 µM, showing relative *M. smegmatis* selectivity of the MAD1 peptide (Figure 4.15B). At higher concentrations (> 5 µM) of the D-enantiomer probes, there was some off-target labelling as previously reported by Medina,\textsuperscript{126} however, since good labelling was seen at 1 µM this still gave a good selectivity window.

![Figure 4.15](image-url)

**Figure 4.15.** A. Specificity of the MAD1-based green probes for *M. smegmatis* compared to Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria. The bacteria were incubated with the **5-CF-D-MAD1** and **NBD-D-MAD1** probes for 1 h, washed, and imaged (*λ_{ex/em} = 480/505 nm*). Filter used for fluorescence imaging (left panel): Alexa 488 for **5-CF-MAD1** and **NBD-MAD1**. Right panels of all the fluorescent images are the brightfield images (Scale bar = 50 µm). Quantification of the fluorescent labelling calculated by ImageJ, Fiji with standard errors (n = 25). Data from Dr Richa Sharma.
Labelling of *Mycobacterium tuberculosis*

The primary objective of this study was to translate into a real-life application and explore its potential for future use. This was performed by the Indian Council of Medical Research-National Institute for Research in Tuberculosis (NIRT) using *Mycobacterium tuberculosis* (*H37Rv*).

Keeping in mind the biomedical application and the similarity in labeling efficacy between the L and D probes on *M. smegmatis*, the D probes were chosen due to their better stability in a biological setting.\(^{182}\) Note: Sulf-Cy5-D-MAD1 was not included in these studies due to the unavailability of a filter on the clinical microscope. After incubation with the probes, *M. smegmatis* was imaged on a fluorescence microscope (40X, EVOS™ M5000 imaging system) using a GFP filter (NBD and 5-CF) or a Texas red filter (MeroCy).

At low probe concentrations (1 µM), there was no labelling of *H37Rv* by NBD-D-MAD1, 5-CF-D-MAD1, or MeroCy-D-MAD1 and thus higher concentrations were explored. Both 5-CF-D-MAD1 and NBD-D-MAD1 at 10 µM, showed labelling of *H37Rv* whereas MeroCy-D-MAD1 did not exhibit any significant labelling. 5-CF-D-MAD1 and NBD-D-MAD1 were observed to stain cords (end-to-end and side-to-side bacterial attachment structures) formed/observed for *Mtb* (Figure 4.16).\(^{183,184}\)
Figure 4.16. Fluorescent labelling of the pathogenic *Mtb* strain *H37Rv* by incubation with 5-CF-D-MAD1 and NBD-D-MAD1 (10 µM) for 1 h, followed by washing with PBS and imaging at $\lambda_{ex/em}$ 488/505 nm. The yellow/red arrows indicate possible cords and white/black arrows individual bacteria. Regions of possible interest have been expanded for clarity (right). Scale bar = 75 µm. Data from NIRT.

It is important to note that at 10 µM of 5-CF-D-MAD1, there is non-specific labelling of other bacteria. However, this may not be a problem in the analysis of sputum samples since sample preparation/decontamination for TB screening typically involves destruction of other microbial species through treatments such as N-acetyl L-cysteine (NALC) coupled with 2% NaOH, or benzalkonium detergents.\(^\text{185}\)
4.4 Conclusions and Future Work

In conclusion, this chapter details the development and evaluation of a small library of fluorescent probes based upon the mycobacterial membrane-associated disruption peptide (MAD1). A comprehensive evaluation of both L- and D-enantiomers of the MAD1 peptides, decorated with four different fluorophores, including solvato-fluorogenic dyes, was conducted. The choice of fluorophores ranged from green (NBD and 5-CF) to far-red (MeroCy and sulf-Cy5) to maximise visualisation while minimising potential interference from autofluorescence. Fluorescent labelling properties were evaluated on \textit{M. smegmatis}, with all the eight probes showing robust and stable labelling, and certain specificity against common, clinically relevant, strains of Gram-negative and Gram-positive bacteria. Two of our D-probes (5-CF-D-MAD1 and NBD-D-MAD1) successfully labelled \textit{Mtb} (using \textit{H37Rv}, the laboratory strain of \textit{Mycobacterium tuberculosis}). These findings underscore the potential utility of the MAD1-based probes for diagnostic approaches for analysis of TB infection. Future work could expand to:

- Investigate the introduction of spacers to increase the distance between the fluorophore and the peptide; potentially improving binding efficiency and specificity.
- Explore the use of solvato-chromic fluorophores, to enhance sensitivity to micro-environment changes (\textit{e.g.} Nile Red, Thioflavin T, and BODIPY-based fluorophores).
- Conducting investigations to assess the selectivity and specificity of the MAD1 probes, in the context of TB positive and negative sputum samples.
Chapter 5. Bioorthogonal labelling with tetrazine fluorogenic probe

5.1 Introduction

Despite ongoing progress in developing fluorophores that exhibit high fluorescence quantum yields/brightness and intrinsic fluorescence switch-on in aqueous buffers, achieving specific labelling of desired targets remains challenging. Bioorthogonal ligation strategies have allowed the spatial visualization of cellular processes by labelling a diverse range of biomolecules such as proteins, nucleic acids, lipids, and glycans within intricate environments of cells and organisms (Figure 5.1). This versatility highlights the impact of these strategies in advancing our understanding of cellular dynamics and holds promise for future biomedical applications.

Figure 5.1. Representation of the most used bioorthogonal reactions for biological labelling. CuAAC: Copper-Catalysed Azide-Alkyne Cycloaddition, SPAAC: Strain-Promoted Azide-Alkyne Cycloaddition, IEDDA: Inverse Electron-Demand Diels-Alder reaction.
To study biological functions with bioorthogonal fluorescent labelling approaches, it is crucial to selectively introduce small, biocompatible “reactive handles” at the active sites of biomolecules, such as azides, alkenes, and alkynes, etc. These reactive handles can undergo specific binding followed by conjugation with complementary partners linked to pro-fluorophores to unveil the functional biomolecule through bioorthogonal labelling reactions (Figure 5.2).

Fluorogenic probes have the potential to reduce nonspecific background fluorescence and eliminate the need for washing out excess probe. This feature is very important, especially when washing or clearance steps are not possible; for example, in vivo imaging.¹⁹³,¹⁹⁴

One of the earliest bioorthogonal transformations for intracellular protein labelling involved the condensation of aldehydes/ketones with α-effect nucleophiles (e.g., alkoxyamines or hydrazines) to form oxime or hydrazone linkages (Figure 5.3).¹⁹⁵,¹⁹⁶ This approach relies on alkoxyamine or hydrazine-functionised fluorophores, which experience fluorescence quenching via internal charge transfer (ICT) processes,¹⁹⁷ and are restored upon interaction with ketones or aldehydes. However, its practical use encounters challenges, including slow reaction rates (< 10⁻³ M⁻¹ s⁻¹) and the need for
high concentrations of the reactants for efficient labelling, leading to increased background fluorescence and toxicity concerns. Moreover, this method is limited to in vitro applications due to its sensitivity to side reactions with other metabolites.\textsuperscript{196}

\[ \text{aldehyde/ketone} + \text{amine} \rightarrow \text{hydrazone} \]
\[ \text{R}_1 = \text{H, alkyl} \quad \text{X} = \text{O, NH} \quad \text{F} = \text{NBD, BODIPY, Coumarin} \]

\textit{examples:}

\begin{align*}
\text{NO}_2 & \quad \text{HN} = \text{NH}_2 \\
\lambda_{\text{abs}} &= 495 \text{ nm} \\
\lambda_{\text{em}} &= 552 \text{ nm} \\
& \quad 32\text{-fold} \uparrow \text{F.I.}
\end{align*}

\begin{align*}
\text{HN} & \quad \text{HN} - \text{HN}_2 \\
\lambda_{\text{abs}} &= 502 \text{ nm} \\
\lambda_{\text{em}} &= 539 \text{ nm} \\
32\text{-fold} & \uparrow \text{F.I.}
\end{align*}

\begin{align*}
\text{HN} & \quad \text{HN} \quad \text{HN} \\
\lambda_{\text{abs}} &= 380 \text{ nm} \\
\lambda_{\text{em}} &= 459 \text{ nm} \\
3\text{-fold} & \uparrow \text{F.I.}
\end{align*}

\textit{Figure 5.3.} The formation of hydrazone/oxime-based fluorescent probe representing the initial bioorthogonal reaction for protein labelling. Fold increase (F.I.) refers to an increase in fluorescence.\textsuperscript{195-198}

In order to mitigate undesired cross-reactivity with endogenous molecules, Bertozzi reported a coumarin-phosphine probe, in which the fluorescence was quenched by the lone pair of the phosphorus via an ICT process.\textsuperscript{199} Upon Staudinger ligation with an azide, the phosphine (donor) is converted into phosphine oxide (acceptor), eliminating quenching and activating fluorescence (Figure 5.4). The probe’s applicability for biomolecule labelling was assessed by reacting a phosphine probe that directly was attached to the fluorophore with an azido-functionalised recombinant murine dihydrofolate (mDHFR) reductase. The results demonstrated efficient labelling for azido-
mDHFR, leading to a 60-fold increase in fluorescence. Despite its higher reaction rates compared to oxime formation, the Staudinger ligation rate remains relatively slow ($\sim 1.5 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$), necessitating the use of high concentrations of reactants (> 250 µM) and resulting in increased background fluorescence.

Figure 5.4. Activation of the coumarin-phosphine probe during the Staudinger ligation with an azide, accompanied by phosphine oxidation, leading to a 60-fold increase in fluorescence.\(^\text{199}\)

An alternative strategy relying on ester cleavage, leading to the separation of a dye and a FRET quencher during the reaction was employed.\(^\text{200}\) Thus, a fluorescein-phosphine probe was developed for the precise detection of glycoproteins within Hela cells. The dye was initially linked to Disperse Red-1 with an azo derivative serving as intramolecular quencher through a FRET mechanism. Upon Staudinger reaction with an azide-functionalised glycoprotein, the quencher is released, interrupting the FRET phenomenon and restoring the fluorescence of the fluorophore (Figure 5.5). The result demonstrated effective \textit{in vitro} labelling with a 170-fold increase in fluorescence. Notably, the fluorescein-phosphine probe showed minimal non-specific background fluorescence, providing an improvement over the earlier coumarin-phosphine probe. Despite various improvements reported in the Staudinger-activated probes,
bioorthogonal reactions with higher reaction rates are still preferred to enable more efficient and rapid labelling.\textsuperscript{201,202} Moreover, phosphine probes are generally susceptible to nonspecific oxidation, leading to high background fluorescence.

**Figure 5.5.** A FRET-based fluorescein-phosphine probe activated by ester cleavage upon the Staudinger ligation with azide, accompanied by the release of the quencher and a 170-fold increase in fluorescence.\textsuperscript{200}

In 2004, the first example of a bioorthogonal reaction using an azide-based fluorogenic probe was reported by Wang, in which a fluorescent probe was generated through copper-catalysed azide-alkyne cycloaddition (CuAAC).\textsuperscript{203} The approach involved the synthesis of eight pro-fluorophores, each featuring coumarins modified at position 3- or 7- with an azide group to quench fluorescence. The subsequent reaction between
the azide and terminal alkynes resulted in the formation of highly fluorescent 1,2,3-triazoles (Figure 5.6). Among these pro-fluorophores, 3-azido-7-hydroxycoumarin was effectively used to selectively label proteins with up to 100-fold increase in fluorescence in both bacterial and mammalian cells.\textsuperscript{204,205} However, despite continuous efforts to improve the biocompatibility of copper-catalysed azide-alkyne click reactions, some limitations remain.\textsuperscript{206} These probes often show low stability in oxidative conditions and require the use of cytotoxic copper catalysts, thereby restricting their application in biological contexts.

![Figure 5.6](image_url)

\textbf{Figure 5.6.} The first example of an azide fluorogenic probe activated through a copper-catalysed azide-alkyne cycloaddition (CuAAC) with a >100-fold increase in fluorescence.\textsuperscript{203}

To address this limitation, Bertozzi \textit{et al.} established the copper-free version of this click reaction, known as strain-promoted azide–alkyne cycloaddition (SPAAC) for glycoprotein labelling using strained cyclooctynes, albeit at much slower reaction rates (Figure 5.7).\textsuperscript{207} Since then, multiple attempts to improve the cycloaddition rate by modifying the electronics of cyclooctyne or increasing ring strain through ring fusions have been reported, showing increased rates up to 1 M\textsuperscript{-1}s\textsuperscript{-1} (Figure 5.7).\textsuperscript{208-213} However, SPAAC probes also encounter challenges due to hydrophobicity and regioselectivity, stemming from the incorporation of bulky cyclooctynes. Cyclooctyne derivatives can also undergo
nucleophilic attack by cellular components like cysteine and glutathione, limiting their compatibility with in vivo labelling.

![Chemical structure diagram]

Figure 5.7. Strain-promoted azide-alkynyl cycloaddition for protein labelling, with the electronic and ring strain effects on the reaction rates.\textsuperscript{208-213}

However, a newer bioorthogonal reaction based on the inverse electron-demand Diels-Alder reaction (IEDDA) of tetrazine ligation is now out-competing the alkyne/azide reaction and allowing wash-free labelling of targets with high selectivity and specificity. This is due to the ability of tetrazine fluorogenic probes to exhibit site-specific fluorescence upon a simple conjugation reaction with dienophile partners (Figure 5.8), making them extremely useful for bioimaging and biosensing applications.\textsuperscript{15,214,215} Tetrazine acting as a quencher, demonstrates its prowess by offering a catalyst-free approach, mitigating concerns related to catalyst-induced artifacts and toxicity. Its rapid reaction kinetics ($10^6 \text{ M}^{-1} \text{s}^{-1}$) with selected dienophiles (\textit{e.g.}, trans-cyclooctene, cyclooctyne, and norbornene, etc.) have been reported to facilitate precise and efficient labelling (Figure 5.8).\textsuperscript{216-220}
Tetrazine is an electron-deficient group exhibiting a characteristic absorption peak around 520 nm and possessing the ability to quench fluorescence with emission wavelengths near 520 nm through various mechanisms. This adds versatility to their applications in fluorescence-based assays and imaging techniques. Commencing with the BODIPY fluorophore, several fluorophore-tetrazine conjugates have been reported (see chapter 3). One of the first examples of site-specific protein labelling using genetically encoded norbornene-containing amino acids via IEDDA reaction with a tetrazine was reported by Chin et al. In this study, norbornene modified GFP was incubated with tetrazine-quenched fluorophores, such as fluorescein, BODIPY, and tetramethyl-rhodamine (TAMRA). The results showed clear labelling in *E. coli* and on the surface of mammalian cells using low concentrations of tetrazine-probes (200 nM), resulting in 5–10 fold increase in fluorescence intensity (Figure 5.9). Despite the promising results observed with these probes, achieving an improvement in fluorescence intensity is still required.
5.2 “Switch on” of tetrazine-quenched fluorophore via IEDDA reaction

Driven by the need for a rapid, clean, and kinetically efficient method for labelling mycobacteria, an effective "switch-on" amplification system was developed with a tetrazine-quenched boron-dipyrrromethene fluorophore. This system capitalizes on the inverse electron-demand Diels-Alder (IEDDA) reaction with the MAD1 peptide, which was functionalised with a norbornene moiety as a “reactive handle”, as depicted in (Figure 5.10). The reactivity of the tetrazine ligation to norbornene was evaluated by monitoring the time-dependent IEDDA reaction by means of HPLC analysis and fluorescence spectroscopy. The labelling efficiency of the MAD1-based fluorogenic probe
was first investigated by checking the fluorescence amplification on resin-beads and further evaluated on *M. smegmatis* showing robust labelling with specificity against Gram-negative (*E. coli*) and Gram-positive bacteria (*B. subtilis*).

![Diagram](image)

**Figure 5.10:** Design of the MAD1 fluorogenic probe.

### 5.2.1 Synthesis of tetrazine-quenched fluorophore

The synthesis of BODIPY-Tetrazine 61 was attempted following the literature procedure outlined by Wombacher. The *meso*-aryl BODIPY precursor was obtained in a one-pot procedure starting by the condensation of commercially available 4-iodo-benzaldehyde 55 with 2,4-dimethyl pyrrole 56 under acidic conditions to form the dipyromethene framework. Subsequent oxidation was achieved under mild conditions, using the slow addition of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Addition of triethylamine and complexation with boron trifluoride diethyl etherate gave the desired iodo-BODIPY (I-BODIPY) 57 in 60% yield. The ethynyl function was further grafted onto the I-BODIPY using a Sonogashira coupling reaction with trimethylsilyl-acetylene (TMSA), catalysed by
palladium (Pd(PPh₃)₂Cl₂) and Copper(I) iodide. The trimethylsilyl group (TMS) was deprotected to afford the acetylene-BODIPY 58 in 84% yield.

**Scheme 5.1.** Synthetic route to acetylene-BODIPY 58.

The synthesis of the corresponding tetrazine 60 was achieved over two steps, using the nickel-catalysed Pinner reaction protocol between the aromatic nitrile 59 and hydrazine monohydrate. The obtained dihydro-s-tetrazine was then oxidised to give iodo-tetrazine 60 as a pink powder in 38% yield. The BODIPY-tetrazine probe (BODIPY-Tz) 61 was then synthesised via a second Palladium-catalysed coupling reaction between 60 and the acetylene-BODIPY 58. The crude product was purified by flash chromatography to provide the BODIPY-Tz 61 as a red powder in 57% yield (Scheme 5.2).

**Scheme 5.2.** Synthetic route to BODIPY-tetrazine probe (BODIPY-Tz) 61.
5.2.2 Synthesis of norbornene modified MAD1 peptide

The norbornene functionalised MAD1 peptide 66 was synthessed by linking norbornene carboxylic acid to the alpha-N-terminus lysine residue of the resin-bound L-MAD1 peptide 31 (see Chapter 4) through an aminomethylbenzoic acid spacer (Scheme 5.3). The spacer was chosen not only to address potential issues that might arise from the direct attachment of a fluorophore to a peptide but also to enhance structural flexibility and minimise steric hindrance for effective bioconjugation.

Commercially available exo-norbornene carboxylic acid 62 was firstly activated via a carbodiimide coupling reagent using N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) to give the active norbornene ester (Nor-NHS) 63 in 82% yield. The active Nor-NHS was then added to Methyl 4-(aminomethyl)benzoate hydrochloride (1 equiv.) in the presence of DIPEA to obtain the ester 64 in 91% yield. After ester hydrolysis, the acid 65 was obtained in 95% yield. The functionalised norbornene acid was then conjugated to the resin-bound L-MAD1 peptide 31 after a second activation reaction to form the desired norbornene modified MAD1 peptide (Nor-MAD1) 66. After the cleavage from the resin using 90% TFA, compound 67 was isolated and purified by semi-preparative RP-HPLC and characterised by HPLC and HRMS (Figure 5.11).
Scheme 5.3. Synthetic route to the norbornene modified L-MAD1 peptide (Nor-MAD1) 67.
5.2.3 Activation of the MAD1 fluorogenic probe

The reactivity of the tetrazine ligation with the norbornene was initially assessed by monitoring the time-dependent increase in fluorescence (Scheme 5.4). This evaluation involved incubating BODIPY-Tz 61 with Nor-MAD1 67 at 37 °C, varying the concentrations of both reactants (5-50 μM) to fine-tune the reaction conditions in order to achieve the maximal signal amplification (Figure 5.12). BODIPY-Tz 61 (5 μM) exhibited a high increase
in fluorescence up to 48-fold after 2 h and increased to some 61-fold after 4 h upon incubation with an excess of Nor-MAD1 67 (50 μM), demonstrating that 10 equivalent excess of norbornene serves as the optimal concentration to enhance the fluorescence “switch-on” and signal amplification.

Scheme 5.4. “Switch on” of the BODIPY-Tz 61 (500 μl, 5 μM) upon the IEDDA reaction with Nor-MAD1 67. Images of the HPLC vial containing BODIPY-Tz 61 (500 μl, 50 μM) before and 4 h after the IEDDA reaction (illuminated with a 254 nm handheld UV light).
**Figure 5.1.2.** Fluorescence increase of the reaction between BODIPY-Tz 61 and Nor-MAD1 67 in PBS, measured over time (1 h, 2 h and 4 h) at 37 °C. A. 61:67 (5 μM:5 μM), B. 61:67 (5 μM:50 μM), C. 61:67 (50 μM:5 μM).

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**Table:**

<table>
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<th>Reaction</th>
<th>Conjugate 1</th>
<th>Conjugate 2</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
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<tr>
<td>A</td>
<td>5 μM</td>
<td>5 μM</td>
<td>3-fold</td>
<td>5-fold</td>
<td>11-fold</td>
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<tr>
<td>B</td>
<td>5 μM</td>
<td>50 μM</td>
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<td>48-fold</td>
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<tr>
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<td>5 μM</td>
<td>15-fold</td>
<td>17-fold</td>
<td>18-fold</td>
</tr>
</tbody>
</table>

*a. The fluorescence intensities were monitored at λ<sub>exc/em</sub> = 509/520 nm, and the fold increase calculated comparing the fluorescence intensities at t = 0 (before the incubation of Nor-MAD1 62).*
Determination of the second-order rate constant and half-life

Second order rate constants ($k_2$) were determined using pseudo-first order conditions. The increasing fluorescence of BODIPY-Tz 61 at $\lambda_{em} = 520$ nm after the IEDDA reactions (A, B and C) with different concentrations of Nor-MAD1 67 were measured over 240 mins to give the pseudo-first-order rate constant ($k_{obs}$) values, enabling the calculation of $k_2$. $k_{obs}$ was determined directly from the slope of the linear plot of natural logarithm of fluorescence intensity ($\ln(F_t)$) versus time (Figure 5.13). The $k_2$ and the half-life ($t_{1/2}$) were then determined using the following equations: $k_2 = k_{obs}/[A]_0$ and $t_{1/2} = 1/k_2[A]_0$, respectively.

Figure 5.13. Plot of normalised natural logarithm of fluorescence intensities of BODIPY-Tz 61 during treatment with Nor-MAD1 67 against time to obtain the pseudo-first-order rate constants, calculate the second order rate constants ($k_2$) and estimated half-life of each reaction. A. 61:67 (5 µM:5 µM), B. 61:67 (5 µM:50 µM), C. 61:67 (50 µM:5 µM).
Having established the best concentrations for the activatable reaction partners (1 to 10 equiv.), the reactivity was explored by closely monitoring the change in peak intensities of both the reactant (BODIPY-Tz) 61, and the formed products 68a–c over time by means of HPLC analysis (Figure 5.14). Thus, BODIPY-Tz 61 (10 µM, 1 equiv.) was incubated at 37 °C with Nor-MAD1 67 (100 µM, 10 equiv.) over 0-24 hours. At regular time points, a sample was taken and analysed by analytical HPLC.

**Figure 5.14.** HPLC traces for the IEDDA reaction between BODIPY-Tz 61 (500 µl, 10 µM) and Nor-MAD1 67 (500 µl, 100 µM) in PBS. The reaction was monitored by analytical HPLC at 495 nm, eluting with a gradient of ACN/H$_2$O 0.1% FA over 0-24 h, based on changes in the BODIPY-Tz 61 peak (grey) and formation of IEDDA products peaks 68a–c (red).
CHAPTER 5

The HPLC results clearly show that the amount of BODIPY-Tz 61 (10 µM- selected to observe the visible detection on HPLC) decreased over time and that the products 68a–c of the cycloaddition were gradually produced. Notably, a slight precipitation was observed after 24 hours, likely due to the limited solubility of the reaction products in PBS.

5.3 Fluorescence amplification on the resin beads

To verify the potential of norbornene-MAD1 bound to polystyrene resin 66 in activating the fluorescence of BODIPY-Tz 61 during the course of the IEDDA reaction, the reaction system of beads containing 66 (10 mg, 0.745 mmol/g, 100-200 mesh) and BODIPY-Tz 61 (100 µM) was mixed in DMSO:PBS (1:1, v/v), using “naked” beads and BODIPY-Tz 66 individually as controls (Figure 5.15). DMSO was added to avoid possible precipitation and the reaction incubated at 37 °C and 400 rpm for 24 hours. The supernatant was then removed, and the beads were washed with PBS and imaged by Zeiss fluorescence microscopy with fluorescein isothiocyanate (FTIC) and yellow fluorescent protein (YFP) filters to capture optimal brightness (Figure 5.15). The beads showed a distinct fluorescence after the IEDDA reaction, demonstrating that the BODIPY-Tz 61 successfully reacted with the norbornene-MAD1 on resin 66. (Figure 5.16).
Figure 5.15. Fluorescence amplification on resin beads based on the IEDDA reaction between BODIPY-Tz 61 and Nor-MAD1 bound-resin 66. The beads were incubated with the BODIPY-Tz 61 (100 µM) for 24 h, washed and imaged using a Zeiss fluorescence microscope. Notably, the polystyrene beads themselves showed some fluorescence intensity before the reaction,226 but a significant increase was observed in fluorescence afterwards. Filters used for fluorescence imaging: FTIC ($\lambda_{ex/em} = 475/530$ nm, green) and YFP ($\lambda_{ex/em} = 490/510$ nm, yellow). Scale bar= 200 µm. Data from Dr Richa Sharma.
Figure 5.16. Fluorescence intensity of the resin beads following the IEDDA reaction between BODIPY-Tz 61 and resin-bound Nor-MAD1 beads 66 calculated by ImageJ, Fiji with standard errors (n = 25). Total beads fluorescence was measured on the FITC channel (green) and the YFP channel (yellow). Data from Dr Richa Sharma.

5.4 Fluorescence amplification on cells

To demonstrate the fluorescence “switch-on” and labelling mycobacteria, *M. smegmatis* were incubated at 37 °C with Nor-MAD1 67 (50 µM) for 3 hours. Subsequently, the supernatant was removed, and followed by addition of the BODIPY-Tz 61 (5 µM) and the cells were analysed by confocal microscopy (Figure 5.17). Labelled cells were imaged at different time points 1, 2, 4 and 24 hours after incubation with BODIPY-Tz 61, which was removed before imaging (no labelling observed before 1 hour) (Figure 5.18). To establish baseline comparisons, untreated *M. smegmatis* bacteria (only bacteria) and those treated with the quenched fluorophore BODIPY-Tz 61 were included as controls.
Figure 5.17. No-wash Labelling of *M. smegmatis* with the MAD1 fluorogenic probe over 1 h, 2 h, 4 h and 24 h (top). The bacteria were incubated with Nor-MAD1 67 (50 μM) for 3 h then treated with BODIPY-Tz 61 (5 μM) and imaged using a confocal microscope. Filters used for fluorescence imaging: BODIPY (λ<sub>ex/em</sub> = 500/580 nm). Scale bar = 50 μm.
Figure 5.18. Time dependent labelling of *M. smegmatis* incubated with the MAD1 fluorogenic probe 68. The bacteria were incubated with Nor-MAD1 67 (50 µM) for 3 h then treated with BODIPY-Tz 61 (5 µM) and imaged over time, with the fluorescence intensity calculated by ImageJ, Fiji with standard errors (n = 25).

To evaluate the specificity, the labelling of two common pathogenic *E. coli* and *B. subtilis* were examined with MAD1 fluorogenic probe by confocal microscopy over 24 hours (Figure 5.19). The MAD1 fluorogenic probe did not show off-target labelling after the addition of BODIPY-Tz 61 (5 µM) to the treated bacteria with Nor-MAD1 (50 µM). Samples were compared with the untreated bacteria and those treated with only quenched fluorophore BODIPY-Tz 61 (5 µM), showing good selectivity of the MAD1 fluorogenic probe 68.
Figure 5.19. Specificity of MAD1 fluorogenic probe 68 against Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria. The bacteria were incubated with Nor-MAD1 67 (50 µM) for 3 h then treated with BODIPY-Tz 61 (5 µM) and imaged using confocal microscope. Filters used for fluorescence imaging: BODIPY (\(\lambda_{\text{ex/em}} = 500/580\) nm). Scale bar = 50 µm.

5.5 Conclusions and Future Work

In conclusion, an intracellular bioorthogonal labelling approach was developed based on the MAD1 fluorogenic probe. This wash-free approach involves the controlled interaction and activation between a quenched fluorophore (BODIPY-Tz), and a norbornene-modified MAD1 peptide (Nor-MAD1) via tetrazine chemistry, allowing activation and amplification of fluorescent signal. The reactivity of the tetrazine ligation to norbornene was evaluated by monitoring the change in peak intensities of both reactants, (BODIPY-
Tz) \textit{61}, and the formed products \textit{68a–c} over time, using HPLC analysis and fluorescence spectroscopy. A fluorescence increase of 61-fold was determined after 4 h of incubation between 1.0 equivalent of BODIPY-Tz \textit{61} and 10 equivalents of Nor-MAD1 \textit{67}. The HPLC analysis unequivocally indicates a gradual reaction of BODIPY-Tz \textit{61} and progressive generation of the cycloaddition products \textit{68a–c} over time. Subsequently, the efficiency of the MAD1-based fluorogenic probe in labelling was proven on resin beads and on \textit{M. smegmatis}. The results revealed a robust and highly specific labelling, showing notable selectivity against common pathogenic Gram-negative bacteria (\textit{E. coli}) and Gram-positive bacteria (\textit{B. subtilis}). The in situ "switch-on" amplification method holds promise for practical applications, providing a biocompatible approach for biomedical applications. Future work could expand to:

Exploring red or near-infrared fluorophores could be beneficial to offer enhanced signal-to-noise ratios and improved penetration depth, rendering them more suitable for \textit{in vivo} biological imaging applications.

Testing the MAD1 fluorogenic probe on \textit{M. tuberculosis} to show the efficiency of the probe against real disease.
Chapter 6. Conclusions

Bioorthogonal reactions, particularly those utilising the reactivity of tetrazines, have resulted in promising advancements with far-reaching implications in biological and biomedical sciences. One limitation of the use of tetrazines in the field has been their laborious synthesis and purification. Thus, the work presented in this thesis has significantly contributed to the synthesis of $s$-tetrazines and their use in both chemical and biological contexts.

The solid-phase synthesis method presented in Chapter 3 stands as a cornerstone achievement in the efficient production of $s$-tetrazines. By circumventing the challenges inherent in traditional solution-based synthesis, this approach offers enhanced versatility providing an expedient route to the mono- or disubstituted $s$-tetrazines from various resin-bound aryl nitriles. This method is compatible with most used resin and linker types in solid-phase synthesis. Furthermore, the product yields are excellent, with only a single purification step in the route. This solid-phase route is also applicable to more complex tetrazines, as was demonstrated by the successful synthesis of a coumarin-linked tetrazine on the $N$-terminus of a resin-bound peptide. This underscores the practical utility of this method in chemical biology applications and paves the way for their broader application in areas such as drug discovery, imaging, diagnostics, and therapeutic interventions.

Expanding upon this groundwork, Chapters 4 and 5 describe the development and evaluation of fluorescently labelled peptides for the detection of mycobacteria. The mycobacterial membrane-associated disruption peptide (MAD1) was used as the
targeting ligand, with various “green” and “red” fluorophores conjugated to the N-terminus lysine residue, with the MAD1 peptide investigated as both the L- and D-enantiomers. All these fluorescent peptides successfully labelled *M. smegmatis* in a robust and stable manner, while demonstrating specificity over common clinically relevant strains of Gram-negative and Gram-positive bacteria. Notably, two of D-enantiomers of the probes (NBD-D-MAD1 and 5 CF-D-MAD1) successfully labelled *M. tuberculosis* cords, which are commonly observed forms in smear tests for the diagnosis of TB. Thus, these fluorescent probes offer promising opportunities for advancing TB research and diagnosis.

Finally, fluorescent labelling of mycobacteria by an *in situ* bioorthogonal IEDDA reaction between a norbornene-modified MAD1 peptide and tetrazine-quenched BODIPY-fluorophore was demonstrated. When *M. smegmatis* was consecutively incubated with the norbornene-modified MAD1 peptide and fluorogenic BODIPY-tetrazine, the IEDDA reaction on the cell surface resulted in a 61-fold increase in fluorescence on the mycobacteria. This approach benefits from increased pathogen selectivity and low background fluorescence due to the “turn-on” nature of the probe.
Chapter 7. Experimental

7.1. General Information

All chemicals including anhydrous solvents, Fmoc protected amino acids and resins were purchased from Sigma Aldrich, Merck, Acros, GL Biochem (Shanghai) Ltd, Fisher Scientific Fluorochem and Apollo Scientific. Commercially available reagents were used without further purification.

$^1$H and $^{13}$C NMR spectra were recorded on an automated Bruker AVA 500 (at 500 and 126 MHz, respectively) or a Bruker AVA 600 (at 600 and 151 MHz, respectively). Chemical shifts are reported in parts per million (ppm) on the $\delta$ scale with respect to the non-deuterated solvent residual peak for $^1$H spectra, and to the deuterated carbon resonance for $^{13}$C. All coupling constants ($J$ values) were measured in Hertz (Hz). Resonances are labelled as singlet (s), doublet (d), triplet (t), and multiplet (m). Low resolution electrospray ionization mass spectrometry (ESI-MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS). High-resolution mass spectra (HR-MS) were measured on (a): Finnigan MAT 900 XLP high resolution double-focusing mass spectrometer or (b): a 12T Bruker Daltonics-Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) using electrospray ionisation at 4.5 kV. The spectra were acquired with a $m/z$ range of 147 to 3000, and a minimal collisional activation of -2 V was used. The ions accumulation was set at 200 ms, and 16 Free Induced Decay (FID) transients were combined to produce a mass spectrum.
at 2 MW resolution. Reactions under microwave irradiation were performed using a Biotage® Initiator™.

Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck) with a forced flow of eluent. Analytical TLC was carried out using commercially available silica gel (F254 plates, Merck) and visualized at 254 nm UV light.

Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 system equipped with a Phenomenex Kinetex 5 μm XB-C18 100 Å LC Column (50 × 4.6 mm) with a flow rate of 1 mL/min. Samples were eluted with a gradient of H₂O/CH₃CN (buffered with 0.1% HCO₂H) from 5% of CH₃CN in H₂O to 95% of CH₃CN over 6 min, then holding at 95% for 3 min, followed by elution at 5% CH₃CN for 1 min, with detection by evaporative light scattering (ELSD) at UV and visible wavelengths. Semi-preparative RP-HPLC was performed on an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 RP column (250 × 9.4 mm, 5 μm) with a flow rate 2.0 mL/min, eluting with a gradient of 5% of CH₃CN in H₂O to 95% of CH₃CN (with 0.1% HCO₂H) over 25 min, followed by 5 min isocratic period. Preparative reverse phase HPLC (RP–HPLC) was performed on an Agilent 1100 system equipped with a Kinetex XB-C18, AXIA Packed reverse-phase column (150 x 21.2 mm, 5 μm, 100A) with a flow rate 10 mL/min, with a gradient of 5% of CH₃CN in H₂O to 95% of CH₃CN (buffered with 0.1% HCO₂H) over 18 min and additional isocratic period of 1 min. UV/Vis absorbance spectroscopy was performed on an Agilent 8453 absorbance spectrometer and fluorescence spectroscopy was performed on a Shimadzu RF-6000.
7.2. General solid-phase synthetic methods for amide tetrazines 4–10

**Rink-amide linker attachment to Aminomethyl ChemMatrix resin.**

4-[(2,4-Dimethoxyphenyl)-(Fmoc-amino)methyl]phenoxyacetic acid (Fmoc-Rink amide-linker, 0.97 g, 1.8 mmol, 3.0 equiv.) and ethyl(hydroxyimino)cyanoacetate (Oxyma, 0.26 g, 1.8 mmol, 3 equiv.) were dissolved in DMF (5 mL, 0.1 M) and the mixture was stirred for 10 min. *N,N’*-Diisopropylcarbodiimide (DIC, 279 µL, 1.8 mmol, 3 equiv.) was added and the solution was stirred for 1 min. This solution was added to the aminomethyl ChemMatrix resin (1.0 g, 0.6 mmol/g, 1 equiv., pre-swollen in DCM) and the mixture was stirred at 50 °C in a DMF bath for 1 h. After cooling down to room temperature, the resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The coupling reaction was monitored by a Kaiser test. To cap any remaining free amines, the resin was treated with a mixture of Ac₂O:Pyridine:DMF (2:3:15, v/v/v) for 30 min, followed by washing with DMF (3 × 10 mL), DCM (3 × 10 mL), MeOH (3 × 10 mL) and Et₂O (10 mL).

**Fmoc deprotection.** A solution of 20% piperidine in DMF (5 mL, 0.1 M) was added to the resin (1.0 g, 0.6 mmol/g, pre-swollen in DCM), and the reaction mixture was shaken for 10 min. The solution was then drained, and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). This procedure was repeated twice.

**Cyanobenzoic acid coupling.** The appropriate cyanobenzoic acid (0.27 g, 1.8 mmol, 3 equiv.) and Oxyma (0.26 g, 1.8 mmol, 3 equiv.) were dissolved in DMF (5 mL, 0.1 M) and stirred for 10 min. DIC (1.8 mmol, 3 equiv.) was added and the solution was stirred for 1 min. The mixture was then added to the resin (1.0 g, 0.6 mmol/g, pre-swollen in DCM),
and the reaction mixture was stirred at 50 °C in a DMF bath for 2 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The coupling reaction was monitored by the Kaiser test.\(^{127}\)

**Dihydrotetrazine formation.** A mixture of 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.) and hydrazine hydrate (1 mL, 0.06 M) was added to the benzonitrile functionalised resin (100 mg, 60 µmol, 1 equiv.) with either DCM or nitrile precursors to form either the mono- or disubstituted dihydrotetrazine with a variety of functional groups. Further details on the scope are given below.

**Oxidation of dihydrotetrazines to tetrazines.** A solution of NaNO\(_2\) (70 mg, 1.0 mmol, 17 equiv.) in water (5 mL, 0.1 M) was added to the resin (100 mg, 60 µmol/g, 1 equiv., pre-swollen in DCM), followed by dropwise addition of aqueous HCl (2 M) until pH ~3 was obtained. The resin beads turned pink-red within 2–4 minutes indicating the formation of the tetrazine (Caution: this procedure must be carried out in a well-ventilated fume hood due to the formation of nitrous fumes at this stage). The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

**Cleavage off the resin.** The resin (100 mg, 60 µmol, 0.1 equiv., pre-swollen in DCM) was shaken for 3 h in a mixture of TFA/DCM (1 mL, 1:1 v/v). The solution was collected by filtration and the resin was rinsed with a portion of the cleavage mixture (0.5 mL). The combined filtrates were diluted with toluene (5 mL) to avoid the degradation of tetrazine due to TFA, when the solvents were evaporated to dryness *in vacuo*.
A mixture of DCM (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) was added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in DCM). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the crude product was collected and purified by column chromatography (Petroleum ether/EtOAc/AcOH 50:50:1, v/v/v) to give compound 4 as a pink solid (10 mg, 86%).

Rf 0.30 (Petroleum ether/EtOAc/AcOH 50:50:1)

$^{1}H$ NMR (500 MHz, DMSO-$d_6$) δ 10.64 (s, 1H), 8.57 (d, $J = 8.5$ Hz, 2H), 8.19 (s, 1H), 8.14 (d, $J = 8.5$ Hz, 2H), 7.58 (s, 1H). $^{13}C$ NMR (126 MHz, DMSO-$d_6$) δ 167.1, 165.2, 158.2, 137.9, 134.3, 128.5, 127.7.

HRMS (ESI) for C$_9$H$_7$O$_1$N$_5$Na$_1$ [M+Na]$^+$ calcd. 224.0543; found 224.0534.
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4-(6-Methyl-1,2,4,5-tetrazin-3-yl)benzamide (5)

A mixture of acetonitrile (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) was added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the crude product was collected and purified by column chromatography (petroleum ether/EtOAc/AcOH = 50:50:1.5, v/v/v) to give compound 5 as a red/pink solid (12 mg, 88%).

R_f 0.25 (petroleum ether/EtOAc/AcOH = 50:50:1.5).

^1H NMR (600 MHz, DMSO-<_d_6_>) δ 8.53 (d, J = 8.5 Hz, 2H), 8.18 (s, 1H), 8.13 (d, J = 8.5 Hz, 2H), 7.56 (s, 1H), 3.02 (s, 3H). ^13C NMR (151 MHz, DMSO-<_d_6_>) δ 167.3, 166.4, 162.9, 137.6, 134.3, 128.5, 127.3, 20.9.

HRMS (ESI) for C_{10}H_{10}N_{5}O_{1} [M+H]^+ calcd. 216.0880; found 216.0883.
A mixture of tert-butylcyanoacetate (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) was added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the resulting crude product was collected and purified by washing and centrifuging with DMSO (3 × 1 mL), acetone (3 × 3 mL) then DCM (3 × 3 mL) and the supernatant solutions were decanted to give compound 6 as a purple solid (14 mg, 86%).

**HRMS** (ESI) for C_{11}H_{10}N_{5}O_{3}[M+H]^+ calcd. 260.0778; found 260.0784.

*Note:* Due to the very poor solubility of compound 6 in all NMR polar or non-polar solvents tried, NMR spectrum could not be recorded.
4-(6-Phenyl-1,2,4,5-tetrazin-3-yl)benzamide (7)

A mixture of benzonitrile (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) was added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the resulting crude product was collected and purified by washing and centrifuging with DMSO (3 × 1 mL), then DCM (3 × 3 mL). The supernatant solutions were decanted to give compound 7 as a purple solid (13 mg, 77%).

\(^1\)H NMR (500 MHz, DMF-d\(_7\)) \(\delta\) 8.70 (d, \(J = 8.5\) Hz, 2H), 8.65 (d, \(J = 8.5\) Hz, 2H), 8.33 (s, 1H), 8.30 (d, \(J = 8.5\) Hz, 2H), 7.82–7.72 (m, 3H), 7.58 (s, 1H).

\(^1^3\)C NMR (126 MHz, DMF-d\(_7\)) \(\delta\) 167.6, 164.1, 163.7, 138.3, 134.8, 132.9, 132.4, 129.6, 129.05, 128.7, 127.9.

HRMS (ESI) for C\(_{15}\)H\(_{12}\)N\(_3\)O\(_1\) [M+H]\(^+\) calcd. 278.1036; found 278.1033.
A solution of 4-methoxybenzonitrile (24 mg, 0.18 mmol, 3 equiv.) in 1,4-dioxane (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) were added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the resulting crude product was collected and purified by washing and centrifuging with hexane (3 × 3 mL) and then MeOH (3 × 3 mL). The supernatant solutions were decanted to give compound 8 was obtained as a pink solid (18 mg, 92%).

\(^1\text{H NMR}\) (600 MHz, DMF-\(d_7\)) \(\delta\) 8.67 (d, \(J = 8.5\) Hz, 2H), 8.61 (d, \(J = 8.5\) Hz, 2H), 8.31 (s, 1H), 8.29 (d, \(J = 8.5\) Hz, 2H), 7.57 (s, 1H), 7.31 (d, \(J = 8.5\) Hz, 2H), 4.00 (s, 3H).

\(^1^3\text{C NMR}\) (126 MHz, DMF-\(d_7\)) \(\delta\) 168.6, 164.7, 164.2, 139.1, 135.9, 130.8, 129.6, 128.8, 128.4, 125.5, 116.1, 56.5.

\textit{HRMS} (ESI) for C\textsubscript{16}H\textsubscript{14}N\textsubscript{5}O\textsubscript{2} [M+H]\(^+\) \textit{calcd}. 308.1142; \textit{found} 308.1139.
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4-(6-(4-Nitrophenyl)-1,2,4,5-tetrazin-3-yl)benzamide (9)

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{N=N} \\
\text{N-N} & \quad \text{N} \\
\text{O} & \quad \text{NH}_2
\end{align*}
\]

A solution of 4-nitrobenzonitrile (26.7 mg, 0.18 mmol, 3 equiv.) in 1,4-dioxane (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) were added to benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the resulting crude product was collected and purified by washing and centrifuging with hexane (3 × 3 mL) and then MeOH (3 × 3 mL). The supernatant solutions were decanted to give compound 9 as a pink solid (14 mg, 70%).

\(^1\text{H NMR}\) (500 MHz, DMF-\(d_7\)) \(\delta 8.70\) (d, \(J = 8.5\) Hz, 2H), \(8.65\) (d, \(J = 8.5\) Hz, 2H), \(8.34 - 8.27\) (m, 3H), \(7.76\) (d, \(J = 8.5\) Hz 2H), \(7.59\) (s, 1H).

\(\text{HRMS (ESI) for of C}_{15}\text{H}_{11}\text{N}_6\text{O}_3\ [\text{M+H}]^+\) calcd. 323.0887; found 323.0885.

\textbf{Note:} Due to the low solubility of compound 9 in DMF-\(d_7\) and other common NMR organic solvents, a \(^{13}\text{C NMR} \) spectrum could not be recorded. Prior to HRMS (ESI) analysis, the IMS-MS was tuned, and a mass calibration was performed using Agilent Tune Mix from the manufacturer (Cat No.: G2421-60001, Agilent Technologies).
A solution of 4-fluorobenzonitrile (22 mg, 0.18 mmol, 3 equiv.) in 1,4-dioxane (2 mL, 0.03 M), 3-mercaptopropionic acid (15.7 µL, 0.18 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.06 M) were added to the benzonitrile functionalised Rink linker bound resin (100 mg, 60 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were diluted with toluene to prevent degradation of the tetrazine by TFA. The solvents and TFA were then removed by evaporation under reduced pressure, and the resulting crude product was collected and purified by washing and centrifuging with hexane (3 × 3 mL), and DCM (3 × 3 mL) then MeOH (3 × 3 mL). The supernatant solutions were decanted to give compound 10 as a pink solid (15 mg, 84%).

\[ \text{1H NMR (500 MHz, DMF-}d_7) \delta 8.72 (d, J = 8.5 \text{ Hz, 2H}), 8.70 (d, J = 8.5 \text{ Hz, 2H}), 8.33 (s, 1H), 8.30 (d, J = 8.5 \text{ Hz, 2H}), 7.61 (s, 1H), 7.58 (d, J = 8.5 \text{ Hz, 2H}). \]

\[ \text{13C NMR (126 MHz, DMF-}d_7) \delta 168.6, 167.9, 167.7, 164.6 (J = 241.3 \text{ Hz}), 139.3, 135.7, 133.5, 131.6, 131.5 (d, J = 9.4 \text{ Hz}), 129.7, 129.5, 128.7, 117.8, 117.6 (d, J = 22.4 \text{ Hz}). \]

\[ \text{19F(H) NMR (400 MHz, DMF-}d_7) \delta 108.2. \]

\[ \text{HRMS (ESI) for } \text{C}_{15}\text{H}_{10}\text{F}_{1}\text{N}_{5}\text{O}_{1}\text{K}_{1} [M+K]^+ \text{ calcd. 334.0501; found 334.0500.} \]
7.3. General solid-phase synthetic methods for carboxyl tetrazines 13–21

**Activation of 2-chloro/hydroxy-trityl chloride linker (CLTR-Cl) polystyrene resin.**

A solution of SOCl₂ (240 μL, 1.2 mmol, 1.3 equiv.) in anhydrous DCM (10 mL, 0.1 M) was added to the commercially available 2-chloro/hydroxy-trityl chloride linker polystyrene resin (1.0 g, 0.95 mmol/g, 1 equiv., pre-swollen in anhydrous DCM) under an Ar atmosphere. The reaction mixture was stirred for 1 h. The solvent was drained, and the resin was washed with anhydrous DCM (3 × 10 mL) and anhydrous DMF (3 × 10 mL) and used immediately. Note: This activation process converts any hydrolyzed sites to the chloride before subsequent esterification.

**Attachment of benzoic or nicotinic acid to the linker.** A solution of either cyanobenzoic or nicotinic acid (2.5 mmol, 3 equiv., 0.2 M) and DIPEA (456 μL, 4.8 mmol, 5 equiv.) in anhydrous DCM/DMF (7:1, v/v) was added to the pre-activated resin (1.0 g, 0.95 mmol/g, pre-swollen in anhydrous DCM), and the reaction mixture was stirred for 1 h. The solution was drained, and the resin was washed with anhydrous DCM (3 × 10 mL) and anhydrous DMF (3 × 10 mL). To cap any unreacted linker, the resin was treated with a mixture of DCM/MeOH/DIPEA (8:1.5:0.5, v/v/v) for 2 × 15 min followed by washing with DMF (3 × 10 mL), iPrOH (3 × 10 mL) and hexane (3 × 10 mL).

**Dihydrotetrazine formation.** A mixture of 3-mercaptopropionic acid (25.3 μL, 0.29 mmol, 3 equiv.) and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl resin (100 mg, 95 μmol, 1 equiv.) with either DCM or nitrile precursors to form either the
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mono- or disubstituted dihydrotetrazines. Further details on the scope of the reaction are given below.

**Oxidation of dihydrotetrazines to tetrazines.** A solution of NaNO₂ (70 mg, 1.0 mmol, 10 equiv.) in water (5 mL, 0.1 M) was added to the resin (100 mg, 95 µmol/g, 1 equiv., pre-swollen in DCM), followed by dropwise addition of 2 M HCl (aq.) until pH ~3 was obtained. The resin beads turned pink-red within 2–4 min indicating the formation of a tetrazine (Caution: this procedure must be carried out in a well-ventilated fume hood due to the formation of nitrous fumes at this stage).

**Cleavage off the resin.** The resin (100 mg, 95 µmol, 1 equiv., pre-swollen in DCM) was shaken for 2 h in 20% HFIP in DCM (1 mL). The solution was collected by filtration and the resin was rinsed with 20% HFIP in DCM (0.5 mL). The solutions were combined and evaporated to dryness *in vacuo*.

**4-(1,2,4,5-Tetrazin-3-yl)benzoic acid (13)**

![Structure of 4-(1,2,4,5-Tetrazin-3-yl)benzoic acid (13)](image)

A mixture of DCM (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in DCM). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following
oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by column chromatography (Petroleum ether/EtOAc/AcOH 50:50:1, v/v/v) to give compound 13 as a pink solid (16 mg, 81%).

Rf 0.20 (Petroleum ether/EtOAc/AcOH 50:50:1).

$^1$H NMR (600 MHz, DMSO-$d_6$) δ 10.66 (s, 1H), 8.61 (d, $J$ = 8.4 Hz, 2H), 8.22 (d, $J$ = 8.4 Hz, 2H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 166.7, 165.1, 158.3, 135.7, 132.7, 130.2, 128.0.

HRMS (ESI) for C$_9$H$_5$N$_4$O$_2$ [M-H]$^-$ calcd. 201.0418; found 201.0408.

Analytical data in agreement with literature.$^{227}$

4-(6-Methyl-1,2,4,5-tetrazin-3-yl)benzoic acid (14)

A mixture of acetonitrile (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by
column chromatography (Petroleum ether/EtOAc/AcOH 50:50:1.5, v/v/v) to give compound 14 as a pink solid (17 mg, 83%).

**R**<sub>f</sub> 0.38 (Petroleum ether/EtOAc/AcOH 50:50:1.5).

**<sup>1</sup>H NMR** (600 MHz, DMSO-<em>d</em><em>6</em>) δ 8.58 (d, <em>J</em> = 8.4 Hz, 2H), 8.20 (d, <em>J</em> = 8.4 Hz, 2H), 3.03 (s, 3H). **<sup>13</sup>C NMR** (151 MHz, DMSO-<em>d</em><em>6</em>) δ 167.4, 166.7, 162.8, 135.7, 134.0, 130.2, 127.6, 20.9.

**HRMS** (ESI) for C<sub>10</sub>H<sub>9</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup> *calcd.* 217.0720; *found* 217.0722.

Analytical data in agreement with literature.<sup>227</sup>

4-(6-Phenyl-1,2,4,5-tetrazin-3-yl)benzoic acid (15)

![Chemical Structure](image)

A mixture of benzonitrile (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by washing and centrifuging with hexane (3 x 1 mL), then diethyl ether (3 x 3 mL). The supernatant solutions were decanted to give compound 15 as a purple solid (21 mg, 80%).
\[ ^1H \text{NMR} \ (500 \text{ MHz, DMSO-}d_6) \ \delta \ 8.65 \ (d, J = 8.5 \text{ Hz, } 2H), \ 8.57 \ (d, J = 8.5 \text{ Hz, } 2H), \ 8.24 \ (d, J = 8.5 \text{ Hz, } 2H), \ 7.77 - 7.68 \ (m, 3H). \]

\[ ^{13}C \text{NMR} \ (126 \text{ MHz, DMSO-}d_6) \ \delta \ 166.7, \ 163.4, \ 163.0, \ 135.6, \ 132.8, \ 131.7, \ 130.3, \ 130.2, \ 129.5, \ 127.7, \ 127.7. \]

HRMS (ESI) for C_{15}H_{9}N_4O_2 [M-H] \text{ calcd.} \ 277.0731; \text{ found} \ 277.0720.

6-(1,2,4,5-Tetrazin-3-yl)nicotinic acid (16)

A mixture of DCM (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 \mu L, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 \mu mol, 1 equiv., pre-swollen in DCM). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 \times 10 mL), DCM (3 \times 10 mL) and MeOH (3 \times 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by column chromatography (Acetone/MeOH 9:1, v/v) to give compound 16 as a pink solid (16 mg, 83%).

Rf 0.31 (Acetone/MeOH 9:1)
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\(^{1}H\) NMR (500 MHz, DMSO-\(d_{6}\)) \(\delta\) 10.75 (s, 1H), 9.35 (s, 1H), 8.67 (d, \(J = 8.0\) Hz, 1H), 8.58 (d, \(J = 8.0\) Hz, 1H). \(^{13}C\) NMR (126 MHz, DMSO-\(d_{6}\)) \(\delta\) 165.7, 164.9, 158.5, 153.2, 150.9, 138.7, 128.7, 124.1.

HRMS (ESI) for \(\text{C}_{8}\text{H}_{6}\text{N}_{5}\text{O}_{2}\) \([\text{M}+\text{H}]^+\) calcd. 204.0516; found 204.0516.

Analytical data in agreement with literature.\(^{227}\)

6-(6-Methyl-1,2,4,5-tetrazin-3-yl)nicotinic acid (17)

![Structural formula](image)

A mixture of acetonitrile (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 \(\mu\)L, 0.29 mmol, 3 equiv.) and hydrazine hydrate (1 mL, 0.1 M) were added to the nitrile functionalised trityl linker bound resin (100 mg, 95 \(\mu\)mol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by preparative reverse phase HPLC and the desired fractions were pooled and lyophilised to afford compound 17 as a purple-red solid (18 mg, 85%).

\(^{1}H\) NMR (500 MHz, DMSO-\(d_{6}\)) \(\delta\) 9.31 (s, 1H), 8.59 (d, \(J = 8.0\) Hz, 1H), 8.53 (d, \(J = 8.0\) Hz, 1H), 3.06 (s, 3H). \(^{13}C\) NMR (126 MHz, DMSO-\(d_{6}\)) \(\delta\) 168.1, 166.6, 163.4, 152.8, 151.4, 151.0 138.8, 123.9, 21.5.
HRMS (ESI) for C_{9}H_{8}N_{5}O_{2} [M+H]^+ calcd. 218.0673; found 218.0673.

Analytical data in agreement with literature.

6-(6-Phenyl-1,2,4,5-tetrazin-3-yl)nicotinic acid (18)

A mixture of benzonitrile (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by washing and centrifuging with DCM (3 × 3 mL), then hexane (3 × 3 mL). The supernatant solutions were decanted to give compound 18 as a purple solid (20.7 mg, 78%).

\[ {^1}H \text{ NMR (500 MHz, DMF-}d_7) \delta 9.47 (s, 1H), 8.77 (d, J = 8.4 Hz, 1H), 8.70 – 8.64 (m, 3H), 7.82 – 7.73 (m, 3H). \]

\[ {^{13}}C \text{ NMR (126 MHz, DMF-}d_7) \delta 167.2, 165.1, 164.7, 153.8, 150.9, 137.9, 134.1, 133.2, 133.1, 130.7, 129.2, 124.7. \]

HRMS (ESI) for C_{14}H_{10}N_{5}O_{2} [M+H]^+ calcd. 280.0829; found 280.0829.
6-(6-(5-Bromopyridin-3-yl)-1,2,4,5-tetrazin-3-yl)nicotinic acid (19)

A mixture of 5-bromo-3-cyanopyridine (53 mg, 0.29 mmol, 3 equiv.) in 1,4-dioxane (2 mL, 0.03 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by column chromatography (DCM/MeOH/AcOH 90:10:1, v/v/v) to give compound 19 as a pink solid (29 mg, 85%).

Rf 0.45 (DCM/MeOH/AcOH 90:10:1).

\[ ^1H \text{ NMR} \ (600 \text{ MHz, DMSO-}d_6) \ \delta \ 9.62 \ (d, \ J = 1.9 \text{ Hz, 1H}), \ 9.36 \ (dd, \ J = 2.0, 0.9 \text{ Hz, 1H}), \ 9.07 \ (d, \ J = 2.3 \text{ Hz, 1H}), \ 9.00 \ (t, \ J = 2.1 \text{ Hz, 1H}), \ 8.63 \ (dd, \ J = 8.0, 0.9 \text{ Hz, 1H}), \ 8.53 \ (dd, \ J = 8.0, 2.0 \text{ Hz, 1H}). \]

\[ ^{13}C \text{ NMR} \ (126 \text{ MHz, DMSO-}d_6) \ \delta \ 166.1, \ 163.4, \ 161.8, \ 153.8, \ 151.4, \ 150.6, \ 147.2, \ 138.0, \ 137.4, \ 129.8, \ 123.8, \ 120.9, \ 118.9. \]

HRMS (ESI) for C_{13}H_7BrN_6O_2 [M-H] calcd. 357.9774; found 357.9753.
6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinic acid (20)

A mixture of 2-cyanopyridine (2 mL, 0.05 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by column chromatography (DCM/MeOH/AcOH 95:5:1, v/v/v) to give compound 20 as a pink solid (24 mg, 90%).

R_f 0.50 (DCM/MeOH/AcOH 95:5:1).

^1H NMR (500 MHz, DMF-d_7) δ 9.46 (dd, J = 2.1, 0.9 Hz, 1H), 9.00 (dt, J = 4.7, 1.8, 0.9 Hz, 1H), 8.82 (d, J = 8.0 Hz, 1H), 8.74 – 8.67 (m, 2H), 8.24 (td, J = 7.7, 1.8 Hz, 1H), 7.82 – 7.76 (ddd, J = 7.7, 4.7, 0.9 Hz, 1H). ^13C NMR (126 MHz, DMF-d_7) δ 173.8, 164.9, 164.8, 152.4, 151.9, 151.8, 146.7, 139.6, 138.8, 137.8, 127.8, 125.7, 125.2.

HRMS (ESI) for C_{13}H_{8}N_{6}O_{2} [M-H] calcd 279.0636; found 279.0618.

Analytical data in agreement with literature. \(^{228}\)

6,6’-(1,2,4,5-Tetrazine-3,6-diyl)dinicotinic acid (21)
A mixture of 6-cyanonicotinic acid (43 mg, 0.29 mmol, 3 equiv.) in 1,4-dioxane (2 mL, 0.03 M), 3-mercaptopropionic acid (25.3 µL, 0.29 mmol, 3 equiv.), and hydrazine hydrate (1 mL, 0.1 M) was added to the nitrile functionalised trityl linker bound resin (100 mg, 95 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). Following oxidation and cleavage, the combined filtrates were collected and evaporated under reduced pressure, and the resulting crude product was purified by washing and centrifuging with DMSO (3 × 1 mL), acetone (3 × 3 mL) then DCM (3 × 3 mL) and the supernatant solutions were decanted to give compound 21 as a pink solid (29 mg, 94%).

^1H NMR (600 MHz, Acetic acid-d4) δ 9.34 (dd, J = 2.1, 0.9 Hz, 2H), 8.59 (dd, J = 8.0, 2.1 Hz, 2H), 8.00 (dd, J = 8.0, 0.9 Hz, 2H). ^13C NMR (151 MHz, Acetic acid-d4) δ 167.0, 151.6, 139.1, 136.6, 128.6, 128.6, 116.1.

HRMS (ESI) for C_{14}H_{8}N_{6}O_{4} [M-H]^− calcd 323.0534; found 323.0514.
7.4 In Situ formation of s-tetrazine on peptide

7-(Diethylamino)-3-iodo-4-methyl-2H-chromen-2-one (23)

![Chemical structure of 23](image)

To a solution of 7-diethylamino-4-methylcoumarin (3.00 g, 13.0 mmol, 1 equiv.) in dioxane (150 mL), a solution of iodine (16.5 g, 65.0 mmol, 5 equiv.) in a mixture of 1,4-dioxane (60 mL) and pyridine (12 mL) was added under Ar atmosphere. The resulting mixture was stirred in the dark for 3 h. A 50 mL saturated solution of sodium thiosulfate was added into the reaction mixture and extracted with EtOAc (3 × 75 mL). The combined organic layers were washed with water, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by recrystallization from hexane:acetone (99:1, v/v) to give the compound 23 as a yellow solid (4.4 g, 96%).

HPLC (350 nm) \( t_R = 7.68 \) min.

1H NMR (500 MHz, CDCl₃) δ 7.38 (d, \( J = 9.1 \) Hz, 1H), 6.51 (dd, \( J = 9.1, 2.5 \) Hz, 1H), 6.50 (d, \( J = 2.5 \) Hz, 1H), 3.34 (q, \( J = 7.1 \) Hz, 4H), 2.53 (s, 3H), 1.14 (t, \( J = 7.1 \) Hz, 6H). 13C NMR (126 MHz, CDCl₃) δ 160.0, 156.8, 155.2, 150.39, 144.0, 126.4, 109.0, 97.1, 83.8, 44.9, 25.1, 12.6.

LCMS (ESI) for C₁₄H₁₆NIO₂ [M+H]+ calcd. 358.1; found 358.0.

NMR and mass data in agreement with literature.124
3-(7-(Diethylamino)-4-methyl-2-oxo-2H-chromen-3-yl)benzonitrile (24)

Iodocoumarin 23 (263 mg, 0.733 mmol, 1 equiv.), 3-cyanophenylboronic acid (215 mg, 1.47 mmol, 2 equiv.), Pd(OAc)$_2$(PPh$_3$)$_2$ (27.4 mg, 0.037 mmol, 0.1 equiv.), and K$_2$CO$_3$ (203 mg, 1.46 mmol, 2 equiv.) were suspended in a mixture of 1,4-dioxane:water (8 mL, 3:1 v/v). The reaction mixture was refluxed for 7 h. The mixture was then cooled to down room temperature and then extracted with DCM (3 × 15 mL), dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (Hexane/EtOAc 6:1 to 4:1, v/v) to give compound 24 as a bright yellow solid (221 mg, 91%).

R$_f$ 0.65 (Hexane/EtOAc 6:1).

**HPLC** (350 nm) $t_R = 4.77$ min.

**$^1$H NMR** (500 MHz, CDCl$_3$) $\delta$ 7.63 – 7.57 (m, 2H), 7.56 – 7.48 (m, 2H), 7.45 (d, $J = 9.1$ Hz, 1H), 6.62 (dd, $J = 9.1$, 2.6 Hz, 1H), 6.51 (d, $J = 2.6$ Hz, 1H), 3.41 (q, $J = 7.1$ Hz, 4H), 2.20 (s, 3H), 1.20 (t, $J = 7.1$ Hz, 6H).

**$^{13}$C NMR** (126 MHz, CDCl$_3$) $\delta$ 161.6, 155.2, 150.8, 149.5, 136.8, 135.4, 134.3, 131.1, 129.2, 126.4, 118.7, 118.5, 112.5, 109.0, 108.9, 97.3, 44.8, 16.4, 12.47.

**LCMS** (ESI) for C$_{14}$H$_{16}$NIO$_2$ [M+H]$^+$ calcld. 333.4; found 333.4.

NMR and mass data in agreement with literature.$^{125}$
A solution of 6-cyanopyridine-3-carboxylic acid 25 (33.1 mg, 3 equiv. per amine, 0.03 M) in anhydrous DMF (3 mL), HSPyU (92.5 mg, 0.22 mmol, 3 equiv.), and DIPEA (21.6 µL, 0.22 mmol, 3 equiv.) were stirred for 2 h at 40 °C (the formation of the NHS ester was monitored by analytical HPLC). After complete conversion, this mixture was added to the MAD1 peptide functionalised polystyrene resin (100 mg, 75 µmol, 1 equiv., pre-swollen in DCM) in presence of DIPEA (21.6 µL, 0.22 mmol, 3 equiv.) and shaken overnight. The solution was then drained, and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The resin (pre-swollen in DCM) was shaken for 3 h in TFA/TIS/DCM (1 mL, 90:5:5 v/v/v). The solution was collected by filtration and the resin was washed with the cleavage cocktail. The combined filtrates were added to cold ether, and the precipitated solid was collected by centrifugation, and washed repeatedly with cold ether (3 × 20 mL). The crude peptide was purified by preparative RP-HPLC to give compound 27 as a white solid (144 mg, 88%).

**HPLC** (254 nm) \( t_R = 3.08 \text{ min.} \)

**HRMS** (ESI) for \( \text{C}_{108}\text{H}_{136}\text{N}_{35}\text{O}_{14} \) [M+H]+ \( \text{calcd. 2147.1001; found 2147.1068.} \)
CM-Tz-MAD1 probe (30)

A mixture of the coumarin-nitrile 24 (76.5 mg, 0.22 mmol, 3 equiv.), 3-mercaptopropionic acid (29.7 µL, 0.23 mmol, 3 equiv.) and hydrazine hydrate (1.0 mL, 0.07 M) was added to the resin-bound nitrile 26 (100 mg, 75 µmol, 1 equiv., pre-swollen in 1,4-dioxane). The reaction mixture was stirred in a sealed vial at 40 °C in a heating mantle for 24 h. The resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). After oxidation and cleavage (following the general solid-phase synthetic method for amide tetrazines described above but using 70% TFA/DCM to ensure complete removal of the sidechain protecting groups), the crude product was then purified by preparative RP-HPLC to give compound 30 as a red-orange solid (128 mg, 69%).

**HPLC** (254 nm) $t_R = 3.56$ min.

**HRMS** (ESI) for $C_{129}H_{156}N_{39}O_{16}$ [M+H]$^+$ calcd. 2507.2586; found 2507.2582.
7.5. General solid-phase synthetic methods for MAD1 fluorescent probes

**Fmoc-Rink linker/amino acid couplings.** A solution of the Fmoc-Rink linker-OH or the appropriate N-Fmoc protected amino acid (2.2 mmol, 3 equiv., Table 5.1) and Oxyma (0.31 g, 2.2 mmol, 3 equiv.) in DMF (5 mL, 0.1 M) were stirred for 10 min, after which DIC (223.8 µL, 2.2 mmol, 3 equiv.) was added and the mixture stirred for 1 min. This mixture was added to the resin (1.0 g, 0.745 mmol/g, 1 equiv., pre-swollen in DCM) and the mixture was stirred at 50 °C in a DMF bath for 1 h. After cooling to room temperature, the resin was drained and washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). To cap any remaining free amines, the resin was treated with a mixture of Ac₂O/Pyridine/DMF (2:3:15, v/v/v) for 30 min, followed by washing with DMF (3 × 10 mL), DCM (3 × 10 mL), MeOH (3 × 10 mL) and Et₂O (10 mL). Each coupling reactions were monitored by a Kaiser test.¹²⁷

**Table 5.1.** Identification and quantification of solid-phase coupling reagents.*

<table>
<thead>
<tr>
<th>Entry</th>
<th>Chemical*</th>
<th>Code</th>
<th>Weight (g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Rink amide linker-OH</td>
<td>RINK</td>
<td>0.54</td>
</tr>
<tr>
<td>2</td>
<td>Fmoc-Trp(Boc)-OH</td>
<td>W</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>Fmoc-Val-OH</td>
<td>V</td>
<td>0.34</td>
</tr>
<tr>
<td>4</td>
<td>Fmoc-His(Trt)-OH</td>
<td>H</td>
<td>0.62</td>
</tr>
<tr>
<td>5</td>
<td>Fmoc-Arg (Pbf)-OH</td>
<td>R</td>
<td>0.65</td>
</tr>
<tr>
<td>6</td>
<td>Fmoc-Lys(Boc)-OH</td>
<td>K</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*a. The L- and D-enantiomers of the MAD1 peptides were synthesised on gram scale using a SPPS approach with natural or unnatural amino acids. *b. Weight calculated equals three equivalents per amine.
Fmoc deprotection. 20% Piperidine in DMF (5 mL) was added to the pre-swollen resin and the reaction mixture was shaken for 10 min. The solution was then drained, and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). This procedure was repeated twice.

5-Carboxyfluorescein diacetate NHS ester coupling: A mixture of N-hydroxysuccinimide ester of 5-carboxyfluorescein diacetate\(^{161}\) (123 mg, 0.22 mmol, 3 equiv. per amine, 0.03 M) in DMF (3 mL) was added to the resin (100 mg, 75 µmol, 1 equiv., pre-swollen in DCM) followed by the addition of DIPEA (21 µL, 0.22 mmol, 3 equiv.) and were stirred for 30 min at 50 °C and then shaken at room temperature overnight. The solution was drained, and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL), and subsequently the resin was shaken for 10 min in 20 % piperidine in DMF at room temperature to remove any esters. The solution was drained, and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

NBD-Cl coupling. NBD-Cl (44 mg, 0.22 mmol, 3 equiv. per amine, 0.03 M) and DIPEA (21 µL, 0.22 mmol, 3 equiv.) in DMF (3 mL) was added to resin (100 mg, 75 µmol, 1.0 equiv., pre-swollen in DCM) and the reaction mixture shaken overnight. The solution was drained, and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

MeroCy-CO\(_2\)H coupling. A mixture of MeroCy-CO\(_2\)H\(^{175,176}\) (108 mg, 0.22 mmol, 3 equiv. per amine, 0.03 M) in DMF (3 mL), HSPyU (90.5 mg, 0.22 mmol, 3 equiv.) and DIPEA (21 µL, 0.22 mmol, 3 equiv.) were stirred for 2 h at 40 °C (the formation of the NHS ester was monitored by analytical HPLC). After completion, the mixture was added to the resin (100
mg, 75 µmol, 1 equiv., pre-swollen in DCM) followed by the addition of DIPEA (21 µL, 0.22 mmol, 3 equiv.) and shaken overnight. The solution was then drained, and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

**Sulfonated Cy5-CO₂H coupling.** A mixture of sulfonated Cy5-CO₂H₁₇₇ (146 mg, 3 equiv. per amine, 0.03 M) in DMF (3 mL), HSPyU (90.5 mg, 0.22 mmol, 3 equiv.) and DIPEA (21 µL, 0.22 mmol, 3 equiv.) were stirred for 2 h at 40 °C (the formation of the NHS ester was monitored by analytical HPLC). After completion, the mixture was added to the resin (100 mg, 75 µmol, 1 equiv., pre-swollen in DCM) followed by the addition of DIPEA (21 µL, 0.22 mmol, 3 equiv.) and shaken overnight. The solution was drained, and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

**Cleavage off the resin and deprotection.** The pre-swollen resin (in DCM) was shaken in TFA/TIS/DCM (95:5:5 v/v/v) for 3 h at a ratio of 100 mg resin to 1 mL of the cleavage cocktail. The filtrate was collected, and the product was precipitated into cold diethyl ether and collected by centrifugation (repeated twice). The crude peptides were dissolved in H₂O/CH₃CN (1:1, v/v) and purified by semi-preparative HPLC, and analysed by HPLC and HRMS.

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The characterisation of all synthesised MAD1 probes is detailed in Chapter 4, accompanied by HPLC and HRMS results provided in the appendices.
(Z)-2-((E)-3-Methoxyallylidene)benzo[b]thiophen-3(2H)-one 1,1-dioxide (41)

A mixture of benzo[b]thiophen-3(2H)-one 1,1-dioxide 39 (364 mg, 2 mmol, 1 equiv.) and 1,1,3,3-tetramethoxypropane 40 (1.65 mL, 1 mmol, 5 equiv.) was stirred with TFA (15 μL, 0.2 mmol, 0.1 equiv.) in a sealed vial and heated under microwave irradiation at 150 °C for 15 min. The reaction was allowed to cool to room temperature and the precipitate was filtered and washed with cold hexane/diethyl ether (30 mL, 3:1 v/v), to give compound 41 as a red solid (372 mg, 74%).

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.05 (d, $J = 7.6$ Hz, 1H), 7.98 (d, $J = 7.6$ Hz, 1H), 7.87 – 7.79 (m, 2H), 7.67 (d, $J = 12.8$ Hz, 1H), 7.45 (d, $J = 12.0$ Hz, 1H), 6.46 (t, $J = 12.4$ Hz, 1H), 3.96 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 178.5, 168.9, 144.3, 143.3, 136.0, 133.98, 133.1, 126.8, 124.7, 121.3, 101.5, 59.0

LCMS (ESI) for C$_{12}$H$_{11}$O$_4$S [M+H]$^+$ calcd. 251.3; found 251.4.

$^1$H NMR and mass data in agreement with literature.\textsuperscript{175}
1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium (44)

A mixture of 6-bromohexanoic acid 43 (390 mg, 2 mmol, 2 equiv.) and 2,3,3-trimethyl-3H-indole 42 (159.2 mg, 1 mmol, 1 equiv.) in acetonitrile (15 mL) was stirred and refluxed for 24 h. The solution was cooled to room temperature and evaporated. In an ice bath, the solid residue was dissolved in dichloromethane (8 mL), then diethyl ether was added to precipitate the product (15 mL). The precipitate was collected by filtration and washed with diethyl ether (3 × 15 mL) to give compound 44 as a grey solid (390 mg, 71%).

$^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.03 (s, 1H), 7.91 (d, $J$ = 8.4 Hz, 1H), 7.83 (dd, $J$ = 8.4, 1.6 Hz, 1H), 4.44 (t, $J$ = 7.7 Hz, 2H), 2.83 (m, 4H), 2.23 (t, $J$ = 7.2 Hz, 2H), 1.87 – 1.80 (m, 2H), 1.55 (m, 8H), 1.45 – 1.37 (m, 2H).$^{13}$C NMR (126 MHz, CD$_3$OD) δ 197.3, 174.3, 149.5, 141.5, 140.9, 126.4, 120.7, 115.0, 54.3, 47.6, 34.3, 33.4, 26.9, 25.4, 24.0, 21.9, 14.1.

LCMS (ESI) for C$_{17}$H$_{25}$NO$_2$ [M+H]$^+$ calcd 275.2; found 275.3.

Analytical data in agreement with literature.$^{176}$
CHAPTER 7

6-((E)-2-(((2E,4Z)-4-(1,1-Dioxido-3-oxobenz[b]thiophen-2(3H)-ylidene)but-2-en-1-ylidene)-3,3-dimethylindolin-1-yl)hexanoic acid (45)

Compound 41 (91.0 mg, 0.36 mmol, 1 equiv.), indolium salt 44 (100 mg, 0.36 mmol, 1 equiv.) and sodium acetate (30.0 mg, 0.36 mmol, 1 equiv.) were mixed with MeOH:DCM (5 mL, 1:1 v/v) in a sealed vial and heated under microwave irradiation at 75 °C for 30 min. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude product was purified by column chromatography (MeOH/DCM 1:15, v/v) to give compound 45 as a blue solid (161 mg, 90%).

Rf 0.39 (MeOH/DCM 1:15).

HPLC (600 nm) tR = 3.95 min

1H NMR (500 MHz, CD3OD) δ 8.04 – 8.03 (m, 1H), 7.94 – 7.88 (m, 3H), 7.86 – 7.79 (m, 2H), 7.60 – 7.53 (m, 1H), 7.42 – 7.41 (m, 1H), 7.38 – 7.33 (m, 1H), 7.18 – 7.15 (t, J = 7.3 Hz, 1H), 6.57 – 6.52 (t, J = 12.6 Hz, 1H), 6.16 – 6.13 (d, J = 13.4 Hz, 1H), 4.02 – 3.99 (t, J = 7.6 Hz, 2H), 2.33 – 2.29 (m, 2H), 1.83 – 1.78 (m, 2H), 1.70 – 1.69 (m, 2H), 1.67 (s, 6H), 1.52 – 1.47 (m, 2H). 13C NMR (125 MHz, DMSO-d6) δ 176.1, 174.7, 171.9, 170.7, 155.9, 144.2,
HRMS (ESI) for C\textsubscript{28}H\textsubscript{30}NO\textsubscript{5}S [M+H]\textsuperscript{+} calcd. 492.1839; found 492.1849.

NMR and mass data in agreement with literature\textsuperscript{173}

\textbf{2,3,3-Trimethyl-3H-indole-5-sulfonate (48)}

![Chemical structure of 2,3,3-Trimethyl-3H-indole-5-sulfonate (48)]

4-Hydrazinobenzene sulfonic acid 46 (5.00 g, 26.5 mmol, 1 equiv.) and 3-methyl-2-butane 47 (8.40 mL, 79 mmol, 3 equiv.) were dissolved in acetic acid (45.0 mL) and refluxed at 120 °C for 4 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in MeOH (5 mL) and stirred with a saturated solution of potassium hydroxide in 2-propanol (15 mL). The precipitate was collected by filtration and washed thoroughly with 2-propanol (4 × 20 mL), then with diethyl ether (4 × 20 mL) to give compound 48 as a yellow solid (4.7 g, 75%).

HPLC (245 nm) \( t_\text{R} = 2.55 \text{ min} \)
\textbf{CHAPTER 7}

$^1\text{H NMR}$ (500 MHz, DMSO-$d_6$) $\delta$ 7.61 (d, $J = 1.7$ Hz, 1H), 7.53 (dd, $J = 7.9$, 1.7 Hz, 1H), 7.33 (d, $J = 7.9$ Hz, 1H), 2.21 (s, 3H), 1.24 (s, 6H). $^{13}\text{C NMR}$ (126 MHz, DMSO-$d_6$) $\delta$ 188.8, 153.6, 145.3, 145.1, 125.1, 119.1, 118.1, 53.2, 22.5 (2C), 15.1.

\textbf{LCMS (ESI)} for C$_{11}$H$_{13}$NO$_3$S $[M+H]^+$ calcd. 240.1; found 240.1.

Analytical data in agreement with literature.$^{176}$

\textit{1,2,3,3-Tetramethyl-3H-indol-1-ium-5-sulfonate (49)}

A mixture of potassium 2,3,3-trimethyl-3H-indole-5-sulfonate 48 (1.00 g, 3.6 mmol, 1 equiv.) and methyl iodide (2.2 mL, 36 mmol, 10 equiv.) in acetonitrile (2.5 mL) was heated at 150 °C for 1 h under microwave irradiation. The reaction solution was cooled to room temperature and decanted. The residue was suspended in diethyl ether (5 mL), filtered, washed with diethyl ether (3 $\times$ 15 mL) and DCM (3 $\times$ 15 mL) to give compound 49 as a yellow-brown solid (724 mg, 79%).

\textbf{HPLC} (245 nm) $t_R = 2.83$ min.
$^{1}H$ NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.98 (s, 1H), 7.83 (d, $J = 8.3$ Hz, 1H), 7.78 (dd, $J = 8.3, 1.6$ Hz, 1H), 3.96 (s, 3H), 2.76 (m, 3H), 1.52 (s, 6H). $^{13}C$ NMR (126 MHz, DMSO-$d_6$) $\delta$ 196.8, 149.3, 141.9, 141.2, 126.1, 120.5, 114.5, 54.0, 34.8, 21.6 (2C), 14.2.

LCMS (ESI) for C$_{12}$H$_{16}$O$_3$NS [M+H]$^+$ calcd. 254.1; found 254.1.

Analytical data in agreement with literature.$^{176}$

2-((1E,3Z)-(5-Carboxypyridin-2-yl)-5-(E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (51)

1,2,3,3-Tetramethyl-3H-indol-1-ium-5-sulfonate 49 (372 mg, 1.47 mmol, 2.2 equiv.), 6-(1-formyl-2-oxoethyl)-3-pyridinecarboxylic acid 50 (129 mg, 0.67 mmol, 1 equiv.) and sodium acetate (346 mg, 4.22 mmol, 6.3 equiv.) was mixed with acetic anhydride/acetic acid (10 mL, 1:1 v/v) in a sealed vial and heated under microwave irradiation at 120 °C for 30 min. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The solid residue was dissolved in MeOH (2 mL), then diethyl ether was added to precipitate the product (15 mL). The precipitate was collected by
filtration and washed with diethyl ether (3 × 15 mL). The crude product was purified by column chromatography (ACN/H$_2$O 7:3, v/v) to give compound 51 as a dark blue solid (890 mg, 90%).

$R_f$ 0.22 (ACN/H$_2$O 7:3).

**HPLC** (650 nm) $t_R$ = 3.0 min

$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.17 (s, 1 H), 8.37 (d, $J = 14.3$ Hz, 2H), 8.33 (dd, $J = 7.9$, 2.1 Hz, 1H), 7.80 (s, 2H), 7.70 (dd, $J = 8.2$, 1.7 Hz, 2H), 7.44 (d, $J = 7.9$ Hz, 1H), 7.25 (d, $J = 8.3$ Hz, 2H), 5.80 (d, $J = 14.3$ Hz, 2H), 3.15 (s, 6H), 1.73 (s, 12H). $^{13}$C NMR (126 MHz, CD$_3$OD) $\delta$ 175.4, 168.7, 155.7, 153.3, 150.8, 144.0, 143.0, 142.3, 141.2, 138.5, 137.8, 127.6, 125.4, 119.9, 110.4, 101.3, 49.4, 30.3, 26.2.

**LCMS** (ESI) for C$_{33}$H$_{34}$N$_3$O$_8$S$_2$ [M+H]$^+$ calcd. 664.2; found 664.2.

NMR and mass data in agreement with literature.$^{177}$
5,5-Difluoro-10-(4-iodophenyl)-1,3,7,9-tetramethyl-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (57)

4-Iodobenzaldehyde 55 (928 mg, 4.0 mmol, 0.5 equiv.) and TFA (60 µL) were added to a solution of 2,4-dimethylpyrrole 56 (820 µL, 8.0 mmol, 1 equiv.) in anhydrous THF (50 mL). The reaction mixture was stirred for 24 h under N₂ atmosphere. To this solution, DDQ (910 mg, 4.0 mmol, 0.5 equiv.) dissolved in anhydrous THF (25 mL) was added dropwise, and reaction mixture was stirred for 4 h. After that, the reaction mixture was cooled to 0 °C in an ice bath and Et₃N (24 mL) was added dropwise. The solution was stirred for 0.5 h, then cooled to 0 °C, and BF₃.OEt₂ (25 mL) was added slowly, and the reaction mixture was stirred for an additional 24 h. The solvent was evaporated under reduced pressure and the residue washed with NH₄Cl (2 × 100 mL), water (2 × 100 mL) and dried over MgSO₄. The crude product was purified by column chromatography (Hexane/EtOAc 100:5, v/v) as an eluent to give compound 57 as a red solid (1.1 g, 60 %).

Rₐ 0.38 (Hexane/EtOAc 100:5).

¹H NMR (500 MHz, CDCl₃) δ 7.85 (d, J = 8.4 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 5.99 (s, 2H), 2.55 (s, 6H), 1.42 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 155.8, 142.9, 140.0, 138.3, 134.5, 131.1, 129.9, 121.4, 94.7, 14.62, 14.57.

LCMS (ESI) for C₁₉H₁₆BF₂IN₂ [M+H]⁺: calcd.: 451.1; found: 451.1.
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$^1$H NMR and mass data in agreement with literature.$^{222}$

10-(4-Ethynylphenyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-4$\lambda^4$,5$\lambda^4$-dipyrrlo[1,2-c:2',1'-f][1,3,2]diazaborinine (58)

To a solution of I-BODIPY 57 (250 mg, 0.5 mmol, 1 equiv.) in anhydrous THF (4 mL), Pd(PPh$_3$)$_2$Cl$_2$ (35 mg, 0.05 mmol, 0.1 equiv.), Cul (9.5 mg, 0.05 mmol, 0.1 equiv.), ethynyltrimethyl silane (105 µL, 0.75 mmol, 1.5 equiv.) and Et$_3$N (1.5 ml) were added, and the mixture was degassed with flow of N$_2$ atmosphere for 15 min. The reaction mixture was stirred at room temperature under N$_2$ atmosphere for 24 h. The solvent was evaporated under reduced pressure and the residue was filtered through a pad of silica. The TMS-BODIPY intermediate (orange solid) was used in the next step without further purification.

To a solution of TMS-BODIPY (200 mg, 0.475 mmol, 1 equiv.) in MeOH (20 mL), K$_2$CO$_3$ (655 mg, 4.75 mmol, 10 equiv.) was added and the reaction mixture was stirred for 2 h. The solvent was evaporated, and the crude product was purified by column
chromatography (Hexane/EtOAc 100:5, v/v) to give compound 58 as a red solid (147 mg, 84 %).

RF 0.43 (Hexane/EtOAc 100:5).

H NMR (600 MHz, CDCl3) δ 7.63 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 5.99 (s, 2H), 3.18 (s, 1H), 2.55 (s, 6H), 1.40 (s, 6H). C NMR (150 MHz, CDCl3) δ 156.0, 143.1, 140.7, 135.8, 133.0, 131.3, 128.4, 123.1, 121.5, 83.0, 78.7, 14.72, 14.68.

LCMS(ESI) for C21H20BF2N2 [M+H]+: calcd.: 349.2; found: 349.2.

NMR and mass data in agreement with literature.223,224

3-(4-Iodophenyl)-6-methyl-1,2,4,5-tetrazine (60)

4-Iodobenzonitrile 59 (100 mg, 0.44 mmol, 1 equiv.) was mixed with Ni(OTf)2 (78 mg, 0.22 mmol, 0.5 equiv.), acetonitrile (230 µL, 4.4 mmol, 0.5 equiv.) and hydrazine monohydrate (1.1 mL, 22 mmol, 50 equiv.) in a sealed vial. The reaction mixture was stirred for 24 h at 60 °C. After cooling, a solution of NaNO2 (607 mg, 8.8 mmol) in water
(5 mL) was added, followed by the dropwise addition of 2 M HCl until pH ~ 3 was reached. The mixture was extracted with EtOAc (3 x 30 mL), the organic layers combined and dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (Toluene/Hexane 4:1, v/v) to give compound 60 as a pink solid (51 mg, 38%).

R_f = 0.43 0.52 (Toluene/Hexane 4:1).

_H NMR (500 MHz, CDCl₃) δ 8.32 (d, J = 8.6 Hz, 2H), 7.95 (d, J = 8.6 Hz, 2H), 3.10 (s, 3H).

_C NMR (125 MHz, CDCl₃) δ 167.7, 163.9, 138.7, 131.4, 129.4, 100.3, 21.3.

LCMS (ESI) for C₉H₈IN₄ [M+H]^+: calcd.: 299.1; found: 299.0.

NMR and mass data in agreement with literature.¹¹⁸
5,5-Difluoro-1,3,7,9-tetramethyl-10-(4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-ethynyl)phenyl)-5H-4-λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (61)

Triethylamine (93 µL, 0.68 mmol, 5.2 equiv.) was dissolved in anhydrous DMF (3 mL) and degassed with the flow of N₂ atmosphere for 20 min. BODIPY-acetylene 58 (65 mg, 0.19 mmol, 1.4 equiv.), tetrazine 60 (40 mg, 0.13 mmol, 1 equiv.), Pd(PPh₃)₂Cl₂ (9.4 mg, 0.013 mmol, 0.1 equiv.) and CuI (2.5 mg, 0.013 mmol, 0.1 equiv.) were mixed and dissolved in the triethylamine/DMF solution. The reaction mixture was further degassed with a flow of N₂ atmosphere for 10 min and stirred for 24 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by column chromatography (toluene) to give compound 61 as a red solid (41 mg, 60%).

Rᵣ 0.45 (Toluene).

HPLC (495 nm) tᵣ = 7.4 min

¹H NMR (500 MHz, CDCl₃) δ 8.62 (d, J = 8.7 Hz, 2H), 7.77 (d, J = 8.7 Hz, 2H), 7.71 (d, J = 8.5 Hz, 2H), 7.33 (d, J = 8.5 Hz, 2H), 6.00 (s, 2H), 3.12 (s, 3H), 2.57 (s, 6H), 1.45 (s, 6H).
13C NMR (126 MHz, CDCl₃) δ 167.3, 163.7, 155.9, 143.0, 140.6, 135.5, 132.5, 132.4, 131.6, 131.2, 128.4, 127.9, 127.2, 123.6, 91.7, 90.1, 21.2, 14.80, 14.78.

HRMS (ESI) for C₃₀H₂₆BF₂N₆ [M+H]^+: calcd.: 519.2275; found: 519.2297.

Analytical data in agreement with literature.¹¹⁸

**Exo-2,5-Dioxopyrrolidin-1-yl-bicyclo[2.2.1]hept-5-ene-2-carboxylate (63)**

A solution of exo-5-norbornenecarboxylic acid (100 mg, 0.73 mmol, 1 equiv.) in anhydrous DCM (5 mL) EDC.HCl (166.5 mg, 0.88 mmol, 1.2 equiv.) and NHS (100 mg, 0.88 mmol, 1.2 equiv.) were added and the reaction mixture was stirred overnight under N₂ atmosphere. The reaction was monitored by analytical RP-HPLC. Upon completion, the solvent was removed under reduced pressure and the residue was purified by column chromatography (DCM) to give compound 63 as a white solid (142 mg, 82%)

R<sub>f</sub> = 0.55 (DCM)

HPLC (254 nm) t<sub>R</sub> = 4.79 min.

1H NMR (500 MHz, CDCl₃) δ 6.21 (dd, J = 5.7, 3.0 Hz, 1H), 6.15 (dd, J = 5.7, 3.0 Hz, 1H), 3.28 (s, 1H), 3.00 (s, 1H), 2.84 (d, J = 1.5 Hz, 4H), 2.51 (ddd, J = 9.0, 4.5, 1.5 Hz, 1H), 2.06
CHAPTEr 7

$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.8, 169.4, 138.7, 135.4, 47.3, 46.8, 46.6, 41.9, 40.5, 31.1, 25.8.

LCMS (ESI) for C$_{12}$H$_{14}$NO$_4$ [M+H]$^+$: calcd.: 236.1.; found: 236.1.

$^1$H NMR and mass data in agreement with literature.$^{225}$

Methyl 4-bicyclo[2.2.1]hept-5-ene-2-carboxamido)methyl benzoate (64)

The NHS ester 63 (80 mg, 0.34 mmol, 1 equiv.) was added to methyl 4-(aminomethyl) benzoate hydrochloride (68.6 mg, 0.34 mmol, 1 equiv.) and DIPEA (178 µl, 1.02 mmol, 3 equiv.) in anhydrous DMF (5 mL). The reaction was stirred under N$_2$ atmosphere for 24 h. The solvent was then removed under reduced pressure and the residue was purified by column chromatography (Hexane/EtOAc 4:1, v/v) to give compound 64 as a white solid (89 mg, 91%).

$R_f = 0.55$ (Hexane/EtOAc 4:1)

HPLC (254 nm) $t_R = 5.07$ min.

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.98 (d, $J = 8.4$ Hz, 2H), 7.32 (d, $J = 8.4$ Hz, 2H), 6.14 (dd, $J = 5.7$, 3.0 Hz, 1H), 6.08 (dd, $J = 5.7$, 3.0 Hz, 1H), 5.97 (t, $J = 5.6$ Hz, 1H), 4.55 – 4.43 (m, 2H),
3.90 (s, 3H), 2.94 (dd, \( J = 16.5, 2.4 \text{ Hz}, 2H \)), 2.05 (ddd, \( J = 8.5, 4.5, 1.7 \text{ Hz}, 1H \)), 1.95 (ddd, \( J = 11.4, 4.5, 3.4 \text{ Hz}, 1H \)), 1.73 (dt, \( J = 8.5, 1.7 \text{ Hz}, 1H \)), 1.46 – 1.29 (m, 2H). \(^{13}\text{C NMR} (126 \text{ MHz, CDCl}_3)\) \( \delta \) 175.8, 167.0, 144.1, 138.5, 136.1, 130.2, 129.4, 127.6, 52.3, 47.4, 46.6, 44.9, 43.5, 41.8, 30.8.

\textbf{LCMS} (ESI) for C\(_{17}\)H\(_{20}\)NO\(_3\) [M+H]\(^+\): \textit{calcd.}: 286.2.; \textit{found}: 286.2.

\textbf{4-(bicyclo[2.2.1]hept-5-ene-2-carboxamido)methyl benzoic acid (65)}

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

Compound \textbf{64} (300 mg, 1.1 mmol, 1 equiv.) was dissolved in MeOH/H\(_2\)O (23 mL, 1.5:1 v/v). LiOH (100 mg, 4.2 mmol, 4 equiv.) was added and the reaction mixture was stirred for 24 h. The mixture was neutralized with 0.15 M HCl (0.1 mL, 4.4 mmol, 4 equiv.) then concentrated under reduced pressure. The acidic aqueous solution was extracted with DCM (3 \( \times \) 20 mL), and the combined organic phase was washed with water (30 mL), dried over Na\(_2\)SO\(_4\) and filtered. The solvent was then removed under reduced pressure to give \textbf{65} as a white solid (285 mg, 95%).

\textbf{HPLC} (254 nm) \( t_R = 4.32 \text{ min.} \)

\textbf{\(^1\text{H NMR} (500 \text{ MHz, DMSO-}\text{d}_6)\) \( \delta \) 12.87 (s, 1H), 8.57 (t, \( J = 6.0 \text{ Hz}, 1H \)), 7.89 (d, \( J = 8.1 \text{ Hz}, 2H \)), 7.36 (d, \( J = 8.1 \text{ Hz}, 2H \)), 6.14 (d, \( J = 1.9 \text{ Hz}, 2H \)), 4.40 – 4.27 (m, 2H), 2.85 (d, \( J = 1.9 \text{ Hz}, 2H \)).}
Hz, 1H), 2.18 – 2.12 (m, 1H), 1.80 (dt, J = 11.3, 3.9 Hz, 1H), 1.65 (d, J = 7.9 Hz, 1H), 1.25 – 1.16 (m, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 174.5, 168.8, 137.7, 136.3, 129.2, 129.0, 127.2, 125.8, 46.8, 45.6, 42.9, 42.0, 41.0, 29.9.

LCMS (ESI) for C$_{16}$H$_{18}$NO$_3$ [M+H]$^+$: calcd.: 272.1; found: 272.1.

**Nor-MAD1 (67)**

Compound 65 (60.9 mg, 3 equiv., per amine, 0.03 M) in anhydrous DMF (3 mL), HSPyU (92.5 mg, 0.22 mmol, 3 equiv.), and DIPEA (21.6 µL, 0.22 mmol, 3 equiv.) were stirred for 2 h at 40 °C (the formation of the NHS ester was monitored by analytical PR-HPLC). After completion, the mixture was added to the L-MAD1 peptide 32 functionalised polystyrene resin 31 (100 mg, 75 µmol, 1 equiv., pre-swollen in DCM) in presence of DIPEA (21.6 µL, 0.22 mmol, 3 equiv.) and shaken overnight. The solution was then drained, and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The resin 66 (pre-swollen in DCM) was shaken 3 h in TFA/TIS/DCM (1 mL, 90:5:5 v/v/v). The solution was collected by filtration and the resin was washed with the cleavage cocktail. The combined filtrates were added to cold diethyl ether, and the precipitated solid was
collected by centrifugation, and washed repeatedly with cold ether (3 × 20 mL). The 
crude peptide was purified by preparative RP-HPLC to give compound 67 as a white solid 
(116 mg, 68%).

**HPLC** (254 nm) \( t_R = 2.97 \text{ min.} \)

**HRMS** (ESI) for \( C_{117}H_{149}N_{34}O_{15} \) \([M+H]^+ \text{ calcd.} 2270.1936; \text{ found} 2270.1942. \)

### 7.6 IEDDA reaction monitoring by HPLC

The IEDDA reaction between **BODIPY-Tz 61** and **Nor-MAD1 67** was monitored using 
analytical HPLC as follows: Stock solutions of **BODIPY-Tz 61** (5 mM in DMSO) and **Nor-
MAD1 67** (4 mM in BPS) were initially prepared and then diluted with BPS to achieve 
concentrations of 10 µM for **BODIPY-Tz 61** and 100 µM for **Nor-MAD1 67**, respectively. 
Equal volumes (500 µL) from both solutions were mixed and heated at 37°C with 400 rpm 
in a thermomixer. At specific time intervals (0, 0.5, 1, 2, 3, 4, 6, and 24 h), 50 µL samples 
were withdrawn and diluted with CH₃CN (100 µL) containing 0.1% HCO₂H. Samples were 
analysed by analytical HPLC using a 10-minute method with a gradient of 5% CH₃CN to 
95% in 6 min, maintaining at 95% for 3 minutes, returning to 5% for 1 minute, and 
detected at 495 nm.

### 7.7 Fluorescence measurements

All MAD1 probes were solubilised in DMSO (1 mM) and diluted to ~10 µM concentrations 
with distilled water or PBS unless otherwise stated. UV/Vis absorption spectra of the
solutions were recorded on an Agilent 8453 spectrophotometer. Steady-state fluorescence measurements were performed on solutions (10 µM, optical density ≤0.1) contained in standard \( l = 1 \) cm disposable polystyrene cuvettes with excitation at the wavelength of the absorption maximum using a Shimadzu RF-6000 spectrofluorometer.

Fluorescence quantum yields were measured using: Fluorescein (\( \Phi_{\text{ref}} = 0.90 \) in NaOH 0.1 M, \( \lambda_{\text{exc}} = 474 \) nm), Rhodamine-6G (\( \Phi_{\text{ref}} = 0.94 \) in EtOH, \( \lambda_{\text{exc}} = 488 \) nm) or Cresyl Violet in MeOH (\( \Phi = 0.54 \)) as references, depending on the optical properties of the probes. The emission quantum yield values \( \Phi_f \) of the sample (s) and reference (ref) were calculated using the following equation:

\[
\Phi_f^s = \Phi_f^{ref} \times \left( \frac{n^s}{n^{ref}} \right)^2 \times \frac{1 - 10^{-A_f^{ref}(\lambda_{exc})}}{1 - 10^{-A_f^s(\lambda_{exc})}} \times \frac{\int_0^{\infty} I_f^{s}(\lambda_{exc}, \lambda_f) \, d\lambda_f}{\int_0^{\infty} I_f^{ref}(\lambda_{exc}, \lambda_f) \, d\lambda_f}
\]

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<thead>
<tr>
<th>( \Phi_f^{[\text{s}] \text{ or r}} )</th>
<th>The emission quantum yield values</th>
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<tbody>
<tr>
<td>( n )</td>
<td>Refractive index of the solvents used</td>
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<td>( A )</td>
<td>Absorbance</td>
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<tr>
<td>( I_f^{\lambda_{exc}, \lambda_f} )</td>
<td>Integral of the fluorescence plot</td>
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### 7.8 Biological studies

For the bacterial assays, \( M. \text{smegmatis}, E. \text{coli} \) (DH5α), \( S. \text{aureus} \) and \( B. \text{subtilis} \) were purchased from ATCC. Microbial culture broths (Middlebrook 7H9, Luria Bertani broth), agar medium (Middlebrook 7H10 and Luria Bertani agar) and supplements to media
(ADC, OADC, glycerol, polysorbate) were purchased from Invitrogen, UK, Fisher Scientific, UK and Merck, UK. Buffers and water were sterilised by autoclaving before use.

**Bacterial culture**

The culture broth for *M. smegmatis* was prepared by supplementing 7H9 medium with 0.2% (v/v) glycerol followed by autoclaving and addition of filter sterilised (0.22 µ, Millipore Inc, USA) 0.05% (v/v) polysorbate 80, and 10% (v/v) Albumin Dextrose Catalase. Agar medium was prepared by autoclaving a mixture of 7H10 medium and 0.5% (v/v) glycerol followed by addition of filter sterilised 10% (v/v) Oleic Albumin Dextrose Catalase. Other media were prepared by suspending media powder in water and autoclaving. All cultures were grown at 37 °C in a shaking incubator (200 rpm).

**Bacteria labelling**

Liquid cultures of *M. smegmatis*, *E. coli* or *B. subtilis* were washed with PBS and diluted to an OD600 of 0.5. Cells were attached to Ibidi 15 well glass bottom slides coated with poly-D-lysine.

**Labelling with MAD1 fluorescent probes.** Different concentrations (1, 5 or 10 µM) of both enantiomers of sulf-Cy5-MAD1, MeroCy-MAD1, 5-CF-MAD1 or NBD-MAD1 probes were added to the bacteria and incubated at 37 °C for 1 h. The solution was removed from the wells and the bacteria imaged under a confocal microscope. A second duplicate set of samples was used, where the bacteria were washed twice with PBS before imaging. Images were acquired using a Leica SP5 confocal spinning disk microscope (filters used
were Cy5 for sulf-Cy5-MAD1, Alexa 594 for MeroCy-MAD1, and Alexa 488 for 5-CF-MAD1 and NBD-MAD1) with the LAS-AF software used to obtain the images that were then analysed by Fiji.

**Bioorthogonal labelling with MAD1 probe.** Nor-MAD1 62 (50 µM) was added to *M. smegmatis* bacteria and incubated at 37 °C for 3 h. The solution was removed from the wells and BODIPY-Tz 56 (5 µM) was added to the bacteria. The cells were imaged at different time points between 0–24 h, with BODIPY-Tz 56 removed before each imaging without washing. Images were acquired using a Leica SP5 confocal spinning disk microscope (used BODIPY filter 500-580 nm) with the LAS-AF software used to obtain the images that were then analysed by Fiji.

**Labelling of Mtb strains with D-MAD1 fluorescent probe.** *H37Rv* strain of *Mtb* was grown in 7H9 Middlebrook media to reach (1.0 OD CFU). The cells were washed with PBS and resuspended in PBS. 100 µL of poly-L-lysine was added to the wells of a 96-well plate and incubated for 30 min and then discarded. *H37Rv* cells were added to the wells and the plate was incubated for 1 h at room temperature. The media was discarded by aspiration and 100 µL MeroCy-D-MAD1, 5-CF-D-MAD1 and NBD-D-MAD1 (1 µM, 2 µM and 10 µM) were added and incubated at 37 °C (walk-in-incubator) for 1 h in the dark. The solutions were removed, and bacteria were imaged on a fluorescence microscope (40X, EVOS™ M5000 imaging system) using a GFP filter (NBD and 5-CF) or a Texas red filter (MeroCy).
Chapter 8. References


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Chapter 9. Appendices

- Published paper.
- HPLC traces for MAD1 Probes.
- HRMS for MAD1 Probes.
Solid-Phase Synthesis of s-Tetrazines

Zainab S. Alghamdi, Maxime Klausen, Alessia Gambardella, Annamaria Lilienkampf, and Mark Bradley*

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ABSTRACT: An efficient synthesis of s-tetrazines by solid-phase methods is described. This synthesis route was compatible with different solid-phase resins and linkers and did not require metal catalysts or high temperatures. Monosubstituted tetrazines were routinely synthesized using thiol-promoted chemistry, using dichloromethane as a carbonyl source, while disubstituted unsymmetrical aryl or alkyl tetrazines were synthesized using readily available nitriles. This efficient method enabled the synthesis of s-tetrazines in high yields (70–94%), eliminating the classical solution-phase problems of mixtures of symmetrical and unsymmetrical tetrazines, with only a single final purification step required, and paves the way to the rapid synthesis of s-tetrazines with various applications in bioorthogonal chemistry and beyond.

1,2,4,5-tetrazines undergo inverse electron-demand Diels–Alder reactions with various dienophiles. They are powerful bioorthogonal cycloaddition reactions due to the rapid reactivity of tetrazines, nontoxic by-products (N₂), and high reaction selectivity. As such, inverse electron-demand Diels–Alder reactions with tetrazines have been used in various biological scenarios such as sensing, imaging, and drug delivery. They have also been extended to applications in coordination chemistry, material science, and natural product synthesis. The clinical potential of bioorthogonal reactions involving tetrazines has been demonstrated with a first in-human inverse electron-demand Diels–Alder cycloaddition between a tetrazine decorated polymer and a trans-cyclooctene protected prodrug of Doxorubicin, allowing drug release at the site of the tumor where the polymer was implanted. A variety of mono- or disubstituted aliphatic and aromatic tetrazines have been used in bioorthogonal reactions, with monosubstituted tetrazines preferred due to greater reactivity, in part due to their small size. Tetrazines also have intrinsic fluorescence (λₑm/λₑm = 520–570 nm) with reasonable quantum yields (up to 0.44) and long fluorescence lifetimes (up to 180 ns). They have also been applied as “absorbers” in FRET pairs. Despite the ever-growing applications of tetrazines, their use has been hampered by laborious synthesis and purification and it is therefore important to develop new synthetic routes that provide easy access to substituted tetrazines with different reactivities. Many routes to aromatic/aliphatic s-tetrazines have been investigated, and the area has been well-reviewed.

Conventional approaches toward s-tetrazines include a two-step synthesis starting from the condensation of hydrazine with aromatic nitrile precursors, followed by oxidation of the resulting 1,2-dihydrotetrazine to the tetrazine (Scheme 1). However, this approach is not suitable for aliphatic or unsymmetrical tetrazines, which are commonly prepared from aromatic or alkyll nitrile precursors and formamidine salts in low yields (<20%) and require several purification steps. Devanar developed an efficient Lewis acid catalyzed (5 mol % of Zn(II) or Ni(II) salts) method for the synthesis of 3-substituted unsymmetrical s-tetrazines (30–70% yield), but this approach requires a large excess of potentially hazardous

Scheme 1. Previously Reported Methods to s-Tetrazines and Our Approach

Previous methods:

a) Pluher

b) Lautens

c) Wep

This work:

unsymmetrical tetrazines, which are commonly prepared from aromatic or alkyll nitrile precursors and formamidine salts in low yields (<20%) and require several purification steps. Devanar developed an efficient Lewis acid catalyzed (5 mol % of Zn(II) or Ni(II) salts) method for the synthesis of 3-substituted unsymmetrical s-tetrazines (30–70% yield), but this approach requires a large excess of potentially hazardous
anhydrous hydrazine (50 equiv). Audibert reported a metal-free approach to monosubstituted tetrazines (40–70% yield), using hydrazine hydrate, sulfur, and dichloromethane (DCM) that acts as the source for the C-3 carbon within the tetrazine ring (Scheme 1). This approach required prolonged microwave irradiation (24 h) and had a relatively limited substrate scope as dichloroethane and dibromomethane both failed to generate tetrazines. Wu reported a scalable and high yielding organocatalytic synthesis to unsymmetrical alkyl and aryl tetrazines (34–75% yield) using the reversible reaction between nitriles and a thiol activator/catalyst, such as 3-mercaptopropionic acid or N-acetyl-L-cysteine. These formed thioimidate esters in situ with subsequent nucleophilic attack by hydrazine leading to regeneration of the thiol and formation of an amidrazine, which then reacted with another equivalent of thioimidate ester to give, after oxidation, the tetrazine (Scheme 1). Recently, Fox developed a one-pot method for the synthesis of 3-thiomethyltetrazines from carbonylic esters, with the 3-thiomethyltetrazines used in thioether reduction or palladium-catalyzed cross-coupling chemistries to generate mono- and disubstituted aliphatic or aromatic tetrazines (60–80% yield). Here, we report an expedient solid-phase synthesis route to both monosubstituted and unsymmetrical disubstituted s-tetrazines, bearing different functional groups, based on the thiol-promoted reaction between supported aryl nitriles and hydrazine. This resin-supported approach to tetrazines uses mild conditions, readily available materials, and was compatible with a variety of resins and linkers routinely used in solid-phase synthesis (Scheme 1).

As a proof-of-concept, thiol-promoted s-tetrazine synthesis on the solid-phase was investigated using a ChemMatrix resin (100 mg, loading 0.5–0.7 mmol/g, 100–200 mesh) functionalized with a Fmoc-Rink amide linker (Scheme 2). After N-Fmoc deprotection (20% piperidine), 4-cyanobenzoic acid (3 equiv) was coupled to the linker using DIC and Oxa-Link as the coupling combination. To form the monosubstituted 1,4-dihydropyrazine 2, the nitrile functionalized resin 1 was degassed and treated with hydrazine hydrate (0.06 M, 1 equiv) and 3-mercaptopropionic acid (3 equiv) in DCM (0.03 M), with the DCM here providing the C-3 carbon of the tetrazine as previously reported. The subsequent oxidation of 2 to the tetrazine was carried out on-resin, using an aqueous solution of NaNO2 (0.1 M) with HCl (2 M). The resin beads turned deep pink within a few minutes confirming the formation of the monosubstituted, resin-bound tetrazine 3a (Scheme 2). Cleavage off the solid support proved to be a crucial step in the synthesis, as tetrazines are prone to degradation under the strongly acidic conditions typically used to cleave acid-labile linkers in solid-phase synthesis (often 90% TFA is used for cleaving the Rink linker). The acid-mediated cleavage of tetrazine 3a from the Rink linker was investigated looking at different reaction times and concentrations of TFA (Table 1). 90% TFA in H2O (entries 1–3) gave 4 in low yields (12–23%) with high levels of degradation and decreased yields due to prolonged exposure with TFA (shown visually by the
CHAPTER 9

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Table 1. Optimization of the Resin Cleavage Conditions for the Efficient Liberation of the Tetrazines

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cleavage Cocktail</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90% TFA/H₂O</td>
<td>3 h</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>90% TFA/H₂O</td>
<td>2 h</td>
<td>15%</td>
</tr>
<tr>
<td>3</td>
<td>90% TFA/H₂O</td>
<td>1 h</td>
<td>23%</td>
</tr>
<tr>
<td>4</td>
<td>70% TFA/H₂O</td>
<td>1 h</td>
<td>45%</td>
</tr>
<tr>
<td>5</td>
<td>70% TFA/H₂O</td>
<td>3 × 1 h</td>
<td>71%</td>
</tr>
<tr>
<td>6</td>
<td>50% TFA/H₂O</td>
<td>3 × 1 h</td>
<td>75%</td>
</tr>
<tr>
<td>7</td>
<td>50% TFA/DCM</td>
<td>1 h</td>
<td>49%</td>
</tr>
<tr>
<td>8</td>
<td>50% TFA/DCM</td>
<td>2 × 1 h</td>
<td>80%</td>
</tr>
<tr>
<td>9</td>
<td>50% TFA/DCM</td>
<td>3 × 1 h</td>
<td>86%</td>
</tr>
</tbody>
</table>

*Cleavage was performed at room temperature on 100 mg of ChemMatrix resin that had been preswollen in DCM, and the crude product was isolated by filtration and concentration. Isolated yield after purification by column chromatography.

change in color from pink to yellow and the low isolated yield after purification. Decreasing the acid concentration to 70% (entry 4) increased the yield significantly (45%), which increased to 71% (entry 5) after 3 × 1 h treatments without noticeable decomposition occurring. Similar yields (75%) were observed after 3 × 1 h treatments with 50% TFA. When water, which is traditionally used as a scavenger in the cleavage of the Rink linker, was removed from the “cleavage cocktail” (entries 7–9), the monosubstituted tetrazine 4 was isolated in 86% yield. Scaling up the reaction using 1 g of resin (with loadings of 0.6 or 1 mmol/g) did not affect the reaction, giving 4 in 88% and 90% yield, respectively (Table S1). The effect of using catalysts, such as sulfur or zinc trflate, was investigated; however, both gave lower yields (45% and 58%, respectively). This efficient solid-phase method was further expanded to the generation of dissubstituted unsymmetrical tetrazines 5–10, with a variety of aliphatic and aromatic nitriles (Scheme 2). Using the optimized conditions, the nitrile functionalized resin 1 was treated with hydrazine hydrate (0.06 M), degassed 3-mercaptopropionic acid (3 equiv), and the selected nitrile, either neat or dissolved in 1,4-dioxane. Note, it was crucial to eliminate any traces of DCM (typically used to swell the resin in solid-phase synthesis) in order to prevent preferential formation of monosubstituted tetrazine 4. This synthetic approach was compatible with nitriles bearing both electron-donating (e.g., methoxy) and electron-withdrawing groups (e.g., nitro). The potential formation of undesired symmetrical dissubstituted s-tetrazine side product(s) typically found in solution-phase synthesis is avoided, due to site isolation on the solid phase and the fact that any side products formed in solution are simply washed away. tert-Butylcyclohexane gave access to carbonyl-functionalized tetrazine 6 with the tert-butyl group removed during the acidic cleavage from the resin, although this compound proved to be poorly soluble. The Rink linker is widely used in solid-phase synthesis, however, it leaves behind a primary amide group after cleavage. Since tetrazines are commonly used in bioconjugation reactions, we expanded this methodology to a linker that would provide a “conjugation handle”, such as a carboxylic acid, upon cleavage, while also expanding the choice of resin being used. Thus, a 2-chlorotriyl chloride linker (CLTR-Cl) attached to a polystyrene resin was explored (Scheme 3). The scope of aryl nitriles was expanded using either 4-cyanobenzoic acid or 6-cyanoisonicotinic acids, which were attached to the trityl linker (loading 0.95 mmol/g) by esterification. Monos- and dissubstituted carbonyl-functionalized phenyl tetrazines 13–15, pyridyl tetrazines 16–18, and dipyrrolidyl tetrazines 19–21 were formed using the same synthetic steps described above but with cleavage off the 2-chlorotriyl linker possible with 20% hexafluorooisopropanol (HFIP) in DCM, giving the tetrazines in excellent 78–94% yields. To explore the efficiency of our method to provide access to ortho- and meta-substituted s-tetrazines, 4-bromo- and 5-bromo-3-cyanopyridine were reacted via hydrazine condensation with the nitrile-functionalized resin 11. As expected, the ortho-functionalized s-dipyrrolidyl tetrazine was not accessible owing to steric and electronic limitations, 34 while the meta-functionalized s-dipyrrolidyl tetrazine 19 was successfully synthesized in 85% yield. Moreover, considering the limited stability of the tetrazines, which is a known obstacle to their use, the tetrazines 4–21 reported here were observed to be robust.

Scheme 3. Solid-Phase Synthetic Route for Tetrazines on a 2-Chlorotriyl Chloride Linker Functionalized Polystyrene Resin Illustrating the Scope of the Reaction

**The inset shows the structures and yields of the isolated products obtained after purification.
for over 1–3 months on the solid support (storage in the dark at -20 °C).

In conclusion, a practical method for the synthesis of s-tetrazines has been developed, with the thiol-promoted pathway yielding mono- or disubstituted tetrazines. The methodology was compatible with different resin-supported aryl nitriles and aliphatic and aromatic acceptor nitriles with either electron-withdrawing or electron-donating groups. The method was versatile, using either DCM as the carbon source for monosubstituted tetrazines or either as a solvent or as a reactant (in dioxane) for the disubstituted derivatives. All tetrazines were synthesized in excellent yields (70–94%) without the need for metal catalysts or high temperatures and notably required only a single purification step after cleavage. This solid-phase approach naturally overcomes the problems typically associated with disubstituted tetrazine synthesis in solution, namely, the formation and separation of the undesired symmetrical, disubstituted adducts. The method was compatible with different types of resins and linkers typically used in solid-phase synthesis. This route paves the way for applications in chemical biology where tetrazines can be synthesized in situ attached to peptides, thus providing a range of chemical handles that can be exploited in bioorthogonal chemistries, and also opens up routes to "resin-" or "n resin" cyclization reactions.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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Figure 9.1. HPLC trace of L-MAD1 32 (top) and D-MAD1 32 (lower).
5-CF-L-MAD1

5-CF-D-MAD1

Figure 9.2. HPLC trace of 5-CF-L-MAD1 (top) and 5-CF-D-MAD1 (lower).
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