Peptide/Protein Stapling and Unstapling:
Introduction and Photorelease of Isobutylene Graft

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Darwin College

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This dissertation is submitted for the degree of Doctor of Philosophy.

May 2019
DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the Physics and Chemistry Degree Committee.

Shuang Sun
Cambridge, May 2019
SUMMARY

Shuang Sun

Peptide/Protein Stapling and Unstapling: Introduction and Photorelease of Isobutylene Graft

Intramolecular side-chain-to-side-chain crosslinking, also termed “stapling”, is an important technology in the development of bioactive peptide-based therapeutics. Among all of the tools, S-alkylation is one of the most flexible approaches, as a wide range of bis-thiol-reactive linkers are commercially available. More importantly, this efficient and mild modification can be applied on large biomolecules by re-bridging their disulfides. At the same time, the handle introduced by the method provides further opportunities for manipulating the bioactivity of the molecule.

The first part of this work describes a new peptide-macrocyclisation strategy with an isobutylene graft. The reaction is mild and proceeds rapidly and efficiently both with linear and cyclic peptide substrates. The resulting isobutylene-grafted peptides possess improved passive membrane permeability, and are stable in human plasma and in the presence of glutathione. This strategy can be applied to bioactive cyclic peptides such as somatostatin. Importantly, we found that structural preorganisation forced by the isobutylene graft leads to a significant improvement in the binding affinity of somatostatin to its receptor.

Next, the same approach was optimised for re-bridging the disulfides of monoclonal antibodies. A ‘one-pot’ stapling strategy using isobutylene motifs was able to stabilise the interchain disulfides of antibodies by avoiding Fab exchange and disulfide shuffling. This general method was applied to an anti-HER2 Fab fragment and full-length IgGs under mild and biocompatible conditions. The binding affinity of the antibody was enhanced, relative to its native form, after stapling. The stapled structure maintained its effector functions and behaved similarly to its native form in vivo.

The last part of the thesis describes a photoactivation decaging method of isobutylene-caged thiols through a UV-initiated thiol-ene reaction. The method was demonstrated with an isobutylene-caged cysteine, cyclic disulfide-peptide, and thiol-containing drug, all of which
were rapidly and efficiently released under mild UV irradiation in the presence of thiol sources and a photoinitiator. Importantly, it was shown that the activity of histone deacetylase inhibitor largazole can be switched off when stapled, but selectively switched on within cancer cells when irradiated with non-phototoxic light. Further optimisation also demonstrated a potential application on isobutylene grafted protein-small molecule conjugates.

Overall, the work presented here offers a new tool for producing cyclic bioactive peptides, stabilising therapeutic antibodies and photoactivation of thiol-containing drugs.

**Keywords**

cyclised peptide, disulfides, isobutylene, stapling, thiol-ene, photoactivation
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I am truly grateful to everything and every moment in Cambridge, the most beautiful and important place in the world.
LIST OF PUBLICATIONS


   Sections from this publication are included in Chapter 3


   Sections from this publication are included in Chapter 4


   Sections from this publication are included in Chapter 4


   Sections from this publication are included in Chapter 2


† These authors contributed equally.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>ADC</td>
<td>Antibody-drug conjugate</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AFCT</td>
<td>Addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>AMCA</td>
<td>Aminomethyl coumarin</td>
</tr>
<tr>
<td>AQA</td>
<td>Acrylamide-azobenzene-quaternary ammonium</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerisation</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>Bhc</td>
<td>6-Bromo-7-hydroxycoumarin-4-ylmethyl</td>
</tr>
<tr>
<td>BLI</td>
<td>Bio-layer interferometry</td>
</tr>
<tr>
<td>BME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>PyBOP</td>
<td>(Benzotriazol-1-yloxy)tris(2-methyloxetane)hexafluorophosphate</td>
</tr>
<tr>
<td>CAAAC</td>
<td>Cysteine-Alanine-Alanine-Alanine-Cysteine peptide</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper(I)-catalysed azide-alkyne cycloaddition</td>
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<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DAR</td>
<td>Drug to antibody ratio</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
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<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
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<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNQ</td>
<td>2-Diazo-1,2-naphthoquinone</td>
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<tr>
<td>DOX</td>
<td>Doxorubicin</td>
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<tr>
<td>DPAP</td>
<td>2,2-Dimethoxy-2-phenylacetophenone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DSF</td>
<td>Differential scanning fluorimetry</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDANS</td>
<td>5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid</td>
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<td>EDCI</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
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<td>EDT</td>
<td>1,2-Ethanedithiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>equiv.</td>
<td>Equivalent</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLP</td>
<td>Glucagon-like peptide</td>
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<tr>
<td>GRF</td>
<td>Growth hormone-releasing factor</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>HIC</td>
<td>Hydrophilic interaction chromatography</td>
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<tr>
<td>HOBt</td>
<td>N-Hydroxybenzotriazole</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>HRMS</td>
<td>High resolution mass spectrum</td>
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<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>iEDDA</td>
<td>Inverse electron demand Diels–Alder</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>LAP</td>
<td>Lithium phenyl-2,4,6-trimethylbenzoylphosphinate</td>
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<tr>
<td>LC–MS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
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<td>Abbreviations</td>
<td>Full Form</td>
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<tr>
<td>MBHA</td>
<td>4-Methylbenzhydrylamine</td>
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<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MMAE</td>
<td>Monomethyl auristatin E</td>
</tr>
<tr>
<td>MMAF</td>
<td>Monomethyl auristatin F</td>
</tr>
<tr>
<td>Mmt</td>
<td>Monomethoxytrityl</td>
</tr>
<tr>
<td>MSN</td>
<td>Mesoporous silica nanostructures</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
</tr>
<tr>
<td>ONB</td>
<td>o-Nitrobenzyl</td>
</tr>
<tr>
<td>PABC</td>
<td>p-Aminobenzyloxy carbonyl</td>
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<tr>
<td>PAMPA</td>
<td>Parallel artificial membrane permeability assay</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Pyridazinedione</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Phth</td>
<td>Phthaloyl</td>
</tr>
<tr>
<td>PI</td>
<td>Photo initiator</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-protein interaction</td>
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<tr>
<td>RCM</td>
<td>Ring-closing metathesis</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>ROESY</td>
<td>Rotating frame nuclear Overhauser effect spectroscopy</td>
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<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Rt</td>
<td>Retention time</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>scFV</td>
<td>Single-chain variable antibody fragment</td>
</tr>
<tr>
<td>sCT</td>
<td>Salmon calcitonin</td>
</tr>
<tr>
<td>SL</td>
<td>Stapled largazole</td>
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<tr>
<td>SPAAC</td>
<td>Strain-promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid-phase peptide synthesis</td>
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<tr>
<td>SSTR2</td>
<td>Somatostatin receptor 2</td>
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<tr>
<td>TBTU</td>
<td>O-(Benztotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethylpiperidinyl-1-oxy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TSE</td>
<td>2-(Trimethylsilyl)ethanol</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>Vazo 44</td>
<td>2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride</td>
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CHAPTER 1 Introduction

In this chapter, the advances in peptide stapling techniques, protein disulfide re-bridging reagents and photoinduced drug delivery systems will be discussed in order to provide an introduction for the subsequent three chapters of this thesis.

1.1. Peptide Stapling Techniques

The term “stapling” was first applied in peptide studies to describe a covalent side-chain crosslinking system to stabilise the α-helix structure.\cite{1} Since their introduction in 2000 by Verdine and co-workers\cite{2}, stapled peptides have evolved into a promising class of bioactive therapeutics, as evidenced by the rapidly increasing number of applications. Stapling can be divided into two main catalogues: one-component stapling and two-component stapling. The direct one-component method utilises intramolecular reactions to staple two amino acids from the linear peptide (Scheme 1.1a), while two-component stapling exploits a bifunctional linker to staple the two residues \textit{via} intermolecular reactions (Scheme 1.1b).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{Generalised scheme of one- and two-component stapling methods.}
\end{figure}

Stapling can improve the drug-like properties of peptides through several means. Firstly, stapled peptides often display enhanced resistance to proteolytic degradation because proteases...
bind substrates in an extended rather than helical conformation, leading to the increased circulation time \textit{in vivo}.\cite{2} Next, such modified peptides often show increased ability to penetrate cellular membranes.\cite{3} Moreover, in some cases the stapled structure mimics the binding site of proteins, which leads to elevated binding affinity of the modified peptide to its biological target.\cite{4} Stapled peptides have exceeded the developmental stage of most \(\alpha\)-helix mimetics, and some of the candidates have successfully completed phase I clinical trials. For example, Aileron Therapeutics is conducting phase I/IIa clinical trials with the ALRN-6924, a p53 hydrocarbon-stapled peptide, for the treatment of various types of solid and hematological cancers.\cite{5-9}

The earliest method for stapling is forming lactam linkages between Lys and Glu/Asp residues.\cite{10} By now the stapling toolbox has been largely expanded, involving carbon-carbon bonds, disulfide bridges, lactam linkages, oximes, hydrazones, triazoles and thioether bonds. In this section, several major stapling techniques using incorporated non-canonical amino acids will be introduced, followed by the stapling on incorporated cysteines or reduced disulfides, with a focus on alkylation and arylation of cysteine. Several reviews from different aspects have been published and well summarised the chemistry and application of different stapling techniques.\cite{11-12}

1.1.1. Lactamisation

Intramolecular amide-bond formation is the earliest report of \(\alpha\)-helix stabilisation (Figure 1.1a). The first determination of a helix stabilising effect for side-chain lactam bridges came from Felix and co-workers, who incorporated Asp, Lys side-chain (i, i+4) linkages, and the reverse Lys, Asp side-chain (i, i+4) linkages, into growth hormone-releasing factor (GRF) analogues to link residues 8 and 12 and residues 21 and 25, respectively.\cite{10} Circular dichroism and molecular dynamics (MD) simulations based on NOE-derived distance constraints demonstrated that the novel cyclic analogue contained a long \(\alpha\)-helical segment even in aqueous solution. At the same time, the bioactivity of the stapled peptide was preserved.\cite{10}

In order to optimise helix stabilisation, different chain lengths and positions were investigated. Lactam bridges linking (i, i+3)\cite{13}, (i, i+4)\cite{14-15}, and (i, i+7)\cite{16}-spaced residue pairs have all proven useful for stabilising \(\alpha\)-helices, and (i, i+3)-linked residues have also been demonstrated to be able to stabilise \(\beta\)-turns\cite{17}. By now, many other forms of lactam stapled peptides were
investigated, such as bicyclic lactam stapled peptides\cite{15,18} (Figure 1.1b) and two component lactamisation stapled peptides\cite{19-20} (Figure 1.1c and d).

![Figure 1.1](image_url) Different forms of lactam stapled peptides. a) One-component lactamisation stapled peptide\cite{10}; b) bicyclic lactam stapled peptide\cite{15}; c) two-component lactamisation with diesters\cite{20}; d) two-component lactamisation with diamines\cite{19}.

In terms of synthetic approaches, the proteogenic amino acids for lactam stapling are easier to obtain. However, an extra orthogonal protecting group strategy is needed to allow for selective deprotection of the amine and carboxyl group before lactamisation. A previous review\cite{12} covers the synthesis of lactam stapled peptides.

The lactam stapled structures are also widely used in protein biology, including studies of protein folding, protein aggregation, peptide ligand-receptor recognition, and the development of more potent peptide therapeutics. However, the lactam bridge is less biologically inert comparing to other stapling linkages and the limited studies cannot confirm that it can improve cell permeability.\cite{21} More studies are needed to firmly judge whether lactam stapled peptides have inherently poorer uptake characteristics.

Overall, lactamisation is a well-developed stapling technique and has been applied to many biological targets and model systems.

1.1.2. Ring-closing metathesis (RCM)

The preliminary study of using RCM reactions for peptide stapling was done by Blackwell and Grubbs in 1998.\cite{22} They reported the solution-phase metathesis between O-allyl serine...
residues and subsequent hydrogenation of a peptide template (Scheme 1.2a).\[^{[22]}\] Although this peptide template was already helical, and stapling did not significantly affect peptide conformation, this pioneer study demonstrated the practicability of conducting metathesis on peptide side-chains. This work was further extended by Verdine and co-workers with the first all-hydrocarbon cross-linking peptide in 2000.\[^{[2]}\] They investigated a large series of α,α-disubstituted non-natural amino acids bearing olefin tethers to optimise length and stereochemistry for ring-closing metathesis across one or two α-helices (Scheme 1.2b).\[^{[2]}\] In addition to the generated α-helices, Verdine and co-workers also found that the RCM stapled B-cell lymphoma 2 (Bcl-2) homology 3 (BH3) peptides could bind to Bcl-2 family targets with greater affinity than the native domain, induce apoptosis in leukaemia cells, and inhibit the growth of human leukaemia xenografts in mice.\[^{[1]}\]

Scheme 1.2 Ring-closing metathesis on peptides. a) RCM stapling of peptides bearing two serine residues by Blackwell and Grubbs;\[^{[22]}\] b) all-hydrocarbon stapling method.\[^{[2]}\]

Since the RCM method was first developed, it has been widely applied to various peptide templates for a diversity of disciplines and human diseases, including cancer, infectious diseases, metabolism and neuroscience. The recent perspective by Walensky and Bird has introduced most applications and progress of hydrocarbon-stapled peptides.\[^{[23]}\]

By now, hydrocarbon-stapling has been one of the most thoroughly investigated peptide stapling strategies. Many encountered challenges in linkage design, cellular uptake, and biological activity have been overcome.
1.1.3. Cycloaddition

As a popular biocompatible ligation method, the Copper(I)-catalysed azide–alkyne cycloaddition (CuAAC) reaction, ‘click’ reaction, is another well-established method for peptide stapling. In 2008 and 2010, D’Ursi and co-workers reported solution-phase CuAAC stapling reactions on a model peptide derived from parathyroid hormone-related protein containing ω-azido- and ω-yl-α-amino acid residues in positions i and i+4 (Scheme 1.3a).[24-25] CD, NMR and MD studies revealed that those cyclic peptides in which the 1,2,3-triazolyl is flanked by a total of 5 or 6 methylene units nicely accommodated α-helical structures.[24] Further linker optimisation was conducted by Wang and co-workers in 2012. They screened different linker length, triazole stapling location and number of staples for BCL9 peptides.[26] Some of the peptides demonstrated improved helical structure with improved binding affinities toward their targets, as well as increased metabolic stabilities.

Scheme 1.3 CuAAC reaction for peptide stapling. a) One-component CuAAC reaction;[24] b) dual CuAAC reaction.[27]

CuAAC reaction has also been used in two-component stapling, utilising a separate bifunctional linker to bridge the two side-chains. In 2008, Bong and co-workers investigated a peptide derived from the GCN4 leucine zipper with azidoalanine residues at (i, i+4) positions. The azido groups reacted with 1,5-hexadiyne either on-resin or in solution phase to give the stapled peptide.[28] After this, Spring and co-workers reported an (i, i+7) double-click stapling method, applied to the inhibition of the p53-MDM2 interaction. They incorporated two
azidoornithine residues into the peptide and reacted with 3,5-diethynylbenzene to generate the bis-diazaole stapled peptide (Scheme 1.3b). After the optimisation of the staple linker by increasing net positive charge, cellular uptake and p53 activation were achieved.

Similar to CuAAC, the copper free strain-promoted azide–alkyne cycloaddition (SPAAC) reaction is another efficient click reaction between alkyne and azides. Spring and co-workers also reported a novel stapling technique based on double SPAAC reactions, and also exploited its biocompatibility for rapid selection of cell-active stapled peptides (Scheme 1.4a). The authors stapled Mdm2-binding peptides in cell culture medium in 96-well plates, and simultaneously evaluated it in a p53 reporter assay. An optimal candidate with α-helices showed improved proteolytic stability and nanomolar binding to Mdm2 in biophysical assays. Recently, they reported another cross-linking reagent based on a diarylethene core that featured two strained cyclooctynes, which is highly reactive towards azides due to additional strain imposed on the cyclooctyne rings by the fused thiophene rings.

As well as azide-alkyne reactions, the photoinduced intramolecular 1,3-dipolar cycloaddition reaction between tetrazoles and alkenes has been investigated by Lin and co-workers (Scheme 1.4b). They introduced an alkene and a tetrazole moiety onto peptide sidechains located at the
i and i+4 positions of Balaram’s 3_{10}-helix. Upon photoirradiation, the fluorescent pyrazoline stapled peptides were generated, which were capable of permeating the HeLa cell membrane.\cite{32}

Later, they also applied this UV-induced reaction to staple peptides dual inhibitors of the p53-Mdm2/Mdmx interactions. The positively charged, stapled peptides showed improved cellular uptake along with a modest \textit{in vivo} activity.\cite{33}

Recently, Baker and co-workers reported UV-induced [2+2] cycloadditions to reconnect the disulfide after reducing the native disulfides and introducing thiomaleimides. As well as polypeptides, this method was also applied to generate the stapled thiol stable Fab fragment, which is reviewed in the next section.\cite{34}

In summary, cycloaddition reactions are a newly emerged strategy in peptide stapling compared to lactamisation and ring-closing metathesis. Stapling conditions, length and positions have been investigated and established. Traditional ‘click’ chemistry will definitely be applied in peptide chemistry more and more frequently due to the high efficiencies and yields of the reactions. UV-induced stapling also appears to be a simple method for stapling. However, further studies and applications with this newly developed method are needed to reveal its practicability for various biological targets.

The following sections will mainly discuss the stapling on incorporated cysteines or reduced disulfide bonds. Proteinogenic cysteine is the most convenient handle for chemical conjugations due to the high nucleophilicity of the thiolate and its unique reactivity.\cite{35} In peptides, cysteine is easy to incorporate into a sequence during solid-phase peptide synthesis (SPPS) or can be engineered into coding sequence, which is one of the advantages of stapling on cysteine over other techniques. Besides, most of the biologically relevant peptides and proteins possess at least one disulfide bond in the structure. Hence, stapling on large biomolecules can be achieved by the direct reduction of disulfide bonds followed by two-component stapling with dual-functional linker.

1.1.4. Thiol-ene/yne coupling

Thiol-ene coupling, the conjugation between a thiol and an alkene, proceeds through two mechanisms, namely photoinitiated free-radical addition and catalysed Michael addition reactions. In both idealised reactions, a single thiol reacts with a single alkene to yield the product. The photo-triggered radical-mediated thiol-ene coupling is a chemoselective
bioconjugation method that has been demonstrated on various peptides and proteins (Scheme 1.5). Several thorough reviews on thiol-ene chemistry and its application have been published.\[^{36-38}\]

**Scheme 1.5** Thiol-ene/yné stapling method. a) One-component thiol-ene stapling method;\[^{39}\] b) thiol-ene stapling with peptides containing an azobenzene-alkyne amino acid;\[^{40}\] c) two-component thiol-ene stapling;\[^{41}\] b) one-component thiol-ene stapling;\[^{42}\]

In 2010, Anseth et al. first applied thiol-ene coupling to staple the peptide Arg-Gly-Asp (RGD), a peptide ligand of the α\(_5\)β\(_3\) integrin (Scheme 1.5a).\[^{39}\] A linear RGD-derived peptide containing additional cysteine and amino acid with an alkene group was built by SPPS, followed by selective deprotection of monomethoxytrityl on sulphydryl. After adding photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP), the peptidyl resin was exposed to 365 nm and converted to the cyclic peptide with 24% and 37% yield, depending on the introduced alkene group. To confirm that the thiol-ene reaction did not exhibit deleterious effects on the activity of the stapled peptides, a competitive binding ELISA was performed. The IC\(_{50}\) of both stapled peptides, 0.20 ± 0.09 and 0.36 ± 0.09 μM, were comparable to reported
literature values\[^{[43]}\], around 0.1 μM. Although the yield of stapling is only 24–37%, this work demonstrates the thiol-ene click photoreaction as a facile method for the rapid synthesis of cyclic peptides.

After this work, Hoppmann and co-workers reported the thiol-ene reaction of the photo-switchable click amino acid (PSCaa) in presence of photoinitiator Irgacure D-2959, 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone, at the (i, i+4) and (i, i+7) positions (Scheme 1.5b). Besides, in the presence of glutathione (GSH), a naturally occurring thiol in living cells, the click reaction took place at relatively high GSH concentrations (0.5 mM) in buffered solution (pH 7.5) indicating that the thiol click reaction occurs preferentially intramolecularly, although the reaction with GSH was dominant when the GSH concentration was more than 1 mM.\[^{[40]}\]

In 2015, Wang and co-workers applied the thiol-ene coupling reaction to staple peptides between two cysteine residues and an α,ω-diene in high yields (Scheme 1.5c). The two cysteines located at (i, i+4), (i, i+7) and (i, i+8) positions.\[^{[41]}\] After scanning five photoinitiators, DPAP was revealed to be the most effective one. This work demonstrated that unprotected peptides could effectively react with various diene linkers of different length and rigidity, or containing different heteroatoms.\[^{[41]}\] To confirm that the two-component thiol-ene coupling stapled peptide has the same structural features of the classic RCM method, they synthesised a hydrocarbon-stapled Axin mimetic analogue inhibiting the Wnt signalling pathway reported by Verdine et al., as well as an unstapled peptide and a thiol-ene coupling stapled analogue. The CD experiments showed that both the thiol-ene coupling stapled peptide (8-carbon linker) and the reported peptide featured strong α-helices. Furthermore, to apply this to bio-functional peptides, they synthesised and analysed a thioether-stapled analogue of a previously reported p53 hydrocarbon-stapled peptide. To this end, two cysteines at an (i, i + 7) location were linked with a 1,9-nonadiene to give a stapled peptide with similar elevated helical configuration, p53/Mdm2 inhibitory bioactivity and cancer cell toxicity to the linear peptide.\[^{[41]}\]

Apart from alkenes, alkynes can also react with thiols via radical pathway. Li et al. reported a one-component photo-induced thiol-yne stapling method on peptides bearing sulfhydryl and alkynyl at (i, i+4) positions (Scheme 1.5d).\[^{[42]}\] Both the Rotating-frame Overhauser effect (ROE) and MD simulation results suggested that the stapled peptides had helical configuration. To demonstrate the biological potential of this new method, the authors synthesised the vinyl sulfide stapled analogues modulating intracellular estrogen receptor (ER)-coactivator
interactions with a nanomolar binding affinity, enhanced serum stability, a diffuse cellular distribution and selective cytotoxicity towards ER-positive MCF-7 cells.

Overall, the thiol-ene/yne coupling reaction for peptide stapling has not been investigated thoroughly. The feasibility of this stapling in aqueous media, which is crucial for many biomolecules, is yet to be confirmed. Besides, the radical generated by UV might also be a barrier to applications involving large biomolecules. However, unlike the S-alkylation of cysteines with electrophilic linkers, the thiol-ene coupling does not rely on the prominent nucleophilicity of the thiolate to proceed, making it more applicable for peptides containing electrophile-sensitive functionalities.

### 1.1.5. s-Tetrazine

In 2010 and 2012, Smith and Brown first applied the dichlorotetrazine reagent to cross-link short peptides by selective removal of the Mmt cysteine side-chain protecting groups, followed by insertion of the S,S-tetrazine ring by treatment with 3,6-dichlorotetrazine under mildly basic conditions.[44-45] Later, they optimised this method to make it applicable to biomolecules.[46] They developed phase-transfer conditions for incorporation of the s-tetrazine moiety into unprotected peptides, possessing spacings (i, i+3 to i+28) between two cysteine residues, prepared by Fmoc-based SPPS. Most of the peptides have good conversions and yields (21–76%).

Furthermore, Smith et al. also found that the tetrazine moiety of a stapled peptide could be released in situ to their thiocyanate counterparts under 312 nm UV irradiation and, in turn, the resulting thiocyanates removed to regenerate the peptide (Scheme 1.6).[46] The relaxation of tetrazine stapled helical structures can be observed via transient 2D IR spectroscopy.[47]
The introduced \( S,S \)-tetrazine staple also provides a handle for inverse electron demand Diels–Alder reactions to incorporate probes and other functionalities. Smith and Brown demonstrated this strategy with tetrazine-stapled somatostatin which was reacted with a fluorescein dye containing an alkyne to produce a diastereomeric mixture of the fluorescein-labelled somatostatin (Scheme 1.7).\textsuperscript{[46]} Therefore, the tetrazine staples can both confine the peptide conformation and introduce photophysical and other potential probes.\textsuperscript{[46]}

\begin{align*}
\text{Scheme 1.6 Peptide stapling and photochemical unstapling of tetrazine staple.}\textsuperscript{[46]}
\end{align*}

\begin{align*}
\text{Scheme 1.7} \ S,S\text{-Tetrazine stapled somatostatin labelled with fluorescein via the inverse electron demand Diels–Alder reaction.}\textsuperscript{[46]}
\end{align*}
1.1.6. S-Alkylation

Alkyl halides are one of the most widely used functionalities for selective cysteine modification. Generally, S-alkylation with electrophiles to form a stable stapling structure can be achieved under physiological and mild aqueous alkaline conditions, compatible with unprotected peptides and other biological functionalities. To assist the solubilisation, organic co-solvent, such as acetonitrile, DMF or DMSO, can also be added. A reducing reagent, such as TCEP, can be used to prevent oxidation or reduce the disulfide bond.

Wooley et al. first reported this method to generate stapled peptides bearing two cysteines located at (i, i+4) or (i, i+7) or (i, i+11) positions, linked by a photosensitive bis-(2-haloacetamide) azobenzene (Scheme 1.8). For peptides with two cysteines located at (i, i+4) or (i, i+7), trans-to-cis photoisomerisation significantly increases the helix content in these two cases. In contrast, the (i, i+11) system is highly helical in the dark-adapted state with trans structure, and the helix content decreases dramatically upon irradiation at 370 nm (trans-to-cis isomerisation). These cross-linker designs significantly expanded the possibilities for photocontrol of peptide and protein structure. After this, Allemann et al. reported highly potent and specific examples that target the anti-apoptotic protein Bcl-xL. Photocontrol of Bcl-xL binding affinity has been achieved by using short BH3 domain peptides for Bak_{72–87} and Bid_{91–111} alkylated with an azobenzene crosslinker through two cysteine residues with different sequence spacings. Giralt and Gorostiza et al. applied this method in living cells. The azobenzene stapled peptides were able to permeate through the cell membrane and modulate their inhibitory PPI function inside the cell via photocontrol. In 2014, Wegner and Heinis et al. utilised this method to isolate a library of light-responsive constrained peptide ligands from a disulfide-free engineered phage display.
Scheme 1.8 Stapling with a photo-switchable azobenzene crosslinker. Stapling of two cysteines located at (i, i+4), (i, i+7) or (i, i+11) positions.

As well as the photo-switchable linker, Wooley et al. also applied other rigid linkers to staple and stabilise the peptides containing two cysteines located at (i, i+11) position.\(^{[54]}\) To develop α-helical calpain probes by mimicking a natural PPI, Greenbaum and co-workers established a new method of stabilising an α-helix in a small peptide by screening 24 commercially available cross-linkers for successful cysteine alkylation in a model peptide sequence containing two cysteines at (i, i+4) position.\(^{[55]}\) The cross-linkers included alkyl bromides, alkyl iodides, benzyl bromides, allyl bromide, maleimides and an electrophilic difluorobenzene (Scheme 1.9). However, only six of them were able to staple peptides smoothly (red structures in Scheme 1.9), with biaryl and simple alkyl halides failing to alkylate the cysteines and other linkers giving a complex mixture of products. Finally, they chose the \(m\)-xylene reagent to prepare conformational stable analogues of a calpastatin fragment. After testing different positions to place the crosslinker, they generated a \(m\)-xylene stapled peptide containing two-turn α-helix
that binds proximal to the active site cleft, resulting in a potent and selective inhibitor for calpain. They further expanded the utility of this inhibitor by developing irreversible calpain family activity-based probes (ABPs), which retained the specificity of the stabilised helical inhibitor.\cite{55}

\[\text{Scheme 1.9} \] Thiol reactive cross-linkers screened by Greenbaum et al.\cite{55} Red structures successfully stapled the peptides.

To create helical peptide inhibitors of therapeutically relevant intracellular PPIs, different series of two component bis-electrophilic linkers have been investigated not only to stabilise the helical conformation of the peptide ligands but also to improve cellular uptake and the resistance towards proteolytic degradation. In 2011, Lin et al. reported the design of bisarylmethylene bromides as a new class of rigid, distance-matching cysteine cross-linkers.\cite{56}

By cross-linking a peptide dual inhibitor of Mdm2/Mdmx containing cysteines at (i,i+7) positions, enhancement in cell permeability was achieved, along with increased helicity and biological activity.\cite{56} Furthermore, they applied this bisaryl crosslinker to staple a Noxa BH3 peptide, an Mcl-1 inhibitor with potential antitumor activity.\cite{57} After some mutations of the sequence to adjust the overall charge, the obtained stapled peptide greatly increased helicity, cell permeability, proteolytic stability, and cell-killing activity in Mcl-1-overexpressing cancer
cells. Besides, the crystal structure of mouse Mcl-1 in complex with Bph-stapled Noxa BH3 peptide revealed an additional π-π interaction between the biphenyl moiety and a His residue of the protein, which means that the crosslinker may also positively contribute to the binding interface. In 2014, they screened several different distance-matching aromatic linkers to construct the (i, i+7) bis-thioether structure of the Noxa peptide.[58] The stapled Noxa BH3 peptides with the flexible linkers gave the highest degree of helicity as well as the most potent inhibitory activity against Mcl-1. However, the stapled peptides with the highest hydrophobicity showed the most efficient cellular uptake.[58] There are many other examples utilising a biphenyl staple, including a monomeric GCN4 peptide[59] and a capsid assembly inhibitor[60].

![Image of different S-alkylation staples screened by the Ruchala group.][61]

More recently, the Ruchala group designed and synthesised a small library of stapled peptides using several different S-alkylation staples (Figure 1.2), to select inhibitors of the p53-Mdm2/Mdmx interactions.[61] The cysteines were located at (i, i+3), (i, i+4), (i, i+5) or (i, i+6) positions. The selected analogue, an o-xylene stapled peptide was tested in vivo showing potent anticancer activity at a low dose (3.0 mg/kg).

As well as being a linker to stabilise the helices of peptides, a peptide staple can also be used as a handle for modification or labelling. In 2015, Dawson et al. developed a ketone staple (Scheme 1.10), which can be modified with diverse molecular tags by aniline-catalysed oxime ligation. They inserted two homocysteines residues at (i, i+4) positions and crosslinked them
with a dichloroacetone linker.\textsuperscript{[62]} The stapled peptide was then further labelled with a variety of aminooxy-tags by aniline-assisted oxime ligation. However, this staple reagent reacted poorly with native cysteine under the same conditions, which largely limited its application.

\begin{center}
\textbf{Scheme 1.10} Peptide stapling with dichloroacetone and subsequent linker functionalisation via aniline-catalysed oxime ligation.\textsuperscript{[62]}
\end{center}

In 2016, Cramer and co-workers reported a method converting the disulfide bond of peptides into methylene thioacetals (\textbf{Scheme 1.11}).\textsuperscript{[63]} The reaction was performed in water/tetrahydrofuran mixed solvent in the presence of base at room temperature with various unprotected bioactive peptides. The described thioacetal modification on the peptide hormone oxytocin increases its serum, pH and temperature stability, while retaining its biological activities.

\begin{center}
\textbf{Scheme 1.11} Thioacetal linkages by methylene insertion into disulfides.\textsuperscript{[63]}
\end{center}
1.1.7. S_N-arylation

S_N-arylation is another emerging peptide stapling method with reactive electrophilic aryl linkers, which allows the effective stapling on cysteine residues under conditions compatible with unprotected peptide fragments.

In 2013, Pentelute et al. reported a perfluoroaryl-cysteine S_NAr chemistry approach for unprotected peptide stapling (Scheme 1.12a).[64] They developed the method on two short peptides containing cysteine residues separated by three amino acids. The stapling reaction was successfully conducted in Tris base solution in DMF, incubating the unprotected peptides with perfluoroaryl linkers, hexafluorobenzene and decafluorobiphenyl, at room temperature. Next, they applied this method to another more complex peptide that is capable of binding the C-terminal domain of an HIV-1 capsid assembly polyprotein (C-CA). The perfluoroaryl-stapled peptide possessed improved target binding, cell internalisation and proteolytic stability of the peptide ligand in comparison to its linear conformation.[64] After that, they applied this method to a general synthetic platform, by which they simultaneously scanned two cysteine residues positioned from (i, i+1) to (i, i+14) sites in a polypeptide, producing 98 macrocyclic products from reactions of 14 peptides with 7 linkers.[65] However, the major drawback of this method is the poor solubility of the linker, requiring pure organic solvents. Although they reported an enzyme-catalysed S_NAr reaction[66], it can only work with N-terminal GSH tag and a C-terminal perfluoroaryl-modified cysteine on the same polypeptide chain.

Scheme 1.12 S_N-arylation stapling methods. a) Perfluorobenzene stapling at cysteines[64]; b) S_NAr stapling at lysines[67].
Recently, the same group reported using the perfluoroaryl compounds to staple unprotected peptides via $S_N$Ar at lysines (Scheme 1.12b).\textsuperscript{[67]} The studies suggested that nitrogen-linked aryl products were more stable to base and oxidation than thiol arylated species. The strategy was tested on a MDM2 peptide and resulted in identification of a nanomolar binder with improved proteolytic stability and cell permeability.

In 2015, the Pentelute group reported that palladium(II) complexes can be used for efficient and highly selective cysteine conjugation reactions that are rapid and robust under a range of bio-compatible reaction conditions (Scheme 1.13).\textsuperscript{[68]} This conjugation reaction can be conducted in aqueous buffer solutions with around 5% organic solvents. As well as applications for bio-conjugation, they also employed this method to staple a model peptide containing two cysteines at (i, i+4) positions using a two-fold excess of a dual-palladium complex containing two electrophilic palladium ligands to a disubstituted benzophenone. The stapling reaction resulted in quantitative formation of the target stapled peptide within 10 min.

Scheme 1.13 $S_N$-arylation stapling via palladium-mediated cysteine arylation.\textsuperscript{[68-69]}

Lately, they expanded this technology by synthesising aryl linkers of varying length, rigidity, and electronic properties (Scheme 1.13) and studied the effect of linkers on the lipophilicity, human serum albumin (HSA) binding, phospholipid affinity, and target binding affinity of these macrocyclic peptides.\textsuperscript{[69]} The palladium reagents were used to macrocyclise two bioactive peptides: NYAD-1 that binds the HIV-1 C-terminal capsid protein (C-CA) with cysteine
residues at the (i, i + 4) positions and a p53/Mdm2 inhibitor analogue with cysteine residues at the (i, i + 7) positions. Although the linkers have significant effect on the physiochemical properties, most of the stapled peptides lost their binding affinities, especially the biphenyl and biaryl ether species.

Overall, although this approach provides facile access to a diverse aryl-linker for stapled peptides, the additional preparation of the palladium crosslinking reagents and the decrease of binding affinity limit this method to produce bioactive peptides.

1.1.8. Outlook

Since the idea was first introduced decades ago, the toolbox for peptide stapling has been significantly expanded yielding improved efficiency, selectivity and yield. Described in this chapter strategies provide easy access to bioactive cyclic peptides as potential therapeutics and research tools for biological investigations. When it comes to bioactive peptide modification, the cysteine alkylation strategy is one of the promising strategies for peptide stapling since it introduces minimal changes to the sequence and structure. Several challenges remain to be overcome before the cysteine stapling strategy becomes the method of choice for peptide stapling. Firstly, efficiency of the stapling reaction is important in order to reduce the oxidation of the thioether linkage and avoid the generation of disulfide bonds. The efficiency largely depends on the reactivity of the stapling reagent and the buffer system, which needs to be carefully optimised. Secondly, a small structure is necessary in order not to cause detrimental effects to the structure and subsequent biological functions. Moreover, the stability of the linkage is also crucial for developing peptide-based therapeutics.

Overall, with more and more novel peptide stapling methods emerging, the future for efficient peptide macrocyclisation and peptide-based therapeutics remains bright.
1.2. Site-selective Re-bridging of Protein Disulfides

In some cases, often termed disulfide re-bridging, stapling can be applied to proteins in order to improve stability, control activity and produce antibody-drug conjugates (ADCs). Disulfide bonds are responsible for the structure, physico-chemical function and biological activity of proteins.[70-71] They are located either in the buried regions of the protein’s folded region or on its solvent-exposed surface.[72] Most biological therapeutics contain at least one solvent-accessible disulfide[73], providing an opportunity for site-selective modifications. Compared to the stapling of peptides, there are some significant hurdles to disulfide re-bridging in proteins. First of all, the reagents need to be water-soluble. In some cases, the aid of a small percentage of organic co-solvent is tolerated, but a high organic to water ratio will cause denaturation and aggregation of the proteins. Secondly, the size of the inserted moiety needs to be comparable to the disulfide bond in order to be able to re-bridge the thiols without twisting the tertiary structure of the protein. Thirdly, high reactivity and site-selectivity is necessary for the reaction to minimise the possible disulfide scrambling and unintended modifications on other residues. Finally, the inserted moiety needs to be compatible with the bio-environment, especially the reactive thiols common in the biological systems. Therefore, most of the previously mentioned stapling techniques for peptides are not easily translatable to proteins.

The modification of disulfide bonds includes several key steps. Firstly, the reduction of the solvent-accessible disulfide bond is achieved by using various reducing agents. In many cases, the mild reductant, tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, is used either before or in situ together with the disulfide re-bridging reagents. Other reductants, such as dithiothreitol (DTT) or β-mercaptoethanol are used when stronger reducing agents are needed. However, these reagents have poor stability in the presence of metal ions[74] and a narrow working pH[75]. More importantly, the unreacted reductants have to be removed immediately after the reduction as the contained thiol groups react with the disulfide re-bridging reagents.[73] After the addition of the disulfide re-bridging reagent, it reacts with one of the reduced thiol groups before completing the re-bridging process by reacting with the second thiol group of the original disulfide bond.

Since the disulfide re-bridging was first developed, the toolbox for this purpose has been largely expanded. In the following sections, a few widely used techniques, including bis-
sulfones, next generation maleimides and pyridazinediones, will be discussed. Several reviews have been published introducing different techniques for disulfide re-bridging.\cite{76-77}

### 1.2.1. Bis-sulfones and allyl sulfones

**Bis-sulfones**

In 2006, the Brocchini group first discovered a method for PEGylation at native disulfide bonds using bis-thiol alkylating reagents.\cite{78} The bis-sulfone reagent can be prepared from \( p \)-acetylbenzoic acid in three steps, and functional groups of interest can be conjugated to the carboxyl group (Scheme 1.14).

![Scheme 1.14 Synthesis of bis-sulfones from \( p \)-acetylbenzoic acid.\cite{79}](image)

The insertion of the covalent three-carbon PEGylated bridge structure was highly selective and efficient, and the PEGylated protein maintained its tertiary structure and biological activity. The stapling proceeded as described in Scheme 1.15. Prior to the re-bridging steps, the bis-sulfone undergoes *in situ* elimination of an equivalent of the sulfinic acid to obtain the respective allyl sulfone at neutral or weakly basic pH.\cite{73} The elimination step has been elucidated by NMR spectroscopy of a PEGylated bis-sulfone.\cite{80} The resulting allyl sulfone is normally comprised of an electron-withdrawing group, an \( \alpha,\beta \)-unsaturated ketone and a \( \alpha,\beta' \)-sulfonyl group. After the native solvent-exposed disulfide is reduced to two reactive cysteines, one of the thiol groups then reacts with the double bond to eliminate the second sulfinic acid,
providing another double bond at the α,β'-position. Then, the second Michael-type addition with the other thiol group of cysteine residue occurs, forming the three-carbon bridge and generating the bisthioether. NMR spectroscopy of a modified somatostatin illustrated the formation of diastereomers during the second addition\[^{[81]}\]. The conjugation is usually carried out \textit{in situ} starting with the bis-sulfones in a weakly basic pH solution. Studies by Weil and co-workers showed that side reactions with water can occur at pH 8 presumably due to the reactivity of the alkene group in the allyl sulfone\[^{[82]}\].

![Scheme 1.15](image)

\textbf{Scheme 1.15} Mechanism of disulfide re-bridging by bis-sulfones to form a three-carbon bridge between two cysteine residues in proteins.

Moreover, functionalisation of the bis-sulfone can be achieved by amide coupling or ester formation to introduce different functionalities, including PEG chains, drugs, fluorescent tags and bioorthogonal handles\[^{[81, 83-85]}\].

For example, human interferon α-2b (IFN) was used in initial studies as a representative of four-helical-bundle proteins with accessible disulfide bonds\[^{[86]}\]. IFN has two solvent-accessible disulfide bonds which can be easily reduced. Brocchini and co-workers demonstrated that single-PEGylated IFN (57%), with a bridge at either Cys1–Cys98 or Cys29–Cys138, can be achieved when stoichiometric amount of bis-sulfone reagent is added. The double-PEGylated
IFN can be made as the major product using two equivalents of the PEG bis-sulfone reagent. The same PEG bis-sulfone reagent was applied to a human CD4 receptor-blocking antibody fragment (Fab) and L-asparaginase. The PEGylated L-asparaginase retained its enzyme activity and immunogenicity after the reaction. The PEG-Fab, re-bridged at the interchain disulfide bond, was as effective as native Fab at blocking HIV-1 entry into CD4+ T-lymphocyte cells. Various Fab fragments from bevacizumab, ranibizumab and trastuzumab were confirmed to be able to react with the PEG bis-sulfone reagent. The PEGylated Fab variants displayed 2-fold reduction in binding affinity. Brocchini and co-workers also designed a dual-bis-sulfone PEG, which linked two Fabs together to give Fab-PEG-Fab (Figure 1.3a), acting as an IgG mimetic, and displayed similar apparent affinities to their parent IgGs. In 2014, Godwin and co-workers applied the bis-sulfone strategy to generate ADCs with trastuzumab and cytotoxic drug MMAE via an enzyme cleavable valine-citrulline dipeptide and self-immolative p-aminobenzyl moiety (Figure 1.3b). A 78% conversion of antibody to ADC with a drug to antibody ratio (DAR) of 4 by hydrophilic interaction chromatography (HIC) analysis, were achieved with no unconjugated antibody remaining.

![Figure 1.3 Applications of bis-sulfone reagents. a) Fab-PEG-Fab conjugate, acting as an IgG mimetic; b) Trastuzumab-MMAE conjugate via valine-citrulline and PABC linker.](image-url)
**Allyl sulfones**

Due to two hydrophobic benzene groups, bis-sulfone reagents usually possess low water-solubility. In most cases, organic-aqueous co-solvents need to be used, which could lead to denaturation of the protein. Therefore, developing water-soluble reagents remains of great interest in the field of disulfide re-bridging. Recently, Weil and co-workers reported water-soluble allyl sulfones as efficient disulfide modification reagents with improved reactivity, no necessary in situ activation, high stability and increased water-solubility (Scheme 1.16). Compared to existing bis-sulfones, the allyl sulfones possess two fewer hydrophobic benzene groups. Therefore, their n-octanol-water partition coefficients (logPo/w) is lower, indicating better water solubility. The reagent was tested using on a variety of peptides and proteins, including the peptide hormone somatostatin (49%), bovine insulin (28%) and lysozyme (19%) with one, three and four disulfide bonds, respectively. The unreacted native protein could be recovered and recycled for a subsequent reaction.

![Scheme 1.16 Synthesis of allyl sulfones with different functionalities.](image)

Overall, the bis-sulfones or allyl sulfones have been widely studied in terms of protein substrates, functionalisation and linker screening. Although the reactions are normally efficient
and fast, the synthesis of the linker, solubility and stability still remain as concerns for broader applications.

1.2.2. Next generation maleimides

In 2010, the Baker group reported the first next generation maleimide to be used for efficient cysteine cross-linking.\[^{93}\] Since then, several substituted maleimides have been developed, including different di-substituted maleimides\[^{94-109}\] and aryloxymaleimides\[^{110}\].

**Di-substituted maleimides**

The earliest example reported by Baker group is the dibromomaleimide, consisting of two bromine groups at 3- and 4-position which enables two addition-elimination reactions to re-bridge a reduced disulfide bond with a rigid two-carbon spacer.\[^{93}\] The di-substituted maleimides can react within a broader pH range (pH 6–8) than the bis-sulfones, which normally require basic pH for the elimination process. The functionalisation of the di-substituted maleimides can be carried out *via* dehydrative cyclisation\[^{111}\] from dibromomaleic anhydride\[^{93}\] or the Mitsunobu reaction from the commercially available dibromomaleimides\[^{95}\] (Scheme 1.17). Baker and co-workers also reported that the dibromomaleimide might cause side reactions when being used with TCEP, yielding a relatively low conversion rate. Therefore, the reaction cannot be performed *in situ* with reducing agents.\[^{95}\] After screening different dihalomaleimides, dimercaptoethanolmaleimide and dithiophenolmaleimide, the dithiophenolmaleimide was proven to be both the most reactive and compatible with TECP, allowing *in situ* reduction and re-bridging.\[^{95}\] Alternatively, only in the presence of dithiophenolmaleimide, benzeneselenol was applied to catalyse the disulfide cleavage and allowed *in situ* bridging.\[^{102}\] Dithiophenolmaleimide can be prepared from dibromomaleimide in one step or from dibromomaleic anhydride in two (Scheme 1.17).
Scheme 1.17 Synthesis of di-substituted maleimides and aryloxymaleimide from bromomaleic anhydride.

The first application of di-substituted maleimides was to label peptide hormone somatostatin with a fluorescein[93] or PEG[95]. Since then, next generation maleimides have been expanded to various applications. The Haddleton group applied dibromomaleimide for efficaciously and selectively decorating a 32-aa hormone peptide, salmon calcitonin (sCT). sCT contains one disulfide bond that was modified with synthetic PEG polymers in less than 15 min using a stoichiometric balance of reagents.[96] Since the functionalisation of PEG with dibromomaleimide using the Mitsunobu conditions is relatively slow with poor yields, the group attempted to synthesise α-functional polymers by functional atom transfer radical polymerisation (ATRP) directly. However, due to polymerisation inhibition of the dibromomaleimide, the direct polymerisation was impossible. Therefore, indirect routes were chosen via both azide and aniline functional initiators, which were converted to 2,3-dibromomaleimides via a dehydrative cyclisation with dibromomaleic anhydride or the CuAAC click reaction to an alkyne functional dibromomaleimide.[96] Subsequently, they reported the direct synthesis of dithiophenolmaleimide functional polymers via ATRP reactions without the need for protecting groups.[112-113]

In addition to the applications in polymerisation and protein PEGylation, the di-substituted maleimides have been widely applied to produce homogeneous ADCs. Several groups, including Baker, Chudasama and Caddick, have contributed to designing homogeneous ADCs with a controlled DAR. The first study published in 2013 described an acid-cleavable linker strategy for antibody-drug conjugation. The interchain disulfide of the trastuzumab Fab fragment was re-bridged with a dithiophenolmaleimide (later hydrolysed to thiomaleamic acid
post conjugation) functionalised with an anti-cancer drug, doxorubicin (DOX). The binding of the conjugates to the antigen HER2 was retained.\[97\]

Later, the same group reported the application of dithiophenolmaleimide on trastuzumab.\[102\] The maleimides were either linked with DOX or possessed an alkyne handle which was then functionalised with DOX (Scheme 1.18a). Through screening the reductant to reagent ratios, the DARs of the conjugates were controlled to 1, 2, 3 and 4. When using TCEP as the reducing agent, the reduction and conjugation can be carried out simultaneously in situ. As well as the native re-bridged product, an unfavoured half-stapled antibody was observed with an intrachain bridge between the hinge cysteines.\[102\] However, due to the strong non-covalent interactions between the two half antibodies, the two parts are held together in vivo with unaffected pharmacological properties.\[105\]

Scheme 1.18 Di-substituted maleimides constructed ADCs. a) Alkyne functionalised dithiophenolmaleimide re-bridging and subsequent CuAAC click reaction with azide-PEG\(_4\)-DOX;\[102\] b) optimised pro-hydrolysis dibromomaleimide containing a C2 spacer with DOX.\[108\]
Subsequently, Chudasama and co-workers studied the stability and *in vitro* efficacy of an industry relevant ADC, trastuzumab conjugated to the anti-cancer drug monomethyl auristatin E (MMAE) via a non-cleavable PEG12 linker. The trastuzumab-MMAE conjugate was shown to selectively target and kill HER2+ cells. At the same time, Jackson and co-workers reported that ADCs derived from interchain cysteine cross-linking with MMAF-dibromomaleimides demonstrated improved homogeneity, pharmacokinetics, efficacy and reduced toxicity *in vivo* compared to analogous conventional heterogeneous ADCs.

Although the hydrolysed conjugates were proven to be more stable than the initial conjugates, the hydrolysing conditions were relatively harsh, usually requiring 72 h incubation at pH 8.4. Therefore, Baker and Chudasama optimised the hydrolysis procedure by screening several different linkers. Dibromomaleimides with electron-withdrawing C-2 linkers were demonstrated to provide an optimised conjugation method for a homogeneous and serum-stable ADCs (Scheme 1.18b). The conjugation reaction and hydrolysis procedure took just over 1 hour, yielding an ADC with a DAR of 4. With a largely shortened process, the homogeneity of the conjugates was also improved by avoiding undesired side-reactions. The conjugates were proven to be completely stable at acidic to neutral pH.

On the other hand, the Baker group utilised a bis-dibromomaleimide cross-linker to produce homogeneous bispecifics that were comprised of two antibody fragments. These two fragments can bind two different antigens to improve their therapeutic efficacy (Scheme 1.19). This method was demonstrated through connecting two antibody fragments, namely disulfide-stabilised single-chain variable antibody fragment (scFv) against a carcinoembryonic antigen (CEA) and Fab antibody fragment against the HER2 antigen. The bispecifics exhibited retention of activity by ELISA and cell binding assays. Recently, they expanded the technology with diiodomaleimides and produced HSA-Fab and HSA-scFv conjugates in a highly efficient manner. Both conjugates retained binding affinity to their target antigens. Moreover, they also synthesised a trifunctional diiodomaleimide linker which was then used to construct a tri-scFvs conjugate. Compared to the monomer, the trimerised scFvs possessed an increased circulatory half-life.
Scheme 1.19 Construction of homogeneous bispecific antibody conjugate, scFv-PEG-Fab, by bis-dibromomaleimide cross-linker.$^{[101]}$

As well as producing ADCs, the conjugation of peptides or proteins with functionalised di-substituted maleimides has also been used for other applications, such as antibody fragment diagnostics$^{[99]}$ and nuclear imaging$^{[104]}$.

**Aryloxymaleimides**

Single-substituted maleimides, such as aryloxymaleimides, have also been reported to be able to re-bridge disulfide bonds to form succinimide bridges (Scheme 1.20a).$^{[110]}$ Moreover, the aryloxymaleimides are compatible with TCEP, allowing in situ reduction and re-bridging. However, the addition-elimination step is slower, and the conversion rate varies, especially for the phenoxymaleimide. This can be potentially solved by incorporating electron-withdrawing groups on the aryloxy ring.
Scheme 1.20 Additional next generation maleimide applications. a) Disulfide re-bridging of anti-CEA ds-scFv with aryloxymaleimides to form the succinimide bridge;\textsuperscript{110} b) the re-bridging of a Fab fragment \textit{via} photo-triggered [2+2] cycloaddition.\textsuperscript{34}

\textbf{Photo-triggered cycloaddition}

As mentioned in the cycloaddition stapling method in the section 1.1.3, Baker and co-workers reported a photochemically triggered disulfide re-bridging method.\textsuperscript{34} This was achieved by the incorporation of bromomaleimides to form thiomaleimides which subsequently undergo [2+2] photo-cycloadditions to generate a cyclobutene containing a rigid two-carbon bridge between the two thiol groups (Scheme 1.20b). In this case, a mixture of the different diastereo- and regioisomers could be formed. The application was demonstrated with octreotide, a small cyclic peptide, and a Fab fragment of trastuzumab. The binding of the photochemically bridged Fab to its HER2 antigen was retained.\textsuperscript{34} However, the functionalisation of maleimides in this application still remains unexplored.

\textbf{1.2.3. Pyridazinediones (PDs)}

Dibromopyridazinediones (diBrPDs), structurally similar to dibromomaleimides, were initially reported by the Caddick group for protein and peptide bioconjugation and can be used for re-bridging the somatostatin disulfide.\textsuperscript{114} The re-bridging reaction performs similarly to the dibromomaleimide, but diBrPDs contains four attachment points instead of three. The PDs can be synthesised from maleic anhydride and disubstituted hydrazines, with further
functionalisation possible by amide coupling (Scheme 1.21a).\textsuperscript{[115]} Recently, Bahou \textit{et al.} presented a new synthetic route from one-pot reaction between dibromomaleic acid and Boc-protected hydrazines to afford the diBrPDs, followed by activation with $N$-hydroxysuccinimide and conjugation with functionalised amines to achieve the bifunctional diBrPDs (Scheme 1.21b).\textsuperscript{[116]}

Scheme 1.21 Synthesis of dibromopyridazinediones (diBrPDs) a) from maleic anhydride and hydrazines, followed by amide coupling\textsuperscript{[115]}; b) from dibromomaleic acid and hydrazines, followed by activating and coupling\textsuperscript{[116]}.

In 2015, Chudasama and Caddick first reported using the bifunctional diBrPDs to selectively re-bridge the interchain disulfides of Fab fragments and trastuzumab.\textsuperscript{[117]} The authors designed and synthesised a diBrPD containing two bioorthogonal handles (Scheme 1.22): a terminal alkyne, which reacts with azides \textit{via} CuAAC, and a strained alkyne, which reacts with azides \textit{via} SPAAC. The successful conjugation was confirmed by SDS-PAGE analysis with a small amount of half-stapled antibody byproduct present, in a similar manner to next generation maleimides re-bridging.\textsuperscript{[102]} The re-bridged antibody was then functionalised with doxorubicin-azide and sulfo-Cy5-azide fluorophore \textit{via} click chemistry. The internalisation studies of conjugates, antibody-PD-AlexaFluor488, demonstrated the selectivity towards the HER2$^+$ cells (BT-474) over HER2$^-$ cells (MDA-MB-468).\textsuperscript{[117]} Later, the Chudasama group...
applied the diBrPDs with a strained alkyne handle to produce multi-porphyrin attached antibody photodynamic therapeutics via SPAAC click chemistry.\textsuperscript{[115]} The antibody-PD-porphyrin exhibited high phototoxicity at 625 nM to eradicate the HER2\textsuperscript{+} cells (90\% kill), leaving HER2\textsuperscript{-} unaffected.\textsuperscript{[115]}

\textbf{Scheme 1.22} Dual functionalisation using bifunctional dibromopyridazinedione, containing a linear alkyne reacted with DOX-PEG\textsubscript{4}-N\textsubscript{3} via CuAAC and a strained alkyne reacted with sulfo-Cy5-N\textsubscript{3} via SPAAC.\textsuperscript{[117]} *Both regioisomers are obtained, although only one is shown.

To overcome the limitation of the requirement for reduction and re-bridging in distinct steps, Chudasama and co-workers developed a novel dithioaryl(TCEP)pyridazinedione, which can perform both disulfide reduction and functional re-bridging (\textbf{Figure 1.4}).\textsuperscript{[118]} The reagent can be prepared from hydrazine to afford the diBrPD, followed by reacting with 4-aminothiophenol and coupling with a mono-acid TCEP derivative to form the designed reagent. These all-in-
one reagents were applied to Fab fragments of full-length trastuzumab. The successful re-bridging and CuAAC reaction of Fab fragments were observed by LC–MS and SDS-PAGE.\textsuperscript{[118]}

![Chemical structure]

**Figure 1.4** Dithioaryl(TCEP)pyridazinedione reduction/conjugation all-in-one reagent and its application.

Later, Chudasama and Baker applied the PD platform to attach MMAE to trastuzumab via either a non-cleavable PEG linker or a cleavable valine-citrulline linker. These ADCs were made alongside dithiophenolmaleimide re-bridged and classical maleimide conjugated ADCs.\textsuperscript{[119]} HIC analysis demonstrated that the 90% of the PD bridged ADCs had a DAR of 4, representing the successful re-bridging of all four interchain disulfides. Both PD-ADCs displayed potent antiproliferative activities against a HER2\textsuperscript{+} cell line. The \textit{in vivo} assay in a mouse xenograft model demonstrated the efficacy of both ADCs and the non-necessity of the cleavable linker.\textsuperscript{[119]}

Another example recently published by Shao \textit{et al.} reported using the synthesised dithiophenolpyridazinedione to attach the anti-tubulin drug, mertansine (DM1), to the trastuzumab via a non-cleavable SMCC linker (\textbf{Figure 1.5}).\textsuperscript{[120]} On the other arm of the PD, PEG\textsubscript{6} and PEG\textsubscript{26} were attached to increase the solubility of the DM1. Therefore, organic solvents can be reduced or even avoided when performing the conjugation to avoid aggregation. Although over 50% of the ADCs are half-stapled forms, as observed from SDS-PAGE and LC–MS, the two ADCs in the \textit{in vivo} assay displayed similar efficacy with the clinically approved Kadcyla\textsuperscript{®}, trastuzumab covalently linked to DM1.\textsuperscript{[120]}
In order to minimise the generation of half-stapled antibody, Chudasama and Baker optimised the reduction/re-bridging procedure by adding reducing agent and diBrPD reagent in tandem, which significantly reduced the portion of half antibody to less than 5%.\[116\]

For certain hydrophobic drugs, in order to avoid aggregate formation and too rapid clearance, the most desirable DAR is two instead of four.\[121\] This challenge has only been addressed by antibody engineering approaches, such as THIOMAbs, containing an engineered cysteine on each light chain. Chudasama and co-workers presented a PEG-linked bis-diBrPD reagent, containing a terminal alkyne handle (Figure 1.6).\[122\] Two bis-diBrPDs can re-bridge all four interchain disulfides, providing only two functional alkyne groups for click reactions with azide compounds, which was confirmed by mass spectrometry. Furthermore, the authors also synthesised a bis-diBrPD, containing two alkyne handles, to achieve an overall DAR of 3.9.\[122\]

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**Figure 1.5** Trastuzumab attached with DM1 via SMCC linker and a PEG spacer on the other arm of PD.\[120\]

**Figure 1.6** Bifunctional dibromopyridazinedione reagent and its application to build a DAR 2 antibody-drug conjugate.\[122\]
Recently, Chudasama and Baker appraised the Fc function of disulfide modified antibodies. They studied both re-bridged and half-stapled structures, along with thiol-capped forms.\textsuperscript{[123]} The natively re-bridged conjugates displayed no apparent decrease in CD16a or neonatal Fc receptor (FcRn) binding affinity and no significant decrease of the antibody-dependent cellular cytotoxicity (ADCC) activity. Meanwhile, the half stapled affected the ADCC activity with a 10 times higher EC\textsubscript{50} value than the native trastuzumab.\textsuperscript{[123]}

1.2.4. Other reagents

Dichlorotetrazine

As mentioned in Scheme 1.6 and Scheme 1.7, Smith and Brown employed the dichlorotetrazine to staple peptides and performed the inverse electron demand Diels–Alder (iEDDA) reaction with the stapled peptides.\textsuperscript{[46]} They also applied this method to re-bridge the disulfide of the thioredoxin (Trx) protein (Scheme 1.23).\textsuperscript{[46]}

Scheme 1.23 Thioredoxin disulfide re-bridging using dichlorotetrazine and the inverse electron demand Diels–Alder reaction with alkyne-containing fluorescein probe.\textsuperscript{[46]}

The unstapled Trx could also be regenerated by irradiating with UV light at 312 nm and subsequent treatment with cysteine and TCEP. However, the regenerated protein did not
display substantial catalytic turnover for the reduction of insulin. Even so, it remained reactive toward electrophiles, such as dichlorotetrazine, when re-subjected to the stapling conditions.\cite{46} This might be owing to the strong power of the UV lamp, which destroys the complex structure. Moreover, the stapled Trx was reacted with fluorescein dye containing an alkyne to generate a fluorescein-Trx via the iEDDA reaction. Although the conjugate was witnessed both by the retention of green colour from fluorescein upon separation by filtration and by MALDI-TOF-MS (Scheme 1.23), the reaction (10 days at room temperature) was not efficient and no conversion rate was reported.\cite{46} In order to produce ADCs, the efficacy of the iEDDA reaction needs to be improved significantly.

**Dichloroacetone**

As mentioned in Scheme 1.10, Assem et al. developed the dichloroacetone re-bridging strategy to build an acetone-linked bridge, followed by modification via oxime ligation.\cite{62} The strategy has been expanded by Stefanetti et al. on diphtheria toxin mutant CRM197, a clinically used carrier in many glycoconjugate vaccines. An acetone-linked bridge was formed selectively at the C186–C201 bond, which is exposed to the solvent, while the other disulfide bond at C461–C471 remained unchanged.\cite{124} The acetone bridge was further modified via oxime ligation to incorporate bioorthogonal handles for the attachment of other payloads, such as antigens (Scheme 1.24). Compared to conjugates at higher antigen loading, the conjugate CRM197-lipopolysaccharide resulted in relatively high anti O-antigen bactericidal antibody titers.\cite{124} However, there are no studies published about using this method for re-bridging the disulfides on antibodies or the stability studies of the conjugates.

**Scheme 1.24** Re-bridging the C186–C201 disulfide of CRM197 with dichloroacetone, followed by oxime ligation.\cite{124}
**Arsenous acid**

In 2015, Wilson et al. reported a trivalent arsenous acid (As$^{\text{III}}$), obtained from $p$-arsanilic acid (As$^{\text{V}}$), as a new disulfide re-bridging reagent.$^{[125]}$ The re-bridging of the C1–C7 disulfide on sCT was completed in less than 2 minutes *in situ* and proceeded stoichiometrically through sequential reduction–conjugation (**Scheme 1.25**)$^{[125]}$ The sCT was released from the conjugate in the presence of a strong chelating reagent, such as ethanedithiol (**Scheme 1.25**). In contrast to the quantitative labelling with maleimide modification reagents, the application on bovine serum albumin was achieved selectively at the disulfide-bond in the presence of the free Cys-43 residue with only 20% labelling.$^{[125]}$ The authors postulated that the formation of the monothiol adduct is entropically unfavourable, particularly in the presence of chelating dithiols. Therefore, the desired re-bridged product is formed.$^{[125]}$ Additionally, the arsenic-functional polymers displayed negligible cytotoxicity relative to free arsenic salts.$^{[125]}$ However, the stability of the conjugates, especially with regards to oxidation, remains a concern when this strategy is used for producing large amounts of biological therapeutics requiring multiple purification steps.

![Scheme 1.25 Re-bridging sCT disulfide with trivalent arsenous acid (As$^{\text{III}}$), followed by releasing with ethanedithiol.$^{[126]}$](image)

**Thiol-yne coupling**

A photochemical method for disulfide re-bridging using the thiol-yne coupling reaction was reported by Bräse and co-workers.$^{[126]}$ The re-construction of Fab disulfides was carried out under UV-light (365 nm) with 6-heptynoic acid and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, a water-soluble radical initiator) (**Scheme 1.26**). However, the yield of the coupling reaction was extremely low, even with small cyclic peptides, and the unavoidable regio- and stereoisomer products could not be separated. Moreover, the reaction with reduced Fab was carried out at a very high antibody concentration (46.1 mg/mL) and only
gave a 40% conversion ratio. At lower concentrations the reaction did not proceed at all.[126] Further optimisation of the reaction needs to be investigated in order to achieve higher yields and broader applications.

Scheme 1.26 Fab reconstruction via thiol-yne coupling with 6-heptynoic acid at 365 nm.[125]

Divinylpyrimidine

Recently, divinylpyrimidine was reported by Spring and Carroll as a disulfide re-bridging reagent for ADCs (Scheme 1.27).[127] The strategy was demonstrated on the RadA enzyme with two engineered cysteines, a Fab fragment and full-length trastuzumab. Different payloads were site-selectively and efficiently conjugated to the trastuzumab with controlled DAR. Moreover, the re-bridged structure was compatible with bioorthogonal reactions, such as CuAAC, enabling further functionalisation of the introduced handle. The conjugates were stable in human plasma supplemented with GSH at 37 °C for two weeks.[127] The produced trastuzumab-MMAE conjugate demonstrated exquisite potency and selectivity towards the HER2+ cells (SKBR3 and BT474) over HER2− cells (MCF7 and T47D).[127]

Scheme 1.27 Antibody disulfide re-bridging using a divinylpyrimidine reagent functionalised with a bioorthogonal handle or payload.[127]
1.2.5. Stability of disulfide re-bridged conjugates

The stability, especially the resistance to biological thiols, is a key feature to be considered when applying disulfide re-bridging strategies to produce therapeutics.

Several studies about bis-sulfones and allyl sulfones investigated the possibility of cross conjugation of the payloads to other thiol containing species including serum albumin.\cite{84, 90} Badescu et al. conducted a serum stability study using an Alexa Fluor 488 labelled bis-sulfone conjugated to trastuzumab. After being incubated in either 50% rat or undiluted human serum for up to 96 h at 37 °C, serum samples were analysed by SE-HPLC, which revealed that the conjugate remained largely unchanged. In comparison, the peak of traditional maleimide conjugate showed a significant decrease in both rat (47%) and human serum (24%).\cite{90} Furthermore, the authors also monitored the change of DAR over time for MMAE-trastuzumab conjugates in an artificial serum composed of 20 mg/mL human albumin solution (HAS) in PBS. After being incubated for 120 h at 37 °C, the DAR of MMAE-bisthioether-trastuzumab remained unchanged at around 2.3, and the DAR of the maleimide conjugate decreased from 3.2 to 1.9.\cite{90} Although Weil and co-workers demonstrated that bisthioether conjugates could be released at high GSH levels (10 mM) inside cancer cells, the GSH level in blood serum is relatively low, preventing the deconjugation of the drug in blood.\cite{84}

Dibromomaleimide-linked conjugates were proven to be cleavable with first-order kinetics both in vitro and in cells.\cite{128} Several studies also confirmed that the hydrolysis of dithiomaleimide bridges to thiomaleamic acid can improve the serum stability.\cite{97, 106, 129} Upon incubation of the dithiomaleimide antibody conjugate in human serum, thiol exchange with albumin’s cysteine 34 occurred within 4 days. In contrast, the maleamic acid conjugate was completely stable, even over a prolonged period of 7 days.\cite{106} Caddick and Smith demonstrated that while the hydrolysed thiomaleamic acid linker was stable at physiological pH and temperature, it can be quantitatively cleaved at lysosomal pH to release a payload.\cite{97} The succinimide-bridged scFv conjugate from aryloxymaleimides was proven to be fully stable in the presence of 100 equiv. of GSH after 48 h at room temperature.\cite{110}

On the other hand, the pyridazinediones-stapled antibodies were shown to have excellent stability for 7 days in conditions mimicking blood plasma, complete stability after 8 months of storage at 4 °C in PBS buffer and stability in a broad pH range (3–9) over a protracted period at 37 °C.\cite{117} Chudasama and Baker investigated the biophysical profile of disulfide modified
antibodies formed from dithiopyridazinediones.\textsuperscript{[123]} The biophysical analysis of these conjugates, including aggregation tendencies, thermal stability and antigen-binding, demonstrated that the natively re-bridged antibody had no negative impact. However, thiol-capped forms showed a decreased thermostability, and the half-stapled conjugate displayed the most aggregation (2\%). These studies confirmed that native disulfide re-bridging could be a promising strategy for producing homogeneous ADCs.\textsuperscript{[123]}

In terms of other stapling techniques, such as the dichloroacetone and arsenous acid, the applications are limited and there are no stability studies reported.

Disulfide re-bridging moieties can not only bring new functionality to proteins, but can also enhance the stability and activity of proteins, including antibody fragments. Spokoyny \textit{et al.} demonstrated that hexafluorobenzene can increase the $\alpha$-helical content of the HER2 affibody fragment while maintained similar binding of the stapled affibody as compared to the alkylated congeners.\textsuperscript{[64]} Previous research from our group showed that oxetane re-bridged Fab fragment of an anti-HER2 antibody possessed enhanced binding affinity to the HER2 receptor, and was fully stable in the presence of glutathione or human plasma.\textsuperscript{[130]}

\subsection*{1.2.6. Outlook}

In summary, disulfide re-bridging has attracted increasing attention for site-selective protein modification. The strategy has been used for the construction of homogeneous ADCs with defined DAR, polymerisation of proteins, and for the study of protein-protein interactions. It has been demonstrated to have several advantages over traditional modification strategies, including high selectivity over other amino acids (especially lysine), wide compatibility with various proteins and good serum stability, as seen for pyridazinedione reagents. However, several critical challenges still remain. Firstly, the selectivity over native free cysteine is still a concern, even though reactive cysteine is not abundant in proteins. Secondly, the accessibility of the disulfides largely affects the activity. Modifying the buried disulfides can achieve site-selective multi-labelling of a single protein. However, this requires the re-bridging to have a high reactivity and a suitable molecular size. Since this field has only been explored for less than two decades, it is well expected that increasingly promising disulfide re-bridging reagents will be developed and applied in many other areas.
1.3. Photo-Triggered Drug Delivery and Activation

In recent decades, precision medicine has attracted attention for the effective treatment of various diseases, especially cancer. Currently, as a result of a lack of selectivity in the pathological sites, the development of new, effective, and safe therapies remains challenging. Among various new methods, the recently developed light-mediated treatment is recognised as an appealing approach to achieve controlled activation of medicine at pathological sites, and could significantly reduce the side effects of chemotherapy.\textsuperscript{[131]} Compared to other exogenous and endogenous stimulus, light possesses several unique advantages, such as spatiotemporal tunability, near-instantaneous release, non-invasiveness and minimal off-target effects.\textsuperscript{[132-134]} Therefore, a large variety of photoresponsive drug delivery and activation systems have been developed to enable on-demand cargo release and activation. So far, in the battle against cancer, several types of light-activated anticancer reagents, which can be switched on conditionally with irradiation, have been investigated.\textsuperscript{[135-140]} The structures of these photocaged drugs include various ultraviolet, near-infrared, or visible responsive moieties,\textsuperscript{[141]} such as \textit{o}-nitrobenzyl,\textsuperscript{[135, 142-144]} coumarinyl ester,\textsuperscript{[145-146]} and metal complexes.\textsuperscript{[147-149]} These photoresponsive structures offer an extensive toolbox for use in cancer therapies and other biological applications.

Based on the mechanism and the structure of the system, most of the direct photoresponsive drug delivery systems can be divided into the following classes: photocleavage, photoisomerisation and photo-induced rearrangement. In the following sections, various direct photoresponsive drug delivery and activation systems will be introduced.

1.3.1. Photocleavage

Photosensitive bonds can be cleaved under specific wavelength irradiation, releasing the bioactive molecule and triggering the downstream biological reactions. This strategy can be particularly useful for site-specific drug delivery with directed light exposure. The commonly used photosensitive cleavable handles includes \textit{o}-nitrobenzyl, coumarinyl and pyrenylmethyl derivatives. The most commonly used exposure wavelength is UV light (<400 nm) due to the sufficient energy of this short-wavelength light to trigger photo reactions.\textsuperscript{[150]} The photosensitive bond can be applied as a direct photocage (Figure 1.7a), a photodegradable
carrier (Figure 1.7b) or a cleavable linker between a bioactive molecule and an intact carrier (Figure 1.7c).

![Diagram of photocleavage strategies](image)

**Figure 1.7** The photocleavage strategies of photo-activated drug delivery systems, including a) the photoliberation of caged species, b) the photodegradation of carrier or c) the photoscission of molecular tethers between an intact carrier and a bioactive molecule. Reproduced from reference\(^\text{[15]}\)

**\(o\)-Nitrobenzyl (ONB) derivatives**

\(o\)-Nitrobenzyl derivatives are the most widely applied photolabile groups for studying biological systems. Photo-induced cleavage of \(o\)-nitrobenzyl groups can be triggered by both UV and NIR light, yielding 2-nitrosobenzaldehyde and a carboxylic acid (Scheme 1.28).

![Scheme 1.28](image)

**Scheme 1.28** Mechanism of photo-induced cleavage of \(o\)-nitrobenzyl derivatives.
The first application of ONB derivatives for biological purpose was published by Hoffman in 1978, demonstrating that ONB-caged adenosine triphosphate (ATP) can be used as a stable source of ATP which can be released from ONB esters upon irradiation.\textsuperscript{152} Since then, the ONB derivatives have been widely employed to release small molecules\textsuperscript{153}, oligonucleotides\textsuperscript{154} and proteins\textsuperscript{155}.

Several reports applied photolabile ONB derivatives to release anti-cancer reagents, such as doxorubicin\textsuperscript{136, 156-157}, paclitaxel\textsuperscript{157-158}, chlorambucil\textsuperscript{159} and camptothecin\textsuperscript{137, 160}. For example, Hartman and co-workers developed an efficient strategy for drug release based on photocaged permeability (Figure 1.8). The anti-cancer reagent, DOX, was conjugated with the scaffold containing the nitroveratryl moiety and a cell impermeable small fluorophore, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS), which prevented the cellular entry of the conjugates. Upon UV irradiation, the DOX was released from the scaffold, allowing cellular entry and therapeutic activity in cancer cells.\textsuperscript{156}

\begin{center}
\includegraphics[width=\textwidth]{Figure1_8.png}
\end{center}

\textbf{Figure 1.8} Photocaged permeability strategy for drug delivery using the nitroveratryl moiety.\textsuperscript{156}

The ONB derivative has also been used to photo-trigger the release of insulin to control blood glucose levels.\textsuperscript{161} Sarode \textit{et al.} conjugated human insulin with an injectable polymer \textit{via} a di-methoxy nitrophenyl ethyl linker (Scheme 1.29). The conjugate was injected into the skin of streptozotocin-induced diabetic rats. The human insulin was released into the bloodstream from polystyrene matrices after a two-minute trans-cutaneous irradiation by a compact LED light \textit{in vivo}.\textsuperscript{161} This method has the potential to achieve the control of blood glucose levels without using insulin pumps and cannulas.\textsuperscript{161}
Scheme 1.29 Synthesis of photoactivated depot (PAD) material using an insoluble but injectable polymer coupled with a strained cyclooctyne, followed by reaction with insulin monoazide containing a light-cleavable di-methoxy nitrophenyl ethyl group.\textsuperscript{[161]}

As well as being used for releasing small molecule therapeutics, Han and co-workers developed a strategy using light to liberate encapsulated small molecules and large proteins from a box-like DNA nanostructures with high spatiotemporal precision (Scheme 1.30).\textsuperscript{[162]} The strategy was demonstrated on a range of molecular sizes, from small molecules to full-sized proteins, including bovine serum albumin (BSA) and streptavidin.\textsuperscript{[162]}

Scheme 1.30 Schematic depiction of a) the conjugation between alkyne functionalised oligonucleotides, cargos and the photolabile linker containing the ONB motif; b) the encapsulation of cargo, the photocleavage reaction, and subsequent cargo release. Reproduced from reference.\textsuperscript{[162]}
Due to a few drawbacks of utilising a UV light source, such as potential photodamage to living tissues and limited penetration depth, many groups have reported various strategies to increase the ONB photocleavage rates at higher wavelengths. For example, Griffin and Kasko conjugated three model compounds, rhodamine, aminomethyl coumarin (AMCA) and fluorescein to PEG macromers through different substituted photolabile ONB groups, followed by polymerising to hydrogel networks, enabling the wavelength-dependent (436, 405, and 365 nm, sequentially) release of three therapeutics (Figure 1.9).\textsuperscript{[163]} The ONB moieties can also respond to NIR light through multiphoton absorption with much lower efficiency than UV-induced conditions.\textsuperscript{[164-165]} Therefore, nitrobenzyl linkers were often combined with upconverting nanoparticles to trigger the photorelease of small molecules, such as DOX\textsuperscript{[166-167]}, Nile Red\textsuperscript{[168]} and fluorouracil\textsuperscript{[169]} or proteins\textsuperscript{[170]}.

\textbf{Figure 1.9} Wavelength-selective release of multiple therapeutics from a single biomaterial. a) 4-(3-(1-Hydroxyethyl)-4-nitrophenoxy) butanoic acid conjugated with rhodamine; b) 3-hydroxymethyl-2-nitrobenzoic acid conjugated with AMCA; c) 4-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy) butanoic acid conjugated with fluorescein; d) fractional release of rhodamine, AMCA, and fluorescein from a hydrogel. Reproduced from reference.\textsuperscript{[163]}
**Coumarin derivatives**

Coumarin derivatives have been widely used as efficient photoresponsive moieties due to several advantages, such as high absorption efficiencies, efficient cleavage and a wide range of photosensitivity. The coumarin moiety usually includes an ester bond which can be cleaved upon UV or NIR irradiation (Scheme 1.31).

![Scheme 1.31](image)

**Scheme 1.31** Schematic representation of photocleavage of coumarinyl esters.

Coumarin compounds can be used as simple cages for photo-mediated drug release. For example, Okamoto and co-workers reported photo-mediated gene activation in zebrafish embryos.[171] In this strategy, the sugar-phosphate backbone of RNA was caged with a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group, which can be cleaved when being exposed to UV light (Scheme 1.32). The authors demonstrated that Bhc-caged green fluorescent protein (GFP) mRNA has severely reduced translational activity *in vitro*, and partial recovery of translational activity was obtained upon UV irradiation.[171]

![Scheme 1.32](image)

**Scheme 1.32** Photo-mediated gene activation using 6-bromo-7-hydroxycoumarin-4-ylmethyl caged DNA/RNA.[171]
As well as being used as simple photocages, coumarin-based photolabile moieties are often applied in combination with mesoporous silica nanoparticles (MSNs)\textsuperscript{172-175}, magnetic nanoparticles\textsuperscript{176}, micelles\textsuperscript{177} and hydrogels\textsuperscript{178} to achieve photo-induced drug release. Chemical modifications of coumarin derivatives can result in absorption shifts to biocompatible wavelengths. For example, 7-amino coumarin compounds can be photocleaved upon exposure to visible light and NIR light source.\textsuperscript{173-175, 179} Zhu et al. reported a MSN-based drug delivery system for regulated anticancer drug release upon the irradiation with either one- or two-photon excitation (Figure 1.10).\textsuperscript{175} The anti-cancer drug, chlorambucil, was conjugated with a 7-amino-coumarin chromophore, followed by conjugation with silane to obtain the silane precursor. Then, the precursor was grafted onto the surface of the MSNs to obtain the final functionalised materials. Within HeLa cells, the controlled release of active chlorambucil reached 40\% within 2 h upon irradiation with NIR light and over 95\% after 3 h. The cell viability decreased when increasing the irradiation time, confirming the controlled release of the drug.\textsuperscript{175}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{FunctionalisedMSN.png}
\caption{Schematic representation of photolysis for drug release using functionalised MSN by one or two-photon excitation. Reproduced from reference.\textsuperscript{175}}
\end{figure}

Moreover, by combining the coumarin compounds with other caging strategies, researchers were able to achieve specific cell or tissue targeting.\textsuperscript{151} Lin et al. constructed a hypoxia-activated photoresponsive drug-release system based on a nitroimidazole-locked coumarin phototrigger for highly discriminating photorelease of anticancer drug (Figure 1.11). In normal aerobic tissues, the photoexcitation of the coumarin dye relaxed via photoinduced electron transfer (PET) to the nitroimidazole electron acceptor, resulting no photocleavage. However, in anaerobic solid tumours, the hypoxia-specific nitro-to-amino reduction activated the
coumarin photocleavage, causing water-assisted photo-heterolysis of the C–O bond to release the caged anti-cancer drugs.[179]

![Diagram of caged and photocleaved drugs](image)

**Figure 1.11** Light-triggered drug released by the tumour hypoxic microenvironment activation. Reproduced from reference.[179]

**Other structures**

Various other photolytic cleavage structures, such as pyrenylmethyl esters[180-181], acridin derivatives[182] and perylene derivatives[183], to name a few, have been reported to achieve the spatiotemporally controlled release of therapeutics (Scheme 1.33). These structures normally contain an ester bond which can be cleaved upon light irradiation, releasing the bioactive drug or linkers containing the carboxylic acid.
Scheme 1.33 Schematic representation of other photo-induced cleavable compounds. a) Pyrenylmethyl ester; b) acridin derivatives; c) perylene derivatives.

1.3.2. Photoisomerisation

Reversible photoisomerisation, a photo-induced molecular switch from one stereoisomer to the other, is another promising photo-controlled drug activation system. Several different ways of applying the photoisomerisation have been exploited (Figure 1.12), including triggering changes in payload release rates through the reversible adjustment of the volume of the delivery vehicle, reversibly activating the molecular bioactivity through conformational changes\cite{184-186}, degrading hydrophobically stabilised carriers\cite{187} and the reversible gating of therapeutics in stable carriers\cite{188-189}. The most commonly investigated examples are azobenzene isomerisation and spiropyran-merocyanine isomerisation.
Figure 1.12 The photoisomerisation strategies of photo-activated drug delivery and activation systems, including a) triggering changes in payload release rates through the reversible volume change of the delivery vehicle, b) the reversible activation of therapeutic activity through conformational changes, c) degrading hydrophobically stabilised carriers or d) reversible gating of bioactive molecules in stable carriers. Reproduced from reference.\cite{151}

*Azobenzene*

The conformation of azobenzene changes from *trans* to *cis* upon UV irradiation (340–380 nm), then the *cis* isomer relaxes to the thermodynamically stable *trans* form upon irradiation at 420–490 nm or in the dark (Scheme 1.34).

Scheme 1.34 Photoinduced azobenzene isomerisation.

The azobenzene photoisomerisation has been extensively applied to nanoparticles and polymer networks to create drug delivery systems. For example, Tan and Yan constructed a photon-manipulated mesoporous silica nanostructures (MSN) drug release system based on azobenzene-modified nucleic acids (Scheme 1.35).\cite{188} The complementary DNA can undergo dehybridisation/hybridisation upon UV/NIR irradiation, resulting in uncapping/capping of
pore gates of MSN. Therefore, by controlling the light irradiation, controlled release of anticancer drug DOX can be achieved.[188]

Scheme 1.35 Schematic representation of azobenzene-modified DNA-controlled reversible release system. Reproduced from reference.[188]

As well as being used in conjunction with delivery vehicles, the azobenzene isomerisation has also been applied to design photoswitchable drugs.[184-186, 189-195] In 2012, Kramer and co-workers reported the molecule acrylamide-azobenzene-quaternary ammonium (AAQ), which replicates the light switching function of opsins, blocking the K+ channels when activated by light and unblocking the channels in the dark.[190] The authors demonstrated that the AAQ could restore light sensitivity to the retina and behavioural responses in vivo with blind mouse models.[190]

The azobenzene moiety has also been applied to develop photoswitchable antibiotics.[184, 192] Feringa and co-workers synthesised a series of azobenzene-containing quinolones, which could be switched on to their active state upon UV irradiation but switched off automatically within hours or under visible light (Figure 1.13).[184] The NMR studies revealed that the compounds were >50% the cis isomers after being irradiated at 365 nm for 10 min. The minimal inhibitory concentration (MIC) of the photoswitchable quinolones after irradiation was significantly lower than before, demonstrating that the quinolones gained antibacterial activity. Importantly, after being kept at room temperature for 3 hours, the irradiated samples completely lost antibacterial activity, which could be useful for reducing antibiotic activity in the environment.
and preventing the development of microbial resistance.\textsuperscript{[184]} Later, the authors reported subsequent modification of the azobenzene moiety which led to structures that allowed the isomerisation to be triggered by visible light.\textsuperscript{[192]}

![Figure 1.13](image)

**Figure 1.13** Photo-control of antibacterial activity. a) A typical quinolone bears a fluorine atom at the R\textsubscript{1}, R\textsubscript{2} and/or R\textsubscript{4} position, a nitrogen-containing, saturated ring at R\textsubscript{3}, and an alkyl moiety at R\textsubscript{5}; b) the photoisomerisation of azobenzene-containing quinolone.\textsuperscript{[184]}

The strategy has also been exploited for developing chemotherapy drugs with photo-controlled activity. In 2015, Trauner and Thorn-Seshold synthesised the photoswitchable chemotherapy drug based on combretastatin A-4 (CA4), one of the most prominent colchicine domain microtubule inhibitors (CDIs).\textsuperscript{[185]} The CA4 pharmacophore has a trimethoxybenzene ring in a *cis* form (**Figure 1.14**), while the *trans* isomer is several orders of magnitude less potent than the *cis* isomer. By replacing the C=C double bond with isosteric N=N bond and adding more functionalities on the aromatic ring, the authors synthesised a series of photoswitchable CA4 analogues (**Figure 1.14**). The studies in cancer cells revealed that these photostatin were up to 250 times more cytotoxic when converted to the *cis* isomers with blue light than the *trans* isomers when kept in the dark.\textsuperscript{[185]} Several other studies also contributed to promote this new promising class of precision chemotherapeutics.\textsuperscript{[193, 196]}
Figure 1.14 Photoswitchable chemotherapy drugs. a) Structures of photostatins, colchicine and combretastatin A-4 (CA4); b) the photoisomerisation of photostatins.\textsuperscript{[185]}

Trauner and co-workers also applied the photoswitch strategy to diabetes drugs to achieve photo-control of insulin release.\textsuperscript{[189]} The authors combined the azobenzene with the third generation sulfonylurea, glimepiride, to obtain the photoswitchable compound JB253 (Figure 1.15). Upon blue light irradiation, JB253 undergoes photoisomerisation to the \textit{cis} isomer and becomes more active, closing the K\textsubscript{ATP} channel, which leads to depolarisation, promotion of Ca\textsuperscript{2+} influx and the exocytosis of insulin (Figure 1.15). Moreover, thermal relaxation to the \textit{trans} isomer makes the compound less active or leads to dissociation, reopening the channel.\textsuperscript{[189]} This potential photo-control of insulin release offers a promising strategy for the treatment of type 2 diabetes mellitus.
**Figure 1.15** Photoswitchable sulfonylureas to control of insulin release. a) The mechanism of photo-control of insulin release by photoswitchable sulfonylureas; b) structures of tolbutamide and glimepiride; c) the photoisomerisation of azobenzene-containing sulfonylureas. Reproduced from reference.[189]

The same group has also extended this strategy to modify the specific glucagon-like peptide-1 receptor (GLP-1R) agonist, liraglutide, with an azobenzene moiety (Figure 1.16).[186] By altering the peptide folding between the *trans* and *cis* isomers with light, the photo-control of GPCR insulin secretion in pancreatic cells can be achieved.[186]
Figure 1.16 Photoswitchable glucagon-like peptide-1 receptor (GLP-1R) agonist. a) The liraglutide NMR structure (PDBID: 4apd); b) the photoswitchable azobenzene-modified liraglutide, LirAzo; c) the photoisomerisation of the azobenzene unit with in the LirAzo peptide.\textsuperscript{[186]}

Substantial efforts have been made to optimise the synthetic routes for azobenzene containing drugs\textsuperscript{[197-198]} and to redshift the absorbance of azobenzene to achieve longer wavelength photoswitches for use \textit{in vivo},\textsuperscript{[199-200]} making the photoisomerisation strategy even closer to the clinical stage.

\textbf{Spiropyran}

Spiropyran (SP) undergoes a reversible ring-opening isomerisation from a hydrophobic structure to a hydrophilic zwitterionic merocyanine (MC) isomer upon UV (365 nm) irradiation, while the reverse isomerisation is triggered by visible light (\textbf{Scheme 1.36}).

\textbf{Scheme 1.36} Photoinduced spiropyran-merocyanine isomerisation.

The structural conversion from hydrophobic to hydrophilic can induce the change of solubility and be exploited for photoinduced drug delivery systems.\textsuperscript{[187, 201-203]} For example, Tong \textit{et al.} reported hybrid spiropyran-indoline-PEG nanoparticles (NP\textsubscript{HS}) for triggered tissue penetration and drug delivery.\textsuperscript{[187]} These NP\textsubscript{HS} can be reversibly converted to amphipathic merocyanine.
with the decrease of the volume from 150 nm to 40 nm upon UV irradiation, which could enhance tissue penetration (Scheme 1.37a). Subsequently, the authors encapsulated rhodamine B, coumarin 6, cyanine 5 (Cy5), paclitaxel, docetaxel, proparacaine, and doxorubicin to examine the drug release properties. Upon UV irradiation (30 s), 29.3% of the encapsulated NP\(_{\text{H}}\)s rhodamine B was released within 1 h, while 7.2% was released in the same period without UV irradiation.\(^{[187]}\) Later, the same group exploited similar nanoparticles loaded with cholesterol for in vivo studies.\(^{[201]}\) Irradiation of subcutaneous HT-1080 tumours in nude mice with docetaxel-encapsulated nanoparticles was a more effective treatment than free docetaxel or non-irradiated encapsulated docetaxel. The authors hypothesised that the enhanced efficacy might be related to the enhanced tumour penetration and decompression of tumour blood vessels (Scheme 1.37b).\(^{[201]}\) In general, this approach may open a new area for the treatment of solid tumours which are difficult to access.

**Scheme 1.37** The application of the spiropyran-merocyanine isomerisation. a) Schematic representation of photoswitching hybrid spiropyran-indoline-PEG nanoparticles composed of spiropyran and DSPE-PEG; b) effects of light triggering of docetaxel containing nanoparticles in the tumour vasculature. Reproduced from reference.\(^{[187, 201]}\)
1.3.3. Photoinduced rearrangement

Photoinduced rearrangement can cause the change of the molecular properties, such as the water solubility. Therefore, it has also been exploited as a carrier for drug release systems.

2-Diazo-1,2-naphthoquinone (DNQ)

One of the most frequently used light-triggered rearrangement utilises a diazocarbonyl compound, which undergoes a Wolff rearrangement mechanism to a ketene intermediate upon photo irradiation, followed by nucleophilic attack by water to give the carboxylic acid (Scheme 1.38). The hydrophobic 2-diazo-1,2-naphthoquinone (DNQ) can be switched to a hydrophilic 3-indenecarboxylic acid structure under light irradiation (Scheme 1.38).[204]

Scheme 1.38 Photoinduced Wolff rearrangement of DNQ.

In 2005, Fréchet and co-workers reported the first demonstration of using DNQ containing polymers as potential drug carriers in drug delivery systems.[205] The DNQ was attached to the end of the hydrophobic tail of the PEG-lipid, followed by the self-formation of micelles in water. The DNQ-modified hydrophobic tails became hydrophilic under infrared radiation via a two-photon process, causing the dissociation of micelles.[205-206] Since then, several DNQ-containing cytocompatible drug carriers have been reported to achieve photo-triggered drug release.[207-210] For example, Liu et al. reported NIR-sensitive polymeric micelles formed from an amphiphilic copolymer containing dextran (Dex), a highly water soluble polysaccharide, and hydrophobic DNQ molecules (Scheme 1.39).[209] The DOX compounds were then encapsulated into the micelles during self-assembly. After being endocytosed into cancer cells,
the micelles could be dissociated under NIR laser light at 808 nm, resulting in the release of DOX inside the cells.\[^{209}\]

Scheme 1.39 Schematic representation of self-assembly and photo-induced Wolff rearrangement of Dex-DNQ amphiphilic copolymer. Reproduced from reference.\[^{209}\]

### 1.3.4. Indirect photoresponsive systems

As well as directly inducing the spatiotemporal drug release, photochemistry can be applied to trigger intermediate signals or molecules causing the transformation of the carriers to release the drugs. Such intermediate signals or molecules include heat, reactive oxygen species, gas and hypoxia-sensitive systems.\[^{211}\] A more detailed introduction to these systems can be found in several published reviews.\[^{151, 211-214}\]

### 1.3.5. Outlook

The photo-induced drug delivery offers a promising strategy for the spatiotemporal controlled treatment of various diseases, which largely reduces the side effects of the cargo drugs. Substantial efforts have been put forth to develop novel photochemistry reactions, design cytocompatible nanoparticles and redshift the absorbance range of the photoresponsive moiety. However, several drawbacks still remain unsolved, such as the stability of the photoresponsive moieties, the limited penetration depth, the repeatability of the systems. In the chemistry side,
future challenges remain in developing new photochemical reactions, which are orthogonal to current systems, to provide multi-responsive activations in one system. Secondly, improved efficiency and reliability of the photoresponsive step is necessary in order to minimise the unconverted species and provide instantaneous and quantitative activation. This can also reduce the damage to normal cells especially when strong short-wavelength is inevitable for some reactions.

Overall, photochemistry and its combination with polymer materials will remain as a promising drug delivery and activation strategy for precise treatments of various diseases.
CHAPTER 2 Peptide stapling strategy with an isobutylene graft

2.1. Introduction

As reviewed in Chapter 1, the chemical toolbox for peptide macrocyclisation, or “stapling”, has been expanded greatly. Among these stapling techniques, the S-alkylation is one of the most flexible approaches, as a wide range of bis-thiol-reactive linkers is commercially available. Besides, cysteine residues can be easily incorporated into the peptide sequence through solid-phase peptide synthesis. This facile incorporation is an important advantage over other stapling approaches based on nonproteinogenic amino acids. Currently, one challenge associated with cysteine macrocyclisation strategies is potential oxidation to form disulfides. Thus, an efficient strategy for cysteine stapling should in principle be compatible with the presence of reducing agents in a mild, one-pot reaction. Concurrently, the graft should be both small and biologically inert.

![Scheme 2.1 Peptide and protein stapling with an oxetane graft.][1]

Previously in the group, we reported a peptide and protein stapling method with an oxetane graft and demonstrated its applicability on biologically relevant peptides and proteins, including somatostatin, the Fab fragment of antibodies and CRM197 (Scheme 2.1).[130] MD simulations revealed that four-membered oxetane ring has an ideal size to be inserted between two cysteines without affecting the structure of the substrates. Besides, the stapled structure possessed enhanced bioactivity and stability to the native form. However, the bis-bromo...
oxetane stapling reaction is relatively slow and requires heating to 37 °C in some cases, which is not compatible with a large number of peptides and proteins and can damage their structure, causing the loss of biological activity. Therefore, a more reactive reagent is still needed to increase the efficiency and yield of the reaction. Herein, we designed a new cysteine cross-linking strategy that allows biocompatible and chemoselective installation of a small and stable isobutylene graft (Scheme 2.2). This stapling strategy offers a facile and efficient tool for peptide macrocyclisation.

Scheme 2.2 Schematic representation of the macrocyclisation of peptides with cysteine residues by using bis-electrophilic isobutylene.

The content in this chapter has been published in ChemBioChem.\textsuperscript{[215]}

2.2. Results and Discussion

2.2.1. Stapling of small molecule and peptides

Firstly, we initiated this investigation by developing the new stapling reaction on a single amino acid. The \textit{N}-Boc-cysteine 2 was used as a model for the cross-linking reaction to generate the stapled cysteine 3. Two parallel reactions with 3-bromo-2-bromomethyl-1-propene (bis-bromo isobutylene 1) or 3-chloro-2-chloromethyl-1-propene (bis-chloro isobutylene) were monitored by TLC under the same condition in DMF with 2.5 equiv. potassium carbonate (Scheme 2.3). The reaction with bis-bromo isobutylene 1 was completed in 2 hours. However, after the same reaction time, the reaction with biso-chloro isobutylene still had more than 50% of the free cysteine remaining. Therefore, the bis-bromo isobutylene 1 was chosen as the stapling reagent for further applications.
Scheme 2.3 Stapling of protected cysteine 2.

To apply this method to polypeptides, a linear pentamer peptide I (Cys-Ala-Ala-Ala-Cys), containing two cysteines at (i, i+4) positions, was prepared by Fmoc-based solid phase peptide synthesis (SPPS) (Scheme 2.4). After purification by HPLC, formation of the oxidised peptides with disulfide bond was observed. Therefore, tris(2-carboxyethyl) phosphine (TCEP) was added to reduce the disulfides, followed by the addition of stapling reagent in the presence of base. The complete conversion of the linear peptide I was observed by LC–MS with the exclusive generation of stapled peptide I’ in 71% isolated yield. Notably, a small portion of dimer product was observed when high concentration of peptide was applied. Therefore, the concentration of the peptide was optimised to be 1 mg/mL. The same procedure was then successfully applied to hepta- and octamer linear peptides, II–IV, containing cysteines at the (i, i+6) and (i, i+7) positions, which were synthesised by collaborators, Dr Francisco Corzana and Ismael Compañó. In each of the cases, the stapling process afforded the desired macrocyclised peptides in 75–81% isolated yields at room temperature.

Scheme 2.4 Macrocyclisation of linear peptides with cysteines at the (i, i + 4), (i, i+6) and (i, i+7) positions. (Peptides II–IV and II’–IV’ were synthesised by Dr Francisco Corzana.)
Finally, because the double-bond of the isobutylene graft could potentially be used as a handle for further conjugation, we evaluated its reactivity under Michael addition thiol-ene and inverse electron demand Diels-Alder conditions. Under the conditions tested, the alkene did not act as a partner for either thiol-ene or inverse electron demand Diels-Alder. This is a significant difference compared to the method described by Dawson and co-workers\(^\text{[62]}\), in which the introduced dichloroacetone moiety can be used for further conjugation via oxime ligation. On the other hand, the isobutylene scaffold is more flexible than the bis(bromomethyl)benzene platform used in the CLIPS macrocyclisation\(^\text{[55]}\); in some cases, this could be a competitive advantage for selecting the bioactive conformation.

2.2.2. Structural and conformational analysis of the stapled peptides

Next, our collaborator, Dr Francisco Corzana, studied the structural changes of the isobutylene-stapled peptide \(\text{IV}'\) by combining NMR spectroscopy data and molecular dynamics (MD) simulations. The 2D ROESY spectra showed substantial differences between the unstapled and stapled peptide in terms of their conformational preferences (Figure 2.1a and b). Clear medium-sized NH–NH NOE crosspeaks, which are characteristic of a predominantly folded conformation in solution, were observed for stapled peptide \(\text{IV}'\); the absence of these NH–NH NOE crosspeaks for unstapled peptide \(\text{IV}\) is in agreement with an extended disposition of the backbone.\(^\text{[216]}\) To obtain an experimentally derived conformational ensemble of compounds \(\text{IV}\) and \(\text{IV}'\), 20-ns MD simulations with time-averaged restraints (MD-tar)\(^\text{[217]}\) were carried out in explicit water with the key experimental distances included as restraints. The MD-tar simulations were performed by using the AMBER 16\(^\text{[218]}\) package with parm14SB and GAFF force fields.\(^\text{[219-220]}\) The good agreement found between the experimental and theoretical distances validates the outcome of the MD-tar calculations. According to these calculations, peptide \(\text{IV}\) is reasonably flexible in solution, presenting a random-coil distribution for its backbone. Conversely, stapled peptide \(\text{IV}'\) is rather rigid, showing a folded backbone held by the isobutylene staple (Figure 2.1c and d). We then estimated the solvent-exposed polar surface area (PSA) of peptides \(\text{IV}\) and \(\text{IV}'\) through the MD simulations. The stapled peptide \(\text{IV}'\) displayed around 15% less PSA than the unstapled species \(\text{IV}\) (Figure 2.1e); this suggests that the folded conformation forced by the isobutylene fragment promotes shielding of the polar backbone amides.
Chapter 2 Peptide Stapling Strategy with an Isobutylene Graft

Figure 2.1 Conformation analysis of stapled and unstapled peptides in solution. Sections of the 500 ms ROESY spectra (400 MHz) of peptides IV (a) and IV’ (b) in H$_2$O/D$_2$O (9:1) at pH 6.5 and 20 °C, showing amide–aliphatic crosspeaks. Structural ensembles obtained for peptide IV (c) and stapled peptide IV’ (d) through 20-ns MD-tar simulations. The backbone is shown in green, and the carbon atoms of isobutylene moiety are in lavender. The numbers indicate the rmsd for heavy-atom superimposition of the backbone with respect to the average structure. e) PSA estimated for peptides IV and IV’ through the MD-tar simulations. (Experiments were performed and analysed by Dr Francisco Corzana.)

Besides, far-UV circular dichroism was performed to study the difference of secondary structures of the unstapled and stapled peptides (Figure 2.2). The spectra of the stapled peptides are nearly identical to the spectra of the unstapled variants. This can be explained by the oxidation of the cysteines to form the disulfide bonds, thus cyclising the linear peptides.
Figure 2.2 Circular dichroism (CD) spectra of the unstapled peptides, II–IV, and stapled peptides, II’–IV’, at room temperature.

2.2.3. Parallel artificial membrane permeability assay (PAMPA)

We then decided to determine the passive membrane permeability of peptides II–IV and their stapled variants experimentally, a key feature for the development of peptide-based therapeutics (Table 2.1).[221-225] The assay was conducted through a commercial service (Pion Inc.).

Table 2.1 The parallel artificial membrane permeability assay (PAMPA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Avg. ( P_e )</th>
<th>SD ( P_e )</th>
<th>Avg. %R</th>
<th>SD %R</th>
<th>Avg. -( \log P_e )</th>
<th>SD ( \log P_e )</th>
<th>Domain/( \text{nm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>7.4</td>
<td>7.6</td>
<td>0.8</td>
<td>7</td>
<td>12</td>
<td>5.12</td>
<td>0.05</td>
<td>245 – 498</td>
</tr>
<tr>
<td>III</td>
<td>7.4</td>
<td>&lt;0.01</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>240 – 400</td>
</tr>
<tr>
<td>IV</td>
<td>7.4</td>
<td>6.0</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
<td>5.22</td>
<td>0.02</td>
<td>245 – 498</td>
</tr>
<tr>
<td>II’</td>
<td>7.4</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>4.96</td>
<td>0.21</td>
<td>245 – 498</td>
</tr>
<tr>
<td>III’</td>
<td>7.4</td>
<td>10</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>4.99</td>
<td>0.02</td>
<td>245 – 498</td>
</tr>
<tr>
<td>IV’</td>
<td>7.4</td>
<td>13</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>4.90</td>
<td>0.18</td>
<td>245 – 498</td>
</tr>
<tr>
<td>Ketoprofen[^{a}]</td>
<td>7.4</td>
<td>1.3</td>
<td>0.04</td>
<td>6</td>
<td>1</td>
<td>5.90</td>
<td>0.01</td>
<td>250 – 360</td>
</tr>
<tr>
<td>Verapamil·HCl[^{a}]</td>
<td>7.4</td>
<td>64</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>4.20</td>
<td>0.02</td>
<td>250 – 320</td>
</tr>
<tr>
<td>Propranolol[^{a}]</td>
<td>7.4</td>
<td>57</td>
<td>3</td>
<td>21</td>
<td>5</td>
<td>4.25</td>
<td>0.03</td>
<td>250 – 360</td>
</tr>
</tbody>
</table>

\(^{a}\) Ketoprofen, Verapamil and Propranolol were used as reference compounds.

\(P_e\) – effective permeability \(\times 10^{-6}\) cm/sec measured directly from assay.

\(pH\) – refers to the values in donor compartment. Acceptor had a special sink buffer (ASB) at pH 7.4.

\(%R\) – membrane retention

Avg – the value is reported as an average of quadruplicates
All the isobutylene-grafted peptides, II’–IV’, possessed improved permeability compared to their linear forms, particularly compound IV’. For all stapled derivatives, we observed values of $-\log P_e<5.0$; this is indicative of highly passively permeable compounds.[226-228] These data highlight the practicability of the method for developing bioactive peptides with favourable properties.

### 2.2.4. Stapling of bioactive peptides in aqueous solution

The feasibility of our stapling approach in aqueous solution, and the impact of the isobutylene scaffold on bioactivity were evaluated further with growth hormone-inhibiting hormone (GHIH) peptides, octreotide and somatostatin (Figure 2.3). Somatostatin and its synthetic analogue, octreotide, are peptide hormones that inhibit the release of growth hormone, insulin and glucagon, and possess one disulfide bond at the $(i, i+11)$ and $(i, i+5)$ positions, respectively.[229] Unlike the disulfide bond, which is sensitive to the biological environment, especially in the presence of biological thiols, the isobutylene-stapled form can improve stability, pharmacokinetics and binding affinity. The reactions were conducted in water with 10% DMF as co-solvent, and afforded the stapled octreotide and somatostatin quantitatively, which demonstrated the selectivity towards cysteine residue (Figure 2.3). This is possible because the isobutylene-stapling method is compatible with TCEP·HCl and can be performed in a one-pot manner.
2.2.5. Stability of stapled somatostatin

The disulfides can be reduced or exchanged by biological thiols, which results in the loss of the efficacy as bio-therapeutics. In contrast, the isobutylene-grafted peptides are inert to thiols and stable in the presence of thiols. To study the stability of the stapled peptide, we incubated the stapled somatostatin with human plasma and glutathione at 37 °C for 48 h. There was no decomposition or reduction as monitored by HPLC after the incubation (Figure 2.4), which is a significant advantage over disulfide-bridged peptides.
Figure 2.4 HPLC traces at 280 nm of stability test of stapled somatostatin. 1 mM Peptide was incubated with 20 mM glutathione (GSH) or human plasma at 37 °C for 48 h.

2.2.6. Evaluation of the binding affinity of the stapled somatostatin

The affinity of the peptide for the somatostatin receptor type 2 (SSTR2) was experimentally determined by tryptophan fluorescence spectroscopy.[230] As shown in Figure 2.5, the fluorescence emission peak of pure SSTR2 solution was at 328 nm. After increasing the concentration of either native or stapled somatostatin, the emission peak of both solutions shifted to 338 nm, with a decrease in intensity. Subsequent addition of somatostatin did not cause any shift in either peak, thus indicating the saturation of SSTR2. The minimum concentration of the somatostatin surrogate required to achieve saturation with 1 μM SSTR2 was 3.5 μM; in contrast, at least 5.5 μM of the native somatostatin was needed. These data suggest that the isobutylene-grafted somatostatin has a higher binding affinity for SSTR2. This improvement in binding activity represents a considerable advantage of the incorporation of the isobutylene graft when compared to other three-carbon grafts, such as the recently reported methylene thioacetal, which led to a decrease in affinity for SSTR2.[63]
Figure 2.5 Tryptophan fluorescence spectroscopy of somatostatin. Blue: 1 μM SSTR2 in buffer; purple: 5.5 μM native somatostatin and 1 μM SSTR2 in buffer; green: 3.5 μM stapled somatostatin and 1 μM SSTR2 in buffer.

2.2.7. Structural and conformational analysis of the stapled somatostatin

The 0.5-μs unrestrained MD simulations performed by Dr Francisco Corzana on both derivatives indicated that the stapled somatostatin is more rigid in solution (Figure 2.6a). Although the circular dichroism (CD) spectra of somatostatin and the corresponding stapled peptide (Figure 2.6b) are rather similar, the peak at 225 nm found in somatostatin might be indicative of the presence of a higher degree of polyproline II helix (PPII) conformation for this peptide.²³¹ MD simulations showed the S–S distance in the isobutylene scaffold to be around 4.2±0.4 Å, larger than the conventional S–S disulfide bond length (ca. 2.0 Å) and the S–S distance in methylene thioacetals (close to 3.0 Å).²³² Hypothetically this would allow the required degree of flexibility to adopt a bioactive conformation. The restrained peptide flexibility and structural preorganisation within the backbone, favoured by the formation of intramolecular hydrogen bonds, might reduce the entropy cost of receptor binding that limits the conformational ensemble and, ultimately, increase the binding affinity compared to those of disulfide cyclised analogues.²³²-²³⁵
Figure 2.6 Conformational analysis of native and stapled somatostatin. a) Structural ensembles obtained for native and stapled somatostatin through 0.5-μs unrestrained MD simulations. The atomic fluctuation (Cα) analysis of both peptides is also shown. The data correspond to the average structure of both molecules throughout the simulations. The backbone is shown in green, and carbon atoms of cysteine isobutylene residues are in lavender. The numbers indicate the rmsd for heavy-atom superimposition of the backbone with respect to the average structure. b) CD spectra of native and stapled somatostatin at room temperature. (The MD studies were performed and analysed by Dr Francisco Corzana.)

2.3. Conclusion

In conclusion, we have demonstrated a robust cysteine macrocyclisation and stapling strategy in which an isobutylene graft is introduced in a one-pot (with TCEP), biocompatible manner. This method was applied to several linear peptides of various sequence composition and two bioactive disulfide cyclic peptides, octreotide and somatostatin. The shielding of the polar backbone of the amides promoted by the isobutylene graft led to highly membrane-permeable peptide macrocycles. Enhanced binding activity, resulting from limited flexibility and structural preorganisation of the peptide backbone, was also observed. We believe that this access to such a “small” site-selectively introduced isobutylene, which is less disruptive than many current bulky grafts, as demonstrated here for linear and cyclic peptides, is likely to find significant use for the peptide drug discovery community by allowing access to structures with improved properties.
CHAPTER 3 One-Pot Re-Bridging of Protein Disulfides Using an Isobutylene Motif

3.1. Introduction

Biological therapeutics (e.g. vaccines, recombinant proteins and monoclonal antibodies) have been one of the fastest growing areas in the pharmaceutical industry and have contributed several marketed drugs to treat various diseases. Between 2008 and 2017, 22% of the drugs that were approved by the US Food and Drug Administration (FDA) were biologics.\[^{236}\] Within this category, monoclonal antibodies (mAbs) have offered successful treatments to a wide range of diseases, including cancer, chronic inflammation, cardiovascular, transplantation and infectious diseases. The technique was first developed in 1975 by Nobel Prize winners, Köhler, Milstein and Jerne.\[^{237}\] To date, over 80 antibody therapeutics have been approved by the European Medicines Agency (EMA) and the FDA with numerous others at various stages of research and development. At the same time, several commercial mAbs will soon have to face the patent expiration worldwide, including the Herceptin™ (trastuzumab), Rituxan™ (rituximab), Avastin™ (bevacizumab), etc., which will offer massive opportunities for the biosimilars market. Therefore, scalable techniques for producing proteins with enhanced stability and activity are high in demand, including not only those for protein conjugate generation but also for chemical stabilisation methods.

Antibodies, immunoglobulins (Ig), are organised into five classes on the basis of the constant region of the heavy chain, IgA, IgD, IgE, IgG and IgM.\[^{238}\] The most relevant for therapeutics is IgG, containing four subclasses, IgG1–4.\[^{239}\] The IgG monomer, a bivalent molecule, comprises two identical heavy chains and two identical light chains that are linked by interchain disulfide bonds. Each of the heavy and light chains contains structural domains that resemble antibody folds made up of two beta sheets linked by intrachain disulfides.\[^{240-241}\] The intrachain disulfides in both heavy and light chains for all four types of IgG subclasses are similar. IgG1 and IgG4 have two interchain disulfides between their heavy chains, whereas IgG2 and IgG3
have four and eleven disulfides, respectively.\cite{237} The interchain disulfides are more solvent exposed and less stable relative to the intrachain disulfides, which are generally buried between the two layers of anti-parallel β-sheet structures within each domain. Therefore, non-standard disulfide bond patterns, trisulfide bonding, and thioether linkage formation may occur after antibody assembly.\cite{242} Fragment antigen binding (Fab) arm exchange, in which half-antibodies from one therapeutic IgG4 molecule exchange with those of another endogenous molecule, is one of these highly undesirable events.\cite{243} This deviation has profound effects on the therapeutic efficacy of these molecules, including adverse events, and the narrowing of pharmacokinetic and pharmacodynamic profiles.\cite{243} The instability of disulfides is also found in IgG2 subclasses where antibody activity is modulated by the disulfide shuffling that occurs in serum.\cite{244-246} Three different isomers exist with different interchain disulfide patterns. Each of the isomers possesses significantly different activities. Although a mAb is one of the most stable and most resistant to changes in its environment relative to other biological therapeutics, even a small deviation can severely affect their quality, efficacy and safety in the clinic. Therefore, close attention should be paid to these deviations when generating and administering mAbs. In this regard, stapled disulfides might be able to make antibody-based therapeutics more robust.

A previously reported oxetane graft was able to stabilise the Fab fragment of trastuzumab and increase its binding affinity to the HER2 antigen. However, due to its poor efficiency, the attempt on full-length antibody failed to re-bridge the disulfide bonds and caused damage to the protein due to high temperature. Therefore, we expect that the much more reactive isobutylene-containing bis-alkylation reagent can provide an efficient strategy to re-bridge the disulfides on proteins, especially antibodies.

Most of the content in this chapter has been published in \textit{Org Biomol Chem}.\cite{247}

\section*{3.2. Results and Discussion}

\subsection*{3.2.1. Model reaction with monomeric proteins}

We commenced our investigation with a monomeric protein, thioredoxin, containing one solvent-exposed disulfide bond. Thioredoxin is in a class of small redox proteins that act as antioxidants by facilitating the reduction of other proteins through cysteine thiol-disulfide exchange.\cite{248} Therefore, when the disulfide is re-bridged with isobutylene graft, the redox
activity is turned off. Herein, the native thioredoxin was treated with TCEP·HCl, followed by
the addition of the stapling reagent 1 (Figure 3.1a). The stapling reaction was monitored by
LC–MS after 24 hours, which indicated successful installation of the isobutylene graft (Figure
3.1b). A small peak with mass around 12k Da was observed (Figure 5.26), while no native
thioredoxin was monitored.

![Figure 3.1](image_url)

**Figure 3.1** Disulfide re-bridging on thioredoxin. a) Schematic representation of the stapling
reaction; b) LC–MS of native thioredoxin (left) and stapled thioredoxin (right).

The thioredoxin activity was measured by the reduction of human insulin with the method
described by Yang and Ma.[249] Thioredoxin can catalyse the reduction of insulin disulfide
bonds by dithiothreitol (DTT). The increase of turbidity from the reduced insulin was detected
(Figure 3.2a). After accounting for insulin reduction by DTT alone, the stapled thioredoxin
showed no synergistic reactivity against insulin, in contrast to the reducing activity of the native
thioredoxin (Figure 3.2b). This means that the stapled thioredoxin cannot reduce the insulin
or be reduced by DTT. Therefore, the study revealed that the reactivity of thioredoxin was
completely blocked after the incorporation of the isobutylene graft.
Figure 3.2 The measurement of thioredoxin bioactivity. a) Schematic representation of the method of the activity study; b) the plotted redox activity of the native thioredoxin and stapled thioredoxin. The curves were baseline corrected by subtracting from insulin reduction by DTT alone. The corrected slopes from the kinetic data (ΔmAU/min), in the linear region, were plotted as a function of concentration of protein.

Next, the feasibility of this stapling method was further demonstrated with diphtheria toxin mutant CRM197, a protein carrier for many clinical vaccines. The toxin contains two disulfide bonds, C186–C201 and C461–C471. However, only the C186–C201 disulfide bond is solvent accessible and can be reduced by reductants (Figure 3.3a).\textsuperscript{[124]} Previously, the \textit{in vivo} studies of the oxetane-stapled CRM197 induced a statistically significant higher level of anti-protein antibodies relative to the unmodified protein.\textsuperscript{[130]} Therefore, we expect that the isobutylene-stapled protein might have similar effects. In a one-pot reaction, TCEP·HCl and stapling reagent 1 were added together to the CRM197 solution in neutral PBS buffer (pH 7.4). After reacting for 2 hours at 25 °C, the LC–MS indicated the emergence of the stapled CRM197 with one modification at 58469 Da (Figure 3.3b). The peak at 58518 Da could be the oxidised product of the stapled CRM197 or the dual stapled product. However, considering the previously reported references, such as the dichloroacetone\textsuperscript{[124]} and oxetane\textsuperscript{[130]} modified CRM197, only the C186–201 disulfide is solvent accessible. Besides, prolonging the reaction time or increasing the C186–201 disulfide is solvent accessible. Besides, prolonging the reaction time or increasing the reaction temperature did not promote the reaction to produce dual stapled product. Therefore, the peak at 58518 Da should be considered as oxidised product of the stapled CRM197.
Chapter 3 One-Pot Re-Bridging of Protein Disulfides Using an Isobutylene Motif

After the successful studies of isobutylene-modification on monomeric proteins, we applied our method to antibodies. In this case, the stapling graft can potentially improve the stability and binding affinity of the antibodies due to being un-reducible and having a more rigid structure.

3.2.2. Stapling of anti-amyloid-β antibody

We commenced our investigation with the designed antibody DesAb-HET, which was kindly provided by Dr Pietro Sormanni. The antibody targets the region 3–9 of human amyloid-β (Aβ42) peptide, the aggregation of which is a hallmark of Alzheimer’s disease. Following the same procedure as above, the native DesAb-HET, containing one disulfide, was successfully re-bridged (Figure 3.4).

In order to investigate the effect on the secondary structure, circular dichroism (CD) was performed with both native and stapled DesAb-HET. The CD spectra displayed no significant
differences between the two proteins (Figure 3.5a), indicating that the stapled DesAb-HET retained the native secondary structure. Then, the activities of the native and stapled DesAb-HET against the in vitro aggregation of Aβ42 were measured by Dr Pietro Sormanni. The anti-aggregation assay displayed a slight decrease of activity of stapled DesAb-HET, compared to the native form. Although the stapled DesAb-HET possessed higher activity after melting than all the other groups, this effect is highly unspecific and undesirable.

Figure 3.5 Structure studies and activity of stapled DesAb-HET. a) CD spectra of native and stapled DesAb-HET at room temperature; b) aggregation kinetics of 1.5 μM of Aβ42 alone (black) and in the presence of 0.25 μM of DesAb-HET in its native form (blue), stapled form (green) and stapled after melting (orange). Error bars are over three technical replicates. The y-axis reports the raw ThT fluorescence data with the minimum of each curve set to zero. (Aggregation kinetics were measured and analysed by Dr Pietro Sormanni.)

3.2.3. Stapling of Fab fragment of anti-HER2 antibody

To verify this method on commercial antibodies, we investigated the application using a Fab fragment of anti-HER2 antibody that consists of one solvent exposed interchain disulfide. HER2 is a member of the human epidermal growth factor receptor family and is overexpressed in around 20–30% of breast-cancer tumours. In recent years, HER2 has become an important biomarker and target of therapy for breast cancer.[251] The first anti-HER2 monoclonal antibody, trastuzumab (Herceptin™), was approved by the FDA to treat HER2 receptor positive breast cancer in 1998. Herein, the Fab fragment, which was kindly provided by Dr Vijay Chudasama, was incubated with TCEP·HCl and the stapling reagent 1 in both neutral PBS buffer (pH 7.4) and alkaline sodium phosphate buffer (NaPi buffer, pH 9.0) at 25 °C for 12 hours (Figure 3.6a). Full conversion of the Fab fragment was observed by LC–MS with the generation of the
expected mass (Figure 3.6b-c). The coincubation with TCEP-HCl and stapling reagent in a ‘one-pot’ manner may avoid disulfide scrambling. Moreover, the chemoselectivity of the disulfide stapling reaction was confirmed through the reaction of the stapled product with Ellman’s reagent [5,5’-dithiobis(2-nitrobenzoic acid)]. We found that the stapled Fab remained unchanged after incubation with Ellman’s reagent at 25 °C for 6 h which demonstrates that all free thiols had reacted (Figure 3.6d).

Figure 3.6 Stapling of Fab fragment of anti-HER2 antibody. a) Schematic representation of the stapling reaction; the LC–MS of (b) the native Fab, (c) the stapled Fab and (d) the reaction of the stapled Fab with Ellman’s reagent.

As mentioned above, disulfide bonds can be reduced and exchanged when circulating inside the human body as a result of the instability of disulfides; therefore, the stability of the stapled Fab was tested against biological thiols, glutathione (GSH) and human plasma. After 24 hours of incubation with GSH or plasma at 37 °C, the peak of the stapled Fab was still clearly shown in LC–MS (Figure 3.7b-c). Peaks with higher mass, such as 48146 Da on the GSH spectrum and 47854 Da on the plasma spectrum, could be the unintended conjugation to small molecules. Importantly, the mass of native Fab was not monitored, which demonstrated that the isobutylene graft remained on the structure. In comparison, the native Fab was fully reduced in the presence of GSH under identical conditions (Figure 3.7a).
3.2.4. Stapling of full-length IgG antibody trastuzumab

The efficient reaction on the Fab fragment demonstrated the potential practicality of this method when applied to antibodies under mild conditions. Therefore, we proceeded with our investigation on full-length trastuzumab antibodies, which contain four interchain disulfide bonds: two between the light and heavy chains, and two between the heavy chains (Figure 3.8a). The reaction was initially conducted at 4 °C to slow down the stapling and to avoid undesired cross-links. However, since only trace amounts of the stapled product were observed after 12 h, the reaction was warmed up to 25 °C. After being incubated 12 hours, the peaks that were related to the reduced heavy and light chains disappeared on LC–MS, which indicates that the fragments have been covalently re-bridged. Next, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted under both non-reducing and reducing conditions. Similar bands were observed under non-reducing conditions (Figure 3.8b, lane 1, native trastuzumab, and lanes 2 and 3, stapled trastuzumab), which shows that the antibody maintained its structural integrity after the stapling process. Under reducing conditions, the native antibody (lane 4) was reduced to heavy (∼50 kDa) and light chains (∼25 kDa). However, the stapled antibody (lane 4 and 5) was not reduced, which confirms that the antibody remained stapled under reducing conditions. Furthermore, the stapled antibody also had a band ∼75 kDa, which corresponds to the half-stapled antibody (i.e. intrachain stapling of the cysteines on the same heavy chain) (Figure 3.8c). Because the two interchain disulfide bonds in the hinge region were close to each other, the stapling between the cysteines on the same heavy chain cannot be avoided. In this case, because it was not at the binding domain of the antibody, the affinity and kinetic properties were unaffected. Besides, due to strong non-
covalent interactions, the two half antibodies can still be held together in vivo, and the pharmacological properties are not affected.[105]

**Figure 3.8** Stapling of trastuzumab and studies of binding affinities. a) The stapling reaction; b) SDS–PAGE gel of native trastuzumab and reduced trastuzumab. The 4–12% Tris-Glycine gel (Invitrogen) has Precision Plus Protein™ all blue pre-stained protein standards in Lane M and was stained with Coomassie. Lane 1, native trastuzumab; lanes 2 and 3, stapled trastuzumab; lane 4, native trastuzumab with NuPAGE® sample reducing agent; lanes 5 and 6, stapled trastuzumab with NuPAGE® sample reducing agent; c) the proposed structure of the half-stapled fragment.

Native mass spectrometry is an emerging technique that allows the investigation of intact protein complexes with high sensitivity and a theoretically unrestricted mass range.[252] Due to the limited mass range of LC–MS equipment, we validated the result using native mass spectrometry (**Figure 3.9** and **Figure 5.37**), which was performed and analysed by Dr Vukosava M. Torres and Dr Carlos Cordeiro at University of Lisbon. The mass of the stapled...
trastuzumab was 204 Da larger than that of native trastuzumab, which indicates that there were approximately four disulfides being stapled.

**Figure 3.9** Native mass spectrometry of the (a) native trastuzumab and (b) stapled trastuzumab. (Performed and analysed by Dr Vukosava M. Torres and Dr Carlos Cordeiro)

### 3.2.5. Evaluation of the binding affinity of stapled trastuzumab

To investigate whether the staple structure affected the binding affinity of the antibodies, biolayer interferometry (BLI) was used to evaluate the binding kinetics of the native and stapled trastuzumab against the HER2 receptor. The binding between the immobilised antibody and the receptor HER2 in solution produced an increase in optical thickness at the biosensor tip, which resulted in a wavelength shift. Such a shift is a direct measurement of the thickness of antibodies and receptors.[130, 253-254] Firstly, the native and stapled antibodies, including Fab fragments and trastuzumab, were biotinylated with a water-soluble N-hydroxysuccinimide ester biotinylation reagent (NHS-PEG₄-Biotin) in order to immobilise the fragments and antibodies on the biosensor (**Figure 3.10a**). Then the biotinylated antibodies were immobilised on the SA biosensors, dipped in the receptor HER2 solution and dissociated (**Figure 3.10b**). As shown in **Figure 3.10c**, the derived $K_D$ constant of the stapled Fab was larger than the $K_D$ of the native Fab fragment, which indicates that the grafted structure slightly lessened the binding affinity. However, the derived $K_D$ constant of the stapled trastuzumab was smaller than the $K_D$ of the native trastuzumab (**Figure 3.10d**), which indicates that the stapled trastuzumab has an improved binding affinity against HER2 receptor relative to the native species. These findings may be due to the structural changes when stapling the disulfides on a Fab, as they are relatively larger than the changes on antibodies.
**Figure 3.10** Kinetic studies of native and stapled antibodies. a) Biotinylation of antibodies with NHS-PEG₄-Biotin; b) schematic representation of the kinetics analysis by using bio-layer interferometry (BLI) with streptavidin (SA) biosensors; $K_D$ constants of c) native and stapled Fab fragments and d) native and stapled trastuzumab.

Moreover, the binding ability of the stapled trastuzumab was also determined by flow cytometric analysis, which was performed by our collaborator, Dr Padma Akkapeddi, at University of Lisbon. The flow cytometry against HER-2 positive human breast cancer cells (SKBR3) and HER-2 negative HPB-ALL cells displayed the selected binding of both antibodies towards HER-2 positive cells. As shown in **Figure 3.11**, the fluorescence-activated cell sorter (FACS) histograms showed that the stapled structure did not disrupt the antigen recognition abilities of the antibody and was in fact equivalent to the native antibody.
Figure 3.11 Flow cytometry analysis that show the binding of the native and stapled antibodies. Grey-unstained cells, orange-native trastuzumab antibody, and red-stapled trastuzumab antibody. (Performed and analysed by Dr Padma Akkapeddi)

3.2.6. Thermostability studies of stapled trastuzumab

The thermostability studies were performed by our collaborator, Dr Marta C. Marques, at University of Lisbon.

Circular dichroism

Circular dichroism (CD) spectroscopy is a versatile technique towards the understanding of the stability and aggregation of monoclonal antibodies. We assessed the thermal stability of trastuzumab samples in solution by recording the CD spectra at various temperatures from 25 °C to 80 °C. Monoclonal antibodies have predominantly β-sheet and random coil conformations. A broad minimum at 218 nm in the far-UV CD spectra is indicative of a substantial presence of β-sheets. Besides the characterisation of the trastuzumab secondary structure, we used CD spectroscopy to assess any conformational change as a result of the stapling chemistry. Heat may induce aggregation under conditions below the melting temperature of an antibody, which is why high temperatures coupled with spectroscopic techniques are often used to provide information about the structure.

Trastuzumab samples showed well-defined antiparallel β-pleated sheets with negative bands at 218 nm and positive bands close to 195 nm (Figure 3.12a-b), which confirms that the secondary structure of the stapled antibody is not affected. Transitions in the CD spectra were
monitored at different temperatures and the results suggest that the stapled trastuzumab displayed a greater degree of stability at higher temperatures (>75 °C). In fact, a higher level of aggregation for native trastuzumab was seen after scanning this sample at 80 °C. Disordered proteins displayed very low ellipticity above 210 nm and negative bands near 195 nm, which was clearly observed in the native trastuzumab CD spectra at 80 °C, in contrast to the stapled trastuzumab that maintained its secondary structure (Figure 3.12c). This difference in molar ellipticity at 80 °C indicates a transition into a random coil for the native trastuzumab. Above 80 °C, both native and stapled antibodies were considerably denatured (Figure 3.12d).

**Figure 3.12** Thermostability studies by circular dichroism. CD scans of (a) native and (b) stapled trastuzumab at different temperatures; c) CD scans of native and stapled trastuzumab at 80 °C and (d) at 25 °C after denaturation. The antibodies were diluted in PBS to a final concentration of 0.4 mg/mL in each sample. The data are the average of ten runs. (Performed and analysed by Dr Marta C. Marques)

**Differential scanning fluorimetry (DSF)**

To support these results, we also analysed the thermodynamic properties of the two antibodies by differential scanning fluorimetry (DSF). In a DSF experiment, temperature transitions are
used to probe the interactions of a protein with Sypro® Orange, a fluorescent dye that binds to specific residues exposed during unfolding.\cite{255-256} By using such a specific dye in DSF, we were able to identify key temperature transitions and protein stability features that allow for the analysis of molecular interactions.\cite{255}

The DSF data consisted of two transitions, centred around 68 and 80 °C, with slightly different corresponding fluorescence intensities (Figure 3.13a). The transition temperatures (Tm\(_1\) and Tm\(_2\)) for native and stapled trastuzumab samples were quite similar (Figure 3.13b), with a variation in the measured Tm\(_1\) < 0.5 °C. The determination of the melting temperatures was performed by the analysis of the first derivatives (Figure 3.13c-d), being Tm\(_2\) = 81.8±0.5 °C and 82.4±0.2 °C for the native and stapled trastuzumab, respectively. The overall DSF patterns were comparable, although with a significantly higher area under the negative peak (−dF/dT) in the first transition of the native trastuzumab (Figure 3.13c). These data suggest that the fluorescent probe undergoes a substantial increase in quantum yield as a result of the interaction with the exposed hydrophobic regions due to the higher degree of denaturation of the native trastuzumab.

**Figure 3.13** Thermal stability studies of trastuzumab by differential scanning fluorimetry (DSF). a) Normalised DSF melting curves of trastuzumab at 1.1 mg/mL in PBS mixed with Sypro® Orange; b) representation of the first derivative of the first (c) and second (d) transitions as a function of temperature (°C). Data are represented as grey and salmon curves for native...
and stapled trastuzumab, respectively. The data are the mean of three independent experiments. The DSF melting curves were normalised to the maximum intensity. (Performed and analysed by Dr Marta C. Marques)

It was previously described that differences between the spectra at the first transition (close to 60 °C) were ascribed mostly to the denaturation of the immunoglobulin Fab segment. Whereas, the gradual change between 70 °C and 80 °C was most likely due to denaturation of the Fc fragment.[257] It has also been reported that in the human myeloma IgG1 two transitions are also detected (melting temperatures around 70 °C for the Fab and Fc fragments and 66 °C and 82 °C for the Fc fragments). Based on these data, it was proposed that the C_{H2} domain in the Fc fragment and the melting of the Fab fragment could be related to the first transition, leaving the second transition to be represented mainly by the unfolding of the C_{H3} domain in the Fc fragment.[258] Taking into account the correlation between the transitions observed and the domains that were related to those transitions, we observed that, for the stapled trastuzumab, the first transition had a smoothed fluorescence intensity, which means that the Fc fragments were likely not fully denatured.

Both CD and DSF data supported that the stapled trastuzumab possessed a slightly higher degree of stability, though the antibodies have closer melting temperatures and, most importantly, the stapling strategy did not compromise the secondary structure of trastuzumab.

### 3.2.7. Effector-mediated functions of the native and stapled antibodies

The effector-mediated functions, including the antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDC), are important mechanisms of action by which therapeutic antibodies can achieve an antitumor effect. Herein, the effector-mediated functions were evaluated with the anti-CD20 mAb, rituximab, which was first approved in 1997 for the treatment of chronic lymphocytic leukaemia and certain types of non-Hodgkin lymphoma. The native rituximab was incubated with TCEP·HCl and 1,3-dibromo-2-methylpropane 1 at 25 °C for 12 h. Revealing similar results to the stapling of trastuzumab, analysis of the SDS–PAGE gel was conducted and showed that the antibody was fully stapled (Figure 3.14). With the stapled rituximab in hand, effector-mediated functions were further analysed. Further effector-mediated functions were measured by Dr Padma Akkapeddi.
Figure 3.14 SDS–PAGE gel of native rituximab and stapled rituximab. The 4–12% Tris-Glycine gel (Invitrogen) has Precision Plus Protein™ all blue pre-stained protein standards in the ‘M’ lane and was stained with Coomassie. Lane 1, native rituximab; lane 2, stapled rituximab; lane 3, native rituximab with NuPAGE® sample reducing agent; lane 4, stapled trastuzumab with NuPAGE® sample reducing agent.

Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is effector-mediated cell death, which is activated when target cells (cancer cells) are co-cultured with peripheral blood mononuclear cells (PBMCs) in the presence of a full-length IgG antibody. Cell death is induced upon binding of the Fc stem of an antibody with FcRn of the immune cells, mainly natural killer (NK) cells. For stapled versus native rituximab, RAJI cells were co-cultured with freshly isolated human PBMCs. Cell death was similar to the native antibody, which suggests that the antibody stapling does not interfere with the Fc part of the antibody and helps it retain the effector functions as the native isoform (Figure 3.15).
Chapter 3 One-Pot Re-Bridging of Protein Disulfides Using an Isobutylene Motif

Figure 3.15 ADCC effect on Raji cells in the presence of native and stapled rituximab when incubated with freshly isolated PBMCs and human serum from healthy donors. Statistical analysis was performed by using the unpaired t-test (* \( p < 0.05 \)). (Performed and analysed by Dr Padma Akkapeddi)

**Complement-dependent cellular cytotoxicity (CDC)**

Antibodies can also activate the classical complement pathway by the interaction with C1q on the C1 complex, forming a membrane attack complex, which causes target cell lysis. We evaluated the CDC effects of rituximab and trastuzumab antibodies. Similar effects to the ADCC assays were seen for complement-mediated cell killing in which RAJI cells for rituximab (Figure 3.16a) and SKBR3 cells for trastuzumab (Figure 3.16b) were co-cultured with 10% normal human serum and complement-mediated cell death was observed. Again, both stapled antibodies maintained the profile as that of the native antibodies.

Figure 3.16 Complement-dependent cellular cytotoxicity assays. a) CDC effect on Raji cells in the presence of native and stapled rituximab when incubated with freshly isolated PBMCs
and human serum from healthy donors; b) CDC effect on SKBR3 cells when incubated with freshly isolated human serum from healthy donors in the presence of native and stapled trastuzumab. Statistical analysis was performed by using the unpaired t-test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (Performed and analysed by Dr Padma Akkapeddi)

### 3.2.8. In vivo pharmacokinetics of native and stapled antibodies in NOD-SCID mice

Because both forms of the antibody (stapled and native) displayed similar antigen binding ability and effector function properties, we investigated the pharmacokinetic potential of these in healthy mice. The experiment was performed and analysed by Dr Padma Akkapeddi. Equally dosed native and stapled trastuzumab were injected into disease free NOD-SCID mice and the stability of these antibodies was evaluated for over 28 days. Blood was drawn at the indicated time points and the plasma was used to assess the levels of intact antibodies using the HER2 extracellular domain capture-based enzyme linked immunosorbent assay (ELISA). The levels of intact antibodies reflect stability and the total antibody clearance rate. Both of the formats (stapled and native) were highly identical, with the stapled antibody only displaying a slightly lower clearance rate at day 7 (Figure 3.17). These results hint towards the plausibility of higher accumulation of the stapled antibody when tested in tumour bearing mice due to the slight delay in the clearance rate.

![Figure 3.17](image.png)

**Figure 3.17** In vivo pharmacokinetics of native and stapled trastuzumab. Equally dosed native and stapled trastuzumab were injected intravenously into NOD-SCID mice. At the indicated
time points, blood was drawn for determination of total antibody levels by using ELISA. (Performed and analysed by Dr Padma Akkapeddi)

3.3. Conclusion

A general disulfide re-bridging strategy for the stabilisation of antibodies with isobutylene motifs has been developed. This strategy can be applied in a rapid and efficient ‘one-pot’ manner on various proteins, antibodies and derivatives, such as Fab fragments. The non-native stapled structure possesses a higher binding affinity than the native form, which was confirmed by both BLI and flow cytometry assays. Comprehensive stability studies were also conducted, which supported the idea that the stapled antibody has an enhanced thermostability and maintains its secondary structure at higher temperatures relative to the native format. ADCC and CDC experiments confirmed that the stapled antibody maintains its effector-mediated functions. Moreover, in vivo pharmacokinetics demonstrated that the stabilities of both native and stapled antibodies in mice are similar. We believe that this efficient technique can be easily applied to antibody therapeutics and will completely remove the potential issue of disulfide exchange and shuffling. Furthermore, as a result of the enhanced stability, this technique may significantly reduce the cost of storing and transporting antibody therapeutics, as well as offer a scalable tool for producing biosimilars of marketed antibodies.
CHAPTER 4 Photoactivation of Thiol-Containing Drugs via Thiol-Ene Strategy

4.1. Introduction

As reviewed in Chapter 1, light-mediated treatment has been demonstrated to be a promising strategy for controlled delivery and activation of medicine at pathological sites, which can reduce the side effects of the active drugs. In the battle against cancer, it has been shown that photo-activated reagents can be delivered or protected with photoresponsive groups, such as ortho-nitrobenzyl, coumarin ester, azobenzene and other metal complexes. However, issues and challenges remain in this field, such as achieving rapid and efficient activation, improving the bioavailability and stability of the prodrug and using non-phototoxic light. Therefore, there is still demand for new designs and developments for photoresponsive reactions and photomediated therapy.

The thiol-ene reaction (Scheme 4.1a), a conjugation between a thiol and an alkene, has been known since the early 1900s. The coupling reaction proceeds through two mechanisms, namely photoinitiated free-radical addition and catalysed Michael-type addition reactions. Owing to several desirable features of the reaction, such as rapid reaction rates, ease of implementation and high conversion rates, the thiol-ene reaction has been extensively used in many applications, including biofunctionalisation, surface and polymer modification, polymerisation. Among these various reactions and structures, the thiol-isobutylene reaction that is mediated by free radicals drew our attention. The isobutylene-bridged polymer networks have been extensively studied to synthesise polymer networks via UV-triggered radical-mediated thiol-ene reactions. The covalently cross-linked network containing the isobutylene structure was first reported by Bowman and co-workers in 2005. This polymer material exhibits stress and/or strain relaxation upon exposure to light, by introducing radicals via photocleavage of residual photoinitiator in the polymer matrix, followed by addition-fragmentation chain transfer (AFCT) of midchain functional groups. Recently,
this method has been used to provide a reactive handle for reversible addition and exchange of biochemical moieties and proteins in the hydrogel under cytocompatible conditions.[263-264] Key to this reaction is the AFCT reaction of the isobutylene moiety, in which the double bond is attacked by the photoinitiated thiol radical in the presence of photoinitiators to release the caged thiol component.

**Scheme 4.1** a) General thiol-ene coupling reaction; b) the thiol-isobutylene decaging reaction.

Therefore, inspired by the AFCT reaction, we hypothesised that the isobutylene structure could be used as a bridging graft to cage thiol-containing drugs and allow further controlled activation of anticancer drugs by means of a radical-mediated thiol-ene mechanism (*Scheme 4.1b*). Moreover, by applying this reaction on proteins, photo-controlling the activity of a protein can be achieved.

Most of the content in this chapter has been published in *Angew Chem Int Ed.*[265]

### 4.2. Results and Discussion

#### 4.2.1. Optimisation of the thiol-ene decaging reaction

Our research commenced with *N*-tert-butoxycarbonyl-L-cysteine methyl ester 2, which was protected with 3-bromo-2-bromomethyl-1-propene 1, as described in Chapter 2. The stapled *N*-Boc-cysteine 3 was then screened under a series of reaction conditions (*Table 4.1*). Firstly, different photoinitiators were tested with thiol sources and stapled cysteine 3 under irradiation at 365 nm. The reactions with the water-soluble photoinitiator, 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (Vazo 44), did not progress after two hours (*Table 4.1*, entry 1–5). Different solvents, from methanol to methanol/aqueous mixture, and different thiol sources failed to promote the reaction. However, another photoinitiator, 2,2-dimethoxy-2-phenyl-
acetophenone (DPAP), successfully initiated the reaction and released the cysteine moiety 2 in dichloromethane (DCM) (Table 4.1, entry 6). The β-mercaptoethanol (BME) was also tested as the thiol compound under the same reaction condition (Table 4.1, entry 8). This reaction released cysteine 2, which suggests that different thiol sources could be used to promote the reaction. However, when N,N-dimethylformamide (DMF) was used as solvent, two mixed disulfides resulting from the reaction between 1-thioglucose and cysteine 2 were observed (Table 4.1, entry 9–10). To evaluate the reaction and calculate the isolated yield, TCEP was added to reduce the disulfides after the reaction. Addition of TCEP before the reaction caused the desulfurisation of cysteine to alanine (Table 4.1, entry 11). Upon adding the TCEP after the reaction, the reaction was complete within 15 minutes and gave a relatively high yield (65%, Table 4.1, entry 12). Moreover, the reaction was also rapid and efficient when glutathione was used as a thiol source in a 1:1 DMF/H2O (Table 4.1, entry 13), demonstrating that the reaction occurs under aqueous conditions.

**Table 4.1 Optimisation of the thiol-ene decaging reaction.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>PI</th>
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<th>Solvent</th>
<th>TCEP</th>
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<th>Yield [%]</th>
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<td>0</td>
</tr>
<tr>
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<td>Vazo 44</td>
<td>NAGSH</td>
<td>MeOH</td>
<td>–</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
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<td>NAGSH</td>
<td>MeOH/buffer 1:1</td>
<td>–</td>
<td>120</td>
<td>0</td>
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<td>–</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
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<tr>
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4.2.2. Unstapling the isobutylene-grafted peptide

Next, the feasibility of our strategy was investigated with an isobutylene cyclised 5-mer peptide that bears two terminal cysteines, described in Scheme 2.4. The short peptide was completely converted into the disulfide derivative within 15 minutes under the optimised reaction condition (DPAP, 4AcGlcSH, 365 nm) (Figure 4.1 and Figure 5.41).

![Figure 4.1](image_url) The decaging reaction of isobutylene-cyclised CAAAC peptide with 1-thio-β-D-glucose tetraacetate.

4.2.3. Control studies and proposed mechanism

To demonstrate that the release of the thiol compound occurs by means of a radical-mediated thiol-ene mechanism, a series of control experiments with stapled cysteine 3 were conducted (Table 4.2 and Figure 5.39). As shown in Table 4.2, the UV irradiation (entry 2), thiol source (entry 3), and photoinitiator are essential for the decaging reaction. Although the condition without thiol source (entry 4) showed trace of unstapled cysteine 2, the free cysteine might come from the starting material of the first stapling reaction, because increasing the irradiation time did not promote the generation of the unstapled cysteine 2. Besides, when the radical scavenger TEMPO, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl, was added to the reaction, the reaction was terminated by forming an intermediate with the DPAP fragmentation radical as confirmed by MS (Figure 5.40). This provides evidence to support a radical-mediated mechanism.
Table 4.2 Control studies of thiol-ene decaging reactions between isobutylene-grafted cysteine and thiol-glucose.

<table>
<thead>
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<th>Entry</th>
<th>Thiol</th>
<th>Stapled Cys 3</th>
<th>UV</th>
<th>DPAP</th>
<th>TEMPO</th>
<th>Conversion [%]</th>
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<td>+</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>3</td>
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<td>-</td>
<td>+</td>
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</tbody>
</table>

<sup>[a]</sup> The formation of the intermediate, 2,2,6,6-tetramethylpiperidin-1-yl benzoate, by the reaction between TEMPO and DPAP was monitored by MS-ESI+. The proposed mechanism for the radical-mediated thiol-ene decaging reaction was shown below (Scheme 4.2). The mechanism was also studied by quantum mechanical calculations using abbreviated thiol models by our collaborator, Dr Gonzalo Jiménez-Osés, at University of La Rioja. After generation of the thiol radical by the photoinitiator under UV irradiation, the isobutylene grafted structure undergoes a fast thiol-ene anti-Markovnikov addition with a calculated activation energy of ΔG‡ ≈ 14 kcal mol⁻¹ at the PCM(H₂O)/M06-2X/6-31++ G(2,p) theory level, to generate a symmetric tertiary-carbon-centred radical intermediate. Then, the unstable radical intermediate undergoes a β-scission at a very similar reaction rate, regenerating the isobutylene linkage and resulting in a mixed caged compound, which can be again attacked by another thiol radical following the same mechanism to release the other unit of the caged thiol compound. The process is nearly thermoneutral and reversible until two decaged radical thiols collapse to form a stable disulfide bond (Scheme 4.2).
The β-scission step was also indirectly confirmed by the thiol-ene reaction of the diethylethylene stapled compound 5 which contains one more carbon on both sides of the double bond, compared to the isobutylene moiety (Scheme 4.3). Therefore, the asymmetric radical intermediate generated via thiol-ene reaction cannot undergo the β-scission and can be quenched to provide the thiol-ene conjugate. Firstly, the N-Boc-cysteine 2 was stapled with 1,5-dibromo-3-methylenepentane 4 to provide the diethylethylene stapled cysteine 5. Then the thiol-ene reaction was performed using catalytic amount of DPAP as the photoinitiator and thioglucose as the thiol source under UV irradiation at 365 nm for 15 min. As expected, instead of releasing the cysteine, the thiol-ene conjugation product 6 was successfully generated with a 71% yield.
Scheme 4.3 Diethylethylene stapling of cysteine 2 and thiol-ene conjugation with thiol glucose.

The same thiol-ene conjugation was observed with diethylethylene-stapled octreotide (Figure 4.2a). The thiol-ene conjugated octreotide-glucose was observed by HPLC and LC–MS after being irradiated with UV for 15 min (Figure 4.2b).

Figure 4.2 Diethylethylene stapling of octreotide and thiol-ene conjugation with thiol glucose.

a) Schematic representation of the reactions; b) the LC–MS of the native octreotide (left), the diethylethylene-stapled octreotide (middle) and the thiol-ene conjugate (right).

Therefore, based on the control studies, the quantum mechanical calculations and the thiol-ene reaction with diethylethylene-stapled products, the proposed mechanism of photo-induced thiol-ene reaction and β-scission of the radical intermediate was confirmed.
4.2.4. Synthesis of the stapled largazole

With this knowledge in hand and in order to demonstrate that this strategy is practical to activate drugs in vitro, we applied our method to the potent histone deacetylase inhibitor (HDAC) largazole. The cyclic depsipeptide largazole is a marine natural product, and its derivatives are recognised as promising potential anticancer therapeutics. Largazole possesses remarkable and preferential growth-inhibitory activity against cancer cell lines relative to corresponding non-transformed cells. The octanoyl tail in largazole has better cell permeability than the active free thiol species, largazole thiol, the latter being formed inside cells by esterase or lipase-based cleavage of the octanoyl residue (Scheme 4.4). The free thiol group binds to the active site Zn²⁺-domain within the HDAC enzyme, and results in a potent inhibitory effect (Scheme 4.4). Therefore, the developed thiol-isobutylene decaging strategy can be used to protect the thiol group, improve the cell-permeability, and allow controlled activation upon UV exposure.

Scheme 4.4 The mechanism of the inhibitory effect of largazole to class I HDACs.

Based on the reported routes, we optimised the total synthesis of largazole and its analogues. The retrosynthetic route adopted involved macrolactamisation of intermediate ABC1, which could be generated by coupling the TSE-protected acid AB1 with N-Fmoc-L-valine, followed by the condensation with thiazoline-thiazole subunit C4 (Scheme 4.5).
Scheme 4.5 Retrosynthetic analysis of largazole and analogues.

The isomeric thiazoline-thiazole fragment C4 was prepared by the sequence shown in Scheme 4.6. The treatment of N-(tert-butoxycarbonyl)-2-aminoethanethioamide C0 with ethyl bromopyruvate formed the thiazole C1 in a 65% yield. Then, the aminolysis of C1, followed by dehydration of the resulting primary amide, generated the nitrile C3 (50%, two steps). The obtained nitrile C3 was condensed with 2-methyl-L-cysteine to form the thiazoline-thiazole carboxylic acid C4 in 100% yield.
Scheme 4.6 Synthesis of the thiazoline-thiazole fragment C4.

The synthesis of the remaining fragment AB1 is displayed in Scheme 4.7. The formation of the aldehyde A2 was achieved through a hetero-Michael addition of trityl mercaptan into acrolein, followed by a Wittig olefination.[270] The auxiliary was prepared from D-phenylalaninol by refluxing with CS₂ under basic aqueous condition to form the (R)-4-benzylthiazolidine-2-thione, followed by acetylation to give the chiral auxiliary in good yield (98%, two steps).[269] Then, the acetate aldol condensation of the aldehyde A2 with the obtained auxiliary was performed to synthesise thiazolidinethione A3, which was then treated with 2-(trimethylsilyl)ethanol (TSE) to provide the TSE-protected acid A4.[270] The obtained fragment A4 was subsequently coupled with N-Fmoc-L-valine to form the fragment AB1.[271]

With all the necessary building blocks in hand, the last few steps included assembly, macrocyclisation and deprotection (Scheme 4.7). The acyclic precursor of the depsipeptide ABC1 was obtained by deprotecting the amine in AB1 and a subsequent (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) mediated coupling with fragment C4.[271] The macrolactamisation was performed in the presence of HATU and HOBt after removing the Boc protecting group under acid conditions. Finally, the removal of the S-trityl protecting group with iPr₃SiH and trifluoroacetic acid (TFA) provided the formation of the largazole thiol.[267]
Based on the method described in Chapter 2, two largazole thiols were grafted together using 3-bromo-2-bromomethyl-1-propene 1 to generate the stapled largazole (Scheme 4.8). At the same time, in order to make a comparison for cell studies, the largazole thiol was acylated to form the largazole.\cite{273}
Since the octanoyl tail improves the cell permeability of the natural compound, as mentioned above, we would like to understand how the stapled structure behaves. Therefore, the parallel artificial membrane permeability assay (PAMPA) was conducted through a commercial service (Pion Inc.). The result of the PAMPA assay indicated that stapled largazole is a highly passively permeable compound ($\log P_e = -5.29$ and $P_e = 5.3 \times 10^{-6}$ cm/sec; Table 4.3).

**Table 4.3** The parallel artificial membrane permeability assay (PAMPA) for stapled largazole.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Avg. $P_e$</th>
<th>SD $P_e$</th>
<th>Avg. %R</th>
<th>SD %R</th>
<th>Avg. $\log P_e$</th>
<th>SD $\log P_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>stapled largazole</td>
<td>7.4</td>
<td>5.3</td>
<td>1.7</td>
<td>10</td>
<td>1</td>
<td>-5.29</td>
<td>240 - 498</td>
</tr>
</tbody>
</table>

$P_e$ – effective permeability ($>10^{-6}$ cm/sec) measured directly from assay.

pH – refers to the values in donor compartment. Acceptor had a special sink buffer (ASB) at pH 7.4.

%R – membrane retention

Avg – the value is reported as an average of quadruplicates
4.2.5. Decaging the isobutylene-caged largazole

Next, the stapled largazole was reacted with 1-thio-β-D-glucose tetraacetate and DPAP under UV irradiation for 15 minutes (Figure 4.3a). Complete conversion of the stapled largazole was observed in the HPLC trace along with the appearance of a largazole thiol signal (Figure 4.3b).

**Figure 4.3** The photoactivation of isobutylene-grafted largazole thiol. a) Thiol-ene decaging reaction of stapled largazole with 1-thio-β-D-glucose tetraacetate; b) HPLC traces at 254 nm of the stapled largazole (blue) and the reaction mixture after 15 min (red).

Next, the growth-inhibitory activity was evaluated with human colon carcinoma cell lines, HCT-116 (Figure 4.4). As expected,[274] largazole (GI<sub>50</sub> 1.4 nM) is more potent than the corresponding free thiol species (GI<sub>50</sub> 185.1 nM) owing to its octanoyl side-chain which improves cell permeability and allows facile presentation of the free thiol within the cell.[267] The stapled largazole (GI<sub>50</sub> 407.7 nM) is a much less potent compound because the thiol group is protected by the isobutylene structure, which prevents binding with the Zn<sup>2+</sup> domain in cells.
Before testing the decaging conditions in cells, we investigated the toxicity of DPAP and phototoxicity of the light in terms of the power and the irradiation time (Figure 4.5). A set of cytocompatible conditions, 15-minute irradiation at 80 W in the presence of 1.5 μM DPAP, was chosen for further investigations.

**Figure 4.5** The DPAP concentration and UV condition screening. a) The cytotoxicity of DPAP without UV; b) the cytotoxicity of DPAP with UV (365 nm, 64 W, 15 min); c) the cytotoxicity of DPAP with UV (365 nm, 80 W, 15 min).

---

**Figure 4.4** a) Growth inhibitory effects and b) GI50 values of largazole, largazole thiol and stapled largazole on HCT-116 colon carcinoma cells.
The decaging reaction of stapled largazole was tested with HCT-116 cells at 150 nM. Since McCoy’s 5A culturing medium contains cysteine and glutathione, no other thiol source was added. The cell viabilities of the three drug groups with/without 365 nm UV irradiation were consistent with the growth-inhibitory assay (Figure 4.6). The UV-irradiated pre-mixed group of stapled largazole and DPAP showed statistically significant lower cell viability than the corresponding non-irradiation group, the stapled largazole group, and the largazole thiol group. However, if the DPAP and stapled largazole were added separately, the difference between the UV and non-UV group was not significant, which might result from the large amount of DMSO used for dissolving two compounds separately.

**Figure 4.6** HCT-116 Cell survival rate under different conditions. SL = stapled largazole, UV condition, 365 nm 80 W, 15 min. Data are representative of three independent tests and analysed by the two-tailed unpaired Student’s t-test (**** p < 0.0001). Error bars reflect one standard deviation from the mean.

To confirm the results, a fluorometric HDAC activity assay was conducted with HCT-116 cell lysates of two control groups and two pre-mixed groups (Figure 4.7). A significant decrease of fluorescence, which indicated the HDAC enzyme activity, was observed in the UV-irradiated pre-mixed group relative to the control groups and the non-irradiation group. Both the cell viability and the enzyme activity results indicated that the stapled largazole was successfully activated by 365 nm UV light.
4.2.6. Unstapling the isobutylene-grafted protein conjugates

With the developed decaging method, we next decided to investigate whether it could be applied to the protein conjugates. As well as using the 3-bromo-2-bromomethyl-1-propene 1 for re-bridging disulfides on peptides, proteins and thiol-containing drugs, we developed a site-selective protein modification method at cysteine residues to incorporate an electrophilic handle for bioconjugation under mild conditions. This method is demonstrated on a variety of proteins containing a solvent-exposed cysteine residue, including an engineered version of the C2A domain of synaptotagmin-I (C2Am) and ubiquitin (Ub-K63C). Chemically distinct protein conjugates are then efficiently formed through further reaction of the electrophilic site with various nucleophiles, such as β-D-thioglucose sodium salt (Scheme 4.9).

Scheme 4.9 Site-selective installation of an electrophilic isobutylene-Br handle on proteins for bioconjugation.

Figure 4.7 HDAC activity assay of control group and UV group. Data are representative of three independent tests and analysed by the two-tailed unpaired Student’s t-test (** p < 0.01). Error bars reflect one standard deviation from the mean. SL = stapled largazole.
The investigation commenced by trialling the previous conditions with DPAP as a photoinitiator and glutathione as thiol sources. However, the reaction did not proceed after 15–30 min at 80 W 365 nm, with a series of oxidation products observed by LC–MS. In order to promote the reaction, a large amount of photoinitiator was needed. However, since the DPAP is not soluble in aqueous solution, increasing the amount of DPAP means increasing the percentage of DMF, which results in precipitation of the protein. Therefore, we tested two different water-soluble photoinitiators, 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (Vazo 44) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). The Vazo 44 failed to promote the reaction and generated oxidised protein. The LAP, however, was able to trigger the release of isobutylene in the presence of glutathione at room temperature. The release of the isobutylene-Br moiety on ubiquitin was successfully achieved within 30 min in PBS pH 7.4 buffer (Figure 4.8a). LC–MS showed the disappearance of the isobutylene-Br-labelled ubiquitin (8700 Da) along with the emergence of ubiquitin (8569 Da; Figure 4.8b).

![Figure 4.8](image)

**Figure 4.8** Photorelease of the isobutylene on ubiquitin. a) Schematic representation of the decaging reaction; b) the LC–MS of the native ubiquitin (left), the Ub-iso-Br (middle) and the reaction mixture (right).

Then, the same condition was applied to C2Am (Figure 4.9a), which was labelled with isobutylene-thioglucose (C2Am-iso-Glu). Although the peak of C2Am-iso-Glu (16471 Da) disappeared after being incubated for 15 min (Figure 4.9b), the newly generated mass peak at 16289 Da did not match the mass of native C2Am (16222 Da). The increase in mass might come from the oxidation of cysteine and methionine, which suggests that a degassing procedure might be necessary in order to prevent oxidation on larger proteins.
Chapter 4 Photoactivation of Thiol-Containing Drugs via Thiol-Ene Strategy

Figure 4.9 Photorelease of the isobutylene-thioglucose on C2Am. a) Unstapling the isobutylene-labelled C2Am; b) the LC–MS of the reduced C2Am (left), the C2Am-iso-Glu (middle) and the reaction mixture (right).

4.3. Conclusion

In summary, we have developed a rapid and efficient thiol-ene-based photoactivation strategy for thiol-containing drugs caged using isobutylene on small molecules or peptides. The radical-mediated reaction, which is triggered by UV light, undergoes a thiol-ene addition step to form an unstable radical intermediate which is further cleaved by β-scission to release the caged thiols. We applied this method to various substrates, such as N-Boc-cysteine, a cysteine-containing peptide and the HDAC inhibitor largazole, and showed that the caged thiol molecules, unlike their free counterparts, displayed high membrane permeability. The successful activation of largazole in HCT-116 cells demonstrates the potential of this strategy for drug delivery and activation in cancer therapy. In order to apply this method in clinic, the condition needs to be further optimised, such as using NIR or visible light, solutions to introduce the photoinitiator. Finally, preliminary tests with proteins demonstrate the potential of this method for controlling the activity of a protein of interest or the release of the toxic payload from antibody–drug conjugates. However, the low efficiency and the oxidation to the cysteine may significantly limit its application and needs to be improved in the future.
CHAPTER 5 Materials and Methods

5.1. General methods

**Solvents and reagents.** Unless otherwise noted, all chemical reactions were carried out under an atmosphere of argon, and all chemicals were purchased as reagent grade from Sigma-Aldrich, Thermo Fisher Scientific, Carbosynth, Fluorochem, Fluka and Alfa Aesar, and used without further purification. Solvents were dried according to standard methods.

**Chromatography.** Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F$_{254}$ plates, using UV at 254 nm or staining with ninhydrin for visualisation. Column chromatography was performed with Material Harvest silica gel 60. Reverse-phase column chromatography was conducted with Varian Bond Elut® C18. The HPLC was conducted on Agilent 1100 Series fitted with G1322A degasser, G1311A pump, G1313A autosampler and G1315 DAD, with YMC-Pack Pro C18 column 120 Å S-5 µm 10 mm × 250 mm (product no. AS12S05-2510WT) for preparative scale. The eluent was solvent B, water with 0.1% trifluoroacetic acid (TFA), and C, acetonitrile with 0.1% trifluoroacetic acid, unless otherwise noted and gradients specific to the compound.

**Protein mass spectrometry.** Liquid chromatography–mass spectrometry (LC–MS) was performed on a Xevo® G2-S QTof or SQ Detector 2 mass spectrometer or SQ connected to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 µm, 2.1 mm×50 mm). The eluent was solvent A, water with 0.1% formic acid and B, 71% acetonitrile, 29% water with 0.075% formic acid, at a flow rate of 0.2 mL/min. The gradient was programmed from solvent A/B (72:28) to 100% B over 25 min, followed by solvent B for 2 min and then up to solvent A/B (72:28) over 18 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L/h. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx 4.1 software (Waters). To obtain the ion series described, the major peak(s) of the chromatogram, marked with blue arrow, were selected for integration.
and further analysis. The parameters used for MassLynx deconvolution of the LC–MS data are the following: input mass (m/z) range: indicated on each of the spectrum; output mass range: indicated on each of the spectrum; output data resolution: 1.0 Da/channel; model: uniform Gaussian with peak width at half height 1.0 Da; minimum intensity ratios: 33% (left and right); complete iteration: convergence.

**Small molecule characterisation.** $^1$H NMR was recorded on Bruker 400-Avance III, DPX-400 or 500-DCH Cryoprobe as appropriate. $^{13}$C NMR were recorded by the Department of Chemistry NMR service. Chemical shifts (ppm) were referenced to the residual proton signal of the solvent. High resolution mass spectra were obtained by the Department of Chemistry Mass spectrometry service with a Thermo Fischer LTQ Orbitrap Discovery and ionised by electrospray (ESI).
5.2. Peptide Stapling Strategy with an Isobutylene Graft

5.2.1. Model reaction with cysteine 2

\[
\text{N-}(\text{tert-Butoxycarbonyl})-\text{L-cysteine methyl ester } 2 \quad (0.5 \text{ g, } 2.12 \text{ mmol}) \quad \text{and } \text{K}_2\text{CO}_3 \quad (0.71 \text{ g, } 5.14 \text{ mmol, 2.5 equiv.}) \quad \text{were dissolved in DMF (25 mL). 3-Bromo-2-bromomethyl-1-propene } 1 \quad (120 \mu\text{L, } 1.05 \text{ mmol}) \quad \text{or 3-chloro-2-chloromethyl-1-propene (120 } \mu\text{L, } 1.05 \text{ mmol) } \text{was added to the stirred solution. After being stirred at room temperature overnight, the reaction mixture was diluted with Et}_2\text{O (125 mL) and washed sequentially with a saturated solution of LiBr (125 mL) and brine (125 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (petroleum ether 40–60/ethyl acetate, 2:1) to provide the final compound } 3 \quad \text{as white solid (0.51 g, 92%). } \]

\[\text{H NMR (500 MHz, CDCl}_3 \delta 5.38 - 5.26 \text{ (m, 1H), 5.04 (s, 2H), 4.51 (d, } J = 7.4 \text{ Hz, 1H), 3.76 (d, } J = 2.9 \text{ Hz, 6H), 3.27 (s, 4H), 3.01 - 2.73 \text{ (m, 4H), 1.44 (s, 18H); } \]

\[\text{C NMR (125 MHz, CDCl}_3 \delta 171.53, 155.10, 139.77, 117.19, 80.17, 53.10, 52.57, 35.65, 33.30, 28.31; HRMS (ESI+) m/z: calcd. for } C_{22}H_{38}N_2O_8S_2Na [M+Na]^+: 545.1962 \text{ found: 545.1949.} \]

5.2.2. Synthesis of linear peptides

**Rink resin amino acid loading.** Rink Amide MBHA Resin (0.2 mmol) was placed in a peptide synthesis vessel and treated with 25% piperidine/DMF (1 mL/3 mL) and rocked gently for 1 h. The resin was washed with DMF (3 mL), DCM (3 mL), DMF (3 mL). Pre-mixed solution of Fmoc-protected amino acid (0.8 mmol, 4.0 equiv.), OxymaPure (142 mg, 1.0 mmol, 5.0 equiv.) and DIC (155 μL, 1.0 mmol, 5.0 equiv.) dissolved in DMF (3 mL) was added to the resin. The contents were rocked gently for 2 h, then drained and the resin washed with DMF (3 mL), DCM (3 mL), DMF (3 mL). The Fmoc deprotection procedure was repeated followed by the coupling of the next amino acid in the sequence to synthesise the desired peptide.
Peptide acetylation. After the terminal peptide was linked, the Fmoc was deprotected with the same method. Then the resin was treated with pyridine/Ac₂O (2 mL/1 mL) and rocked gently for 1 h. Then the resin was washed with DMF (3 mL), DCM (3 mL), DMF (3 mL).

Peptide cleavage. The resin-bound peptide was treated with TFA/H₂O/EDT/TIS (2.82 mL/75 µL/75 µL/30 µL) and stirred under a nitrogen atmosphere for 2 hours. Et₂O (3 mL) was added to precipitate the peptide I. The white precipitate was collected by vacuum filtration and the solids wash with additional Et₂O. The crude peptide was dried and purified by HPLC, conducted on Agilent 1100 Series fitted with G1322A degasser, G1311A pump, G1313A autosampler and G1315 DAD, with YMC-Pack Pro C18 column (120 Å, S-5 µm, 10 mm × 250 mm, product no. AS12S05-2510WT) for preparative scale, with a flow rate of 3 mL/min.

Peptides II-IV were synthesised following a similar protocol, employing a stepwise microwave assisted solid-phase peptide synthesis on a Liberty Blue synthesiser. In these cases, the peptides were purified by HPLC using a Phenomenex Luna C18(2) column (100 Å, 10 µm, 250 mm × 21.2 mm) and a dual absorbance detector, with a flow rate of 20 mL/min.

Peptide I. HPLC: Rt = 10.27 min (Grad: water 0.1% TFA/acetonitrile (95:5) → (38.3:61.7), 20 min, λ = 212 nm). HRMS (ESI+) m/z: calcd. for C₁₇H₂₈N₆O₆S₂Na [M+Na]⁺ 499.1404 found: 499.1392.

![HPLC chromatogram at 212 nm of short peptide I. The signal at 9.25 min is the disulfide form of I, and the signal at 10.27 min is the linear form of I.](image-url)
Figure 5.2 HRMS ESI+ of peptide I.

Peptide II. HPLC: Rt = 9.37 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for C_{35}H_{53}N_{6}O_{10}S_{2} [M+H]^+ 809.3321, found 809.3316.
Chapter 5 Materials and Methods

Figure 5.3 HPLC chromatogram at 212 nm of peptide II.

Figure 5.4 HRMS ESI+ of peptide II.

Peptide III. HPLC: Rt = 8.92 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for C_{35}H_{53}N_{8}O_{10}S_{2} [M+H]^+ 809.3321, found 809.3315.
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Figure 5.5 HPLC chromatogram at 212 nm of peptide III.

Figure 5.6 HRMS ESI+ of peptide III.

**Peptide IV.** HPLC: Rt = 7.07 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for $C_{35}H_{54}N_{9}O_{9}S_{2}[M+H]^+$ 808.3480, found 808.3462.
### Chapter 5 Materials and Methods

#### 5.2.3. Stapling of peptides

**General procedure for stapling.** The linear peptide (0.02 mmol, 1.0 equiv.) was dissolved in 10 mL of DMF and K$_2$CO$_3$ (0.10 mmol, 5.0 equiv.) and tris(2-carboxyethyl)phosphine (TCEP·HCl, 0.02 mmol, 1.0 equiv.) were then added. The solution was stirred for 1 h at room temperature. 3-bromo-2-bromomethyl-1-propene 1 (0.025 mmol, 1.25 equiv.) was then added and stirred for additional 12 h. The crude peptide was purified by reversed-phase HPLC to obtain the corresponding stapled derivative. In all cases the yield was ≥ 75%. Finally, they were purified by HPLC.

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**Figure 5.7** HPLC chromatogram at 212 nm of peptide IV.

**Figure 5.8** HRMS ESI+ of peptide IV.

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<table>
<thead>
<tr>
<th>Formula</th>
<th>m/z</th>
<th>z</th>
<th>[err] ppm</th>
<th>Mean err [ppm]</th>
<th>mSigma</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$<em>{35}$H$</em>{53}$N$_9$O$_9$S$_2$</td>
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<td>1+</td>
<td>3.9</td>
<td>3.6</td>
<td>0.7</td>
</tr>
<tr>
<td>C$<em>{30}$H$</em>{54}$N$_9$O$_9$S$_2$</td>
<td>858.3480</td>
<td>2+</td>
<td>2.2</td>
<td>2.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Peptide I’. HPLC: Rt = 13.05 min (Grad: water 0.1% TFA/acetonitrile (95:5) → (38.3:61.7), 20 min, λ = 212 nm). HRMS (ESI+) m/z: calcd. for C_{21}H_{34}N_{6}O_{6}S_{2}Na [M+Na]^+ 553.1873 found: 553.1885.

![Peptide Structure](image)

**Figure 5.9** HPLC chromatogram at 212 nm of peptide I’. 
Figure 5.10 HRMS ESI+ of peptide I’.

Peptide II’. HPLC: Rt = 10.47 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for C_{39}H_{57}N_{8}O_{10}S_{2} [M+H]^+ 861.3634, found 861.3630.
Chapter 5 Materials and Methods

**Figure 5.11** HPLC chromatogram at 212 nm of peptide II’.

**Figure 5.12** HRMS ESI+ of peptide II’.

**Peptide III’**. HPLC: Rt = 10.37 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for C_{39}H_{57}N_{8}O_{10}S_{2} [M+H]^{+} 861.3634, found 861.3634.
Figure 5.13 HPLC chromatogram at 212 nm of peptide III’.

Figure 5.14 HRMS ESI+ of peptide III’.
**Peptide IV**'. HPLC: Rt = 8.07 min (Grad: water 0.1% TFA/acetonitrile (70:30) → (63.5:36.5), 11 min, λ = 212 nm). HRMS ESI+ (m/z) calcd. for C_{39}H_{58}N_{9}O_{9}S_{2} [M+H]^{+} 860.3793, found 860.3794.

![Peptide IV']

**Figure 5.15** HPLC chromatogram at 212 nm of peptide IV'.

![HPLC Chromatogram](image-url)
Figure 5.16 HRMS ESI+ of peptide IV'.

**Synthesis of stapled octreotide.** To a solution of 625 μg/mL octreotide (2.5 mg, 2.5 μmol, 1.0 equiv.) in H₂O was added TCEP·HCl (1.1 mg, 3.75 μmol, 1.5 equiv.) and K₂CO₃ (1.7 mg, 12.0 μmol, 7.5 equiv.). The mixture was stirred at room temperature for 1 h. A solution of 3-bromo-2-bromomethyl-1-propene 1 (1.1 mg, 5.0 μmol, 2.0 equiv.) in DMF (0.55 mL) was added and stirred for 24 h at room temperature. After concentrated, the reaction mixture was analysed by HPLC. HPLC: Rt = 14.683 min (Grad: water 0.1% TFA/acetonitrile (73:27), 15 min, λ = 280 nm).
Figure 5.17 HPLC chromatogram at 280 nm of (a) native octreotide and (b) stapled octreotide.

**Synthesis of stapled somatostatin.** To a solution of 1 mg/mL somatostatin (6.5 mg, 4.0 μmol, 1.0 equiv.) in H₂O was added TCEP·HCl (1.7 mg, 6.0 μmol, 1.5 equiv.) and K₂CO₃ (1.7 mg, 12.0 μmol, 3.0 equiv.). The mixture was stirred at room temperature for 1 h. A solution of 3-bromo-2-bromomethyl-1-propene 1 (1.7 mg, 8.0 μmol, 2.0 equiv.) in DMF (0.6 mL) was added and stirred for 24 h at room temperature. After concentrated, the residue was purified by HPLC to obtain the stapled somatostatin (6.8 mg, 100%) and analysed by LC–MS. HPLC: Rt = 15.006 min (Grad: water 0.1% TFA/acetonitrile (73:27), 15 min, λ = 280 nm). HRMS (ESI+) m/z: calcd. for C₈₀H₁₁₁N₁₈O₁₉S₂ [M+H]^+ 1691.7709 found: 1691.7663. Flow rate: 3.5 mL/min.
Figure 5.18 HPLC chromatogram at 280 nm of (a) native somatostatin and (b) stapled somatostatin.
Figure 5.19 LC–MS data and processing of native somatostatin. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to peptide ions (retention time range: 7.821–8.289 min) is combined and reported as m/z.
Figure 5.20 LC–MS data and processing of stapled somatostatin. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to peptide ions (retention time range: 7.312–8.065 min) is combined and reported as m/z.

5.2.4. Circular dichroism spectroscopy

Peptide solutions were prepared from aqueous peptide stock solutions of accurate molecular concentrations determined by NMR. The final concentration of the peptide samples was 1 mM in pure water. CD measurements were performed on an Aviv Model 410 spectrometer, which was routinely calibrated with (1S)-(−)-10-camphorsulfonic acid. Spectra were recorded at 298 K with a 0.1 cm quartz cell over the wavelength range 250–190 nm at 50 nm/min, with a bandwidth of 1.0 nm, the response time of 1 s, resolution step width of 1 nm and sensitivity of 20–50 Mdeg. Each spectrum represents the average of 3 scans.

5.2.5. Tryptophan fluorescence spectroscopy

Fluorescence spectroscopy was used to determine the dissociation constants of SSTR2 against Somatostatin and Stapled Somatostatin. All experiments were carried out in a Cary Eclipse Fluorescence Spectrophotometer (Varian) at 25 °C with SSTR2 at 1 µM, and concentrations of
peptides varying from 0.1 to 7 μM in 25 mM Tris, 150 mM NaCl, pH 7.5 buffer. Fluorescence emission spectra were recorded in the 300-400 nm range with an excitation wavelength of 280 nm, with slit width of 5 nm. The data analysis was performed in Prism (GraphPad software) considering a model with a single binding site.

5.2.6. Stability of stapled octreotide and somatostatin

A 100 μL aliquot of stapled octreotide or somatostatin (around 1 mM) in H2O was treated with 5.3 μL of a 20 mM GSH solution (to 1 mM) or 1 μL of reconstituted human plasma (Sigma-Aldrich), and the resulting mixture vortexed for 30 s and then shaken at 37 ºC. After 48 h, an aliquot of each reaction mixture was analysed by HPLC. No significant degradation of stapled peptide was observed at 48 h.
Figure 5.21 HPLC chromatogram at 280 nm of isobutylene stapled octreotide stability test. a) Stapled octreotide; b) stapled octreotide with GSH for 48 h at 37 °C; c) stapled octreotide with plasma for 48 h at 37 °C.
Figure 5.22 HPLC chromatogram at 280 nm of isobutylene stapled somatostatin stability test. 
a) stapled somatostatin; b) stapled somatostatin with GSH for 48 h at 37 °C; c) stapled 
soxynstatin with plasma for 48 h at 37 °C.
5.3. One-Pot Re-Bridging of Protein Disulfides Using an Isobutylene Motif

5.3.1. Model reaction with monomeric proteins

**Thioredoxin**

To a 0.5 mL Eppendorf containing thioredoxin solution (10 μL, 5 mg/mL, 4.3 nmol, 1.0 equiv.) dissolved in 90 μL PBS buffer pH 7.4 was added TCEP·HCl in H₂O (2.15 μL, 20 mM, 21.5 nmol, 5.0 equiv.) and 3-bromo-2-bromomethyl-1-propene 1 in DMF (4.3 μL, 20 mM, 86 nmol, 20.0 equiv.) was added and the reaction mixture was stirred for 12 h at 25 °C. A 10 μL aliquot was analysed directly by LC–MS and full conversion was observed. Small molecules were removed from the reaction mixture by loading the sample onto a Zeba™ Spin Desalting Column previously equilibrated with 1× PBS buffer at pH 7.4. The sample was eluted via centrifugation (2 min, 1000 × g). The protein solution was then flash frozen with liquid nitrogen and stored at –20 °C.
Figure 5.23 LC–MS data and processing of native thioredoxin (left peak). a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 8.984–9.740 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Figure 5.24 LC–MS data and processing of native thioredoxin (right peak). a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 9.991–10.579 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
**Figure 5.25** LC–MS data and processing of stapled thioredoxin (right peak). a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 10.663–11.335 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Figure 5.26 LC–MS data and processing of stapled thioredoxin (left peak). a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 9.487–10.243 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

**Measurement of thioredoxin bioactivity.** The standard thioredoxin assay mixture, prepared in 200 μL overall volume, contained 50 mM NaPi buffer, pH 7.0, 1 mM EDTA, 0.15 mM human insulin, 1 mM DTT. The amounts of native thioredoxin *E. coli*, stapled thioredoxin, and native thioredoxin were varied and protein concentrations were determined by BCA protein assay. Samples were run in duplicate and the increase in turbidity from the reduction of insulin was detected at 650 nm at room temperature by a SPECTROstar® Nano reader (BMG Labtech).
The kinetic curves were baseline corrected by subtracting from insulin reduction by DTT alone. The corrected slopes from the kinetic data (ΔmAU/min) in the linear region were plotted as a function of protein concentration via Prism (GraphPad software).

**CRM197**

To a solution of CRM197 in PBS buffer pH 7.4 (20 μL, 20 μM, 0.4 nmol, 1.0 equiv.) was added TCEP·HCl in H₂O (0.4 μL, 10 mM, 4 nmol, 10.0 equiv.) and 3-bromo-2-bromomethyl-1-propene 1 in DMF (0.8 μL, 10 mM, 8 nmol, 20.0 equiv.). The reaction was stirred at 25 °C for 2 hours. A 10 μL aliquot was analysed directly by LC–MS and full conversion was observed.
Figure 5.27 LC–MS data and processing of native CRM197. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 11.418–13.853 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

Calcd. Mass: 58413
Observed Mass: 58415
Figure 5.28 LC–MS data and processing of stapled CRM197. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 12.090–14.105 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass; d) zoom-in range of the spectrum.
5.3.2. Stapling of anti-amyloid-β antibody

To a solution of DesAb-HET in NaPi buffer pH 7.4 (100 μL, 20 μM, 2 nmol, 1.0 equiv.) was added TCEP·HCl in H₂O (1 μL, 20 mM, 20 nmol, 20.0 equiv.) and 3-bromo-2-bromomethyl-1-propene 1 in DMF (1 μL, 20 mM, 20 nmol, 20.0 equiv.). The reaction was stirred at 37 ºC for 6 hours. A 10 μL aliquot was analysed directly by LC–MS and full conversion was observed. Small molecules were removed from the reaction mixture by loading the sample onto a Zeba™ Spin Desalting Column (Thermo Scientific) previously equilibrated with pH 7.4 PBS solution buffer. The sample was eluted by centrifugation (2 min, 1000 × g). The protein solution was then flash frozen with liquid nitrogen and stored at –20 ºC.
Figure 5.29 LC–MS data and processing of native DesAb-HET. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 3.218–4.032 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
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Figure 5.30 LC–MS data and processing of stapled DesAb-HET. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 2.992–3.755 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

**Circular dichroism spectroscopy**

Protein concentrations were determined by nanodrop. The final concentration of the protein samples was 0.2 mg/mL in 1× PBS pH 7.4 buffer. CD measurements were performed on an Aviv Model 410 spectrometer, which was routinely calibrated with (1S)-(+)10-camphorsulfonic acid. Spectra were recorded at 298 K with a 0.1 cm quartz cell over the
wavelength range 260–200 nm at 50 nm/min, with a bandwidth of 1.0 nm, response time of 1 s, resolution step width of 1 nm and sensitivity of 20–50 Mdeg. Each spectrum represents the average of 3 scans.

5.3.3. Stapling of Fab fragment of anti-HER2 antibody

To a solution of anti-HER2 Fab in PBS buffer pH 7.4 (100 μL, 20 μM, 2 nmol, 1.0 equiv.) was added TCEP·HCl (1 μL, 20 mM, 20 nmol, 10.0 equiv.) and 3-bromo-2-bromomethyl-1-propene 1 in DMF (2 μL, 20 mM, 40 nmol, 20.0 equiv.). The reaction was stirred at 25 °C for 12 hours. A 10 μL aliquot was analysed directly by LC–MS and full conversion was observed. Small molecules were removed from the reaction mixture by loading the sample onto a Zeba™ Spin Desalting Column (Thermo Scientific) previously equilibrated with pH 7.4 PBS solution buffer. The sample was eluted by centrifugation (2 min, 1000 × g). The protein solution was then flash frozen with liquid nitrogen and stored at –20 °C. (The Fab fragment of the anti-HER2 antibody was kindly provided by Dr Vijay Chudasama.)
Figure 5.31 LC–MS data and processing of native Fab. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 5.709–6.885 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Figure 5.32 LC–MS data and processing of stapled Fab. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 5.961–7.053 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

### 5.3.4. Ellman’s test of stapled Fab

To the solution of the stapled Fab in 1× PBS buffer (10 μL, 20 μM, 0.2 nmol, 1.0 equiv.) was added 2 μL of a solution of Ellman’s reagent (50 mM, 100 nmol, 500 equiv.). After incubated at 25 ℃ for 6 h, a 10 μL aliquot of the solution was analysed directly by LC–MS.
Figure 5.33 LC–MS data and processing of the Ellman’s test reaction with stapled Fab. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 5.709–7.221 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

5.3.5. Stability of stapled Fab

To the solution of native and stapled Fab in 1× PBS buffer (10 μL) was added either GSH (1 μL, 60 mg/mL in H2O) or human plasma (1 μL) and incubated at 37 °C. After 24 h, a 10 μL aliquot of each was analysed directly by LC–MS.
Figure 5.34 LC–MS data and processing of stapled Fab, being incubated with glutathione under 37 °C for 24 h. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 6.213–7.641 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
**Figure 5.35** LC–MS data and processing of native Fab, being incubated with glutathione under 37 °C for 24 h. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 5.625–7.892 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Figure 5.36 LC–MS data and processing of native Fab, being incubated with glutathione under 37 °C for 24 h. a) Total ion count trace of liquid chromatography step; b) the peak
corresponding to protein ions (retention time range: 4.064–12.133 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass; d) zoom-in range of the spectrum.

### 5.3.6. Stapling of IgG antibodies

To a solution of IgG antibody in PBS buffer pH 7.4 (50 μL, 6.7 μL, 0.33 nmol, 1.0 equiv.) was added TCEP·HCl in H₂O (0.85 μL, 10 mM, 0.85 nmol, 25.0 equiv.) and 3-bromo-2-bromomethyl-1-propene 1 in DMF (0.85 μL, 10 mM, 0.85 nmol, 25.0 equiv.). The reaction mixture was incubated at 25 °C for 12 h. Excess reagents were removed by diafiltration into PBS buffer by using Vivaspin 500 sample concentrators (GE Healthcare, 5 min, 15,000 × g, 30 kDa MWCO). The antibody solution was then flash frozen with liquid nitrogen and stored at –20 °C.

### 5.3.7. SDS-PAGE gels

NuPAGE® Novex 4–12% Bis-Tris protein gels were performed following standard protocol. A Precision Plus Protein™ all blue pre-stained protein standard (10–250 kDa) was co-run to estimate protein weights. Non-reduced samples (7.5 μL) were mixed with NuPAGE® LDS sample buffer (2.5 μL, 4X) and heated at 90 °C for 10 min. Reduced samples (6.5 μL) were mixed with NuPAGE® LDS sample buffer (2.5 μL, 4X) and NuPAGE® reducing agent (1.0 μL, 4X), then left at 25 °C for 10 min before heated at 90 °C for 10 min. The gel was run at a constant voltage of 200 V for 35 min in 1× NuPAGE® MES SDS running buffer. The gel was fixed with H₂O/MeOH/AcOH (50:42:8) for 1 h before being stained with Coomassie Brilliant Blue.
5.3.8. Native mass spectrometry of the stapled trastuzumab

Figure 5.37 Native mass spectrometry analysis of the stapled trastuzumab. a) Offset isolated species and reported as m/z; b) deconvoluted mass spectrum; c) and d) peak intensities of the mass spectrum.

5.3.9. BCA protein assay

To determine the concentration of proteins, bicinchoninic acid protein assays (BCAs) were conducted by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) with diluted BSA as the standard. 5 μL of each sample and standard was pipetted into microplate wells. Then, 100 μL of the working reagent was added to each well. After being shaken at 37 °C for 30 min, the absorbance was detected at 562 nm at room temperature by using a SpectraMax® i3x multi-mode microplate reader. A standard linear fitted curve was prepared by plotting the average blank-corrected measurement for each standard vs. its concentration using Prism (GraphPad software). Then the standard curve was used to determine the protein concentration of each sample.
5.3.10. Biotinylation of trastuzumab

To use the streptavidin biosensor to determine the $K_D$, the trastuzumab and stapled trastuzumab were conjugated to EZ-Link™ NHS-PEG$_4$-Biotin (Thermo Fisher Scientific). To a solution of the corresponding protein (20 μL, 20 μM in PBS) was added the biotin linker (20 μL, 10 equiv, 200 μM in PBS). After being shaken at 25 °C for 30 min, the non-reacted NHS-PEG$_4$-Biotin was removed by diafiltration into PBS buffer by using Vivaspin 500 sample concentrators (5 min, 15,000 × g, GE Healthcare, 30 kDa MWCO).

5.3.11. Bio-layer interferometry

Binding assays were performed on an Octet® Red96 System (fortéBIO). Ligand immobilisation, binding reactions, regeneration and washes were conducted in wells of black polypropylene 96-well microplates. Fab, trastuzumab and corresponding stapled compounds (20 nM) were immobilised on SA biosensors in PBS with 0.1% bovine serum albumin (BSA) and 0.02% Tween 20 at 30 °C. Binding analyses were carried out at 25 °C, 1000 rpm in PBS with 0.1% BSA and 0.02% Tween 20, with a 600 s of association followed by a 2200 s of dissociation with different concentrations of recombinant HER2 receptor to obtain the association curve. Glycine pH 2.0 was used as a regeneration buffer. Data were analysed by using Data Analysis (fortéBIO) with Savitzky–Golay filtering. Binding data were fitted to a 2:1 heterogeneous ligand model and steady state analysis was performed to obtain the binding constant ($K_D$).

Figure 5.38 Stacked raw data and fitting curves of BLI.
5.3.12. Circular dichroism of antibodies

The secondary structures of native and stapled trastuzumab were determined by CD spectroscopy. The purified antibodies were dissolved in PBS at the final concentration of 0.4 mg/mL, and the CD spectra were recorded on a Jasco J-815 CD spectropolarimeter (Hachioji, Tokyo, Japan) by using quartz cuvettes of 0.1 mm (Hellma GmbH & Co, Müllheim, Germany). Temperature scans were measured at 25, 37, 55, 75 and 80 °C. Temperature regulation was carried out with a PTC-348WI thermocouple (JASCO). In the 190 to 260 nm wavelength region 5 or 10 scans were accumulated with sampling velocity of 200 nm/min, data pitch of 0.5 nm, data integration time of 1 s, and 1 nm bandwidth. Signal from the buffer blank was subtracted from the sample scan and experimental instrument-related baseline drift was corrected by subtracting the average of the signal between 250 and 260 nm to each spectrum. (Experiments were performed by Dr Marta C. Marques.)

5.3.13. Differential scanning fluorimetry (DSF)

The experiment was performed by using a 7500 Fast Real Time PCR System (Applied Biosystems, Warrington, Cheshire, U.K.), with an excitation range of 510–530 nm and the samples were subjected to the heating cycle as described previously. Briefly, 2 µL of native and stapled trastuzumab at an initial concentration of 7.6 µM were mixed with a freshly dilution of SYPRO Orange® (Invitrogen) and diluted PBS to a final volume of 25 µL. The PCR-plate was kept on ice during the preparation to prevent protein denaturation and to equilibrate the sample for the starting temperature of the assay. The measurements were performed in triplicate. The fluorescence emission signal at 567–596 nm was used for data analysis. (Experiments were performed by Dr Marta C. Marques.)

5.3.14. Cell culturing and flow cytometry studies

Human Burkitt Lymphoma cells (RAJI), HPB-ALL and breast cancer cells (SKBR3) were routinely cultured at 37 °C with 5% CO₂ in RPMI-1640 and or DMEM medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 2 mM L-glutamine. Binding of stapled and native Rituximab and Trastuzumab antibodies to the surface CD-20 and HER2 receptors was analysed by flow cytometry. In brief, cells were incubated with respective stapled and native antibodies at 4 °C for 30 min. Cells were then washed with ice-cold PBS and the primary
antibody was detected by using a commercial Goat alexa 647-conjugated anti-human (H+L) antibody (Thermo Fisher Scientific). Samples were acquired with a FACS Fortessa I/II (BD Bioscience) and analysed with FlowJo (Tree Star). (Experiments were performed by Dr Padma Akkapeddi.)

5.3.15. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDC)

ADCC assay was performed by using fresh PBMCs from a healthy volunteer. In brief, the target Burkitt lymphoma cells (RAJI) were stained with Calcein–AM (Molecular Probes) at a concentration of 2 μM per 1x10^6 cells, at 37 °C for 30 min in phenol red free RPMI medium. Cells were thoroughly washed and incubated with PBMCs at an Effector:Target ratio of 50:1 in presence and absence of respective antibodies for 4 h at 37 °C, 5 % CO_2 in 96-well round-bottom plates. After the incubation time, the plates were centrifuged and 100 μL of the supernatant was transferred and the fluorescence was read at an excitation of 485 nm and emission of 520 nm. Percentage of cytotoxicity was calculated by using the following formula:

\[
\% \text{ dead cells} = \left( \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \right) \times 100
\]

Wherein maximal release was triggered by the addition of 4% Triton-X.

For CDC cells were incubated with 10% Normal Human Serum for a period of 24 h and the assay was developed as mentioned in the ADCC methodology. (Experiments were performed by Dr Padma Akkapeddi.)

5.3.16. Antibody pharmacokinetics in vivo

NOD SCID mice of the age 8–12 weeks (n = 12 per group; n = 4 per time points per group) were administered a 5 mg/kg IV bolus injection of the native and the stapled trastuzumab antibody. Blood was collected at the selected time points for up to 28 days. Blood samples were processed to collect plasma at the aforementioned time points and stored at –80 °C until further processing. The total antibody concentration in plasma was determined by ELISA against recombinant HER2 protein. (Experiments were performed by Dr Padma Akkapeddi.)
5.4. Photoactivation of Thiol-Containing Drugs via Thiol-Ene Strategy

5.4.1. Synthesis of thiol sugars NAGSH and 4AcNAGSH

\[ 1-S\text{-Acetyl-1-thio-}\left(3,4,6\text{-tri-O-acetyl-2-acetamido-2-deoxy-}\beta\text{-D-glucopyranoside}\right) \text{(GlcN3)} \]

\( \text{d-Glucosamine hydrochloride (20.0 g, 93 mmol, 1.0 equiv.) was suspended in anhydrous methanol (20 mL). Sodium methoxide (6.0 g, 111 mmol, 1.2 equiv.) was added in several portions and the mixture stirred vigorously at room temperature for 1 h. Then the mixture was filtered and washed with MeOH. Acetic anhydride (12 mL, 127 mmol, 1.4 equiv.) was added to the filtrate at 0 °C and warmed to room temperature. After 12 hours, the reaction was concentrated to ~20 mL, and the product was precipitated by the addition of diethyl ether (100 mL). The solid was isolated by filtration and dried under vacuum. Acetyl chloride (40 mL, 563 mmol, 6.0 equiv.) was added slowly and the mixture stirred vigorously at 0 °C. After the addition, the reaction was heated to reflux. After 3 hours, the reaction was cooled to room temperature, diluted with CH\(_2\)Cl\(_2\) (200 mL), and poured into a beaker of ice (~200 mL). The organics were washed with sat. aq. NaHCO\(_3\) (2 × 200 mL), dried over MgSO\(_4\), filtered, and concentrated. The dark residue was dissolved in ethyl acetate and filtered through a short column of silica, eluting with ethyl acetate. The product was precipitated by the addition of cold Et\(_2\)O and petrol. The resulting powder was isolated by filtration and dried under vacuum. The crude anomeric chloride GlcN2 was used in the final step without purification. A portion of the anomeric chloride (1.5 g, 4.1 mmol, 1.0 equiv.) was dissolved in DMF (20 mL). Potassium thioacetate (2.3 g, 20.5 mmol, 5.0 equiv.) was then added. The reaction was flushed with nitrogen and stirred at room temperature for 3 hours. After this time, no starting material was detected by TLC. The reaction mixture was diluted with ethyl acetate (250 mL) and washed sequentially with sat. aq. NaHCO\(_3\) (200 mL), H\(_2\)O (200 mL), and brine (200 mL). The organic layer was dried over MgSO\(_4\), filtered, and concentrated under reduced pressure to give a brown solid. This solid was recrystallised from CH\(_2\)Cl\(_2\) and petroleum ether 40–60 to afford the desired compound GlcN3 as white crystals (0.75 g, 45%).} \] 

\[ ^1\text{H NMR (400 MHz, CDCl}_3\text{) } \delta \]
6.03 (d, $J = 9.8$ Hz, 1H), 5.23 – 5.03 (m, 3H), 4.21 (dd, $J = 12.5$, 4.7 Hz, 1H), 4.07 (dd, $J = 12.6$, 2.2 Hz, 1H), 3.79 (ddd, $J = 9.6$, 4.6, 2.2 Hz, 1H), 2.34 (s, 3H), 2.04 (s, 3H), 2.01 (d, $J = 1.4$ Hz, 6H), 1.89 (s, 3H). The preparation followed the reference and the characterisation was in agreement with those reported.[276]

*1-Thio-2-acetamido-2-deoxy-β-D-glucopyranose (NAGSH)*

![Figure showing the structure of NAGSH](image)

GlcNAc thioacetate (754 mg, 1.9 mmol, 1.0 equiv.) was dissolved in anhydrous methanol (25 mL). Sodium methoxide (200 mg, 3.8 mmol, 2.0 equiv.) was added at room temperature. Then the reaction was stirred for 1 hour before quenching with DOWEX-50WX8 (H⁺ form) until pH 7. The resin was removed by filtration and washed with methanol (50 mL). The filtrate was evaporated to provide the desired unprotected thiol sugar NAGSH (494 mg, 1.9 mmol, 100%) as a syrup. ¹H NMR (400 MHz, D₂O) δ 4.60 (d, $J = 10.1$ Hz, 1H), 3.81 (dd, $J = 12.4$, 1.6 Hz, 1H), 3.73 – 3.60 (m, 2H), 3.47 – 3.35 (m, 3H), 1.96 (d, $J = 3.1$ Hz, 3H). The preparation followed the reference and the characterisation was in agreement with those reported.[276]

*(3,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-isothiouronium chloride (GlcN4)*

![Figure showing the structure of GlcN4](image)

Glcpyranosyl chloride GlcN2 (3.7 g, 10.0 mmol, 1.0 equiv.) and thiourea (1.5 g, 20.0 mmol, 2.0 equiv.) were dissolved in acetone (30 mL) under Ar. The reaction mixture was heated to 60 °C. After 2 hours, a white solid precipitated. The precipitate was removed by filtration, the filtrate was returned to reflux and this process was repeated until the solid ceased to precipitate. The solids were combined and recrystallised from acetone/petroleum ether 40–60 to afford the desired compound GlcN4 (3.44 g, 7.8 mmol, 78 %) as a white crystalline solid. ¹H NMR (400 MHz, CDCl₃) δ 5.58 (d, $J = 9.4$ Hz, 1H), 5.18 – 5.02 (m, 2H), 4.57 (t, $J = 9.7$ Hz, 1H), 4.30 – 4.13 (m, 2H), 3.68 (ddd, $J = 9.5$, 4.8, 2.3 Hz, 1H), 2.57 (d, $J = 9.3$ Hz, 1H), 2.10 (s, 3H), 2.04
(s, 3H), 2.03 (s, 3H), 1.98 (s, 3H). The preparation followed the reference and the characterisation was in agreement with those reported.\textsuperscript{[277]}

\textit{1-Thio-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-\textbeta-D-glucopyranose (4AcNAGSH)}

The isothiouronium chloride sugar GlcN4 (3.44 g, 7.78 mmol, 1.0 equiv.) and sodium metabisulfite (1.78 g, 9.34 mmol, 1.2 equiv.) were added to a stirred mixture of CH\textsubscript{2}Cl\textsubscript{2} (25 mL) and H\textsubscript{2}O (15 mL). The mixture was heated to reflux under Ar. After 3 hours, TLC indicated the formation of product with complete consumption of the starting material. The reaction mixture was cooled to room temperature and the phases separated. The aqueous layer was re-extracted with CH\textsubscript{2}Cl\textsubscript{2} (2 × 40 mL). The combined organic layers were washed with H\textsubscript{2}O (40 mL), brine (40 mL), dried over MgSO\textsubscript{4}, filtered and concentrated in vacuum. The resulting white solid was crystallised from ethyl acetate/petroleum ether 40–60 to afford the protected thiol sugar 4AcNAGSH (1.7 g, 4.8 mmol, 61 %) as a white solid. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 5.72 (d, \(J = 9.4\) Hz, 1H), 5.06 – 5.15 (m, 2H), 4.59 (t, \(J = 9.8\) Hz, 1H), 4.24 (dd, \(J = 12.5\) Hz, 1H), 4.08 – 4.17 (m, 2H), 3.69 (ddd, \(J = 9.6\) Hz, 4.6 Hz, 2.1 Hz, 1H), 2.57 (d, \(J = 9.3\) Hz, 1H), 2.03, 2.05, 2.10 (3s, 9H), 1.99 (s, 3H); HRMS (ESI\textsuperscript{+}) m/z: calcd. for C\textsubscript{14}H\textsubscript{22}NO\textsubscript{8}S [M+H\textsuperscript{+}]: 364.1061 found: 364.0970. The preparation followed the reference and the characterisation was in agreement with those reported.\textsuperscript{[277]}

5.4.2. Unstapling the stapled cysteine 3

DPAP (1.5 mg, 6 \(\mu\)mol, 0.3 equiv.) and 1-thio-\textbeta-D-glucose tetraacetate (21.9 mg, 60 \(\mu\)mol, 3.0 equiv.) was dissolved to a solution of stapled cysteine 3 (10.5 mg, 20 \(\mu\)mol, 1.0 equiv.) in DMF (1.5 mL). Then, the solution was irradiated with the UV reactor (LZC-ORG, fitted with
10 × 8 W UVA lamps). When the TLC indicated the disappearance of starting material, the reaction was removed from the reactor. After being stirred in air for one hour, TCEP·HCl (11.5 mg, 40 μmol, 2.0 equiv.) was added into the solution. After 1 h, the mixture was concentrated under reduced pressure and purified by column chromatography (petroleum ether 40–60/ethyl acetate, 3:1) to provide the free cysteine 2 as white solid.

### 5.4.3. Control studies of thiol-ene unstapling reaction

The controlled experiments were performed with the same procedure mentioned above while the relative compounds or step were not performed as stated in Figure 5.39.

![Figure 5.39 Thin-layer chromatography (TLC) of control studies.](image)

DPAP (1.5 mg, 6 μmol, 0.3 equiv.) and 1-thio-β-D-glucose tetraacetate (21.9 mg, 60 μmol, 3.0 equiv.) was dissolved to a solution of stapled cysteine 3 (10.5 mg, 20 μmol, 1.0 equiv.) in DMF (1.5 mL). To the reaction mixture, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 1.4 mg, 90 μmol, 4.5 equiv.) was added, followed by the irradiation with the UV reactor. After 15 mins, the TLC and the MS spectrum indicated no conversion of the stapled cysteine 3 or the generation of the decaging product 2.
Figure 5.40 MS-ESI+ for TEMPO control experiment.

5.4.4. Unstapling short peptide

DPAP (0.6 mg, 2.4 μmol 1.0 equiv.) and 1-thio-β-D-glucose tetraacetate (4AcGlcSH, 8.9 mg, 24 μmol, 10.0 equiv.) were dissolved in a solution of stapled CAAAC (1.3 mg, 2.4 μmol, 1.0 equiv.) in DMF. Then the UV light was turned on. When the LC–MS indicated the disappearance of starting material, the UV light was turned off. Next, the solvent was removed by air flush and injected into HPLC. HPLC: Rt = 9.262 min (Grad: water 0.1% TFA/acetonitrile (95:5) → (38.3:61.7), 20 min, λ = 212 nm). HRMS (ESI+) m/z: calcd. for $C_{17}H_{28}N_6O_6S_2Na \ [M+Na]^+$: 499.1404 found: 499.1401.
Figure 5.41 HPLC chromatogram at 212 nm of unstapled short peptide.

5.4.5. Diethylethylene stapling and thiol-ene reaction

Diethylethylene-stapled cysteine 5

\[
\text{N-}(\text{tert}-\text{Butoxycarbonyl})-\text{L-cysteine methyl ester 2 (0.47 g, 2 mmol, 2 equiv.) and potassium carbonate (0.71 g, 5 mmol, 5.0 equiv.) were dissolved in DMF (25 mL). 1,5-Dibromo-3-methylenepentane (0.24 g, 1 mmol, 1 equiv.) was added to the stirred solution. The reaction was stirred at room temperature overnight. After this, the reaction was diluted with diethyl ether (125 mL) and washed sequentially with a saturated solution of LiBr (125 mL) and brine (125 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The resulting residue was purified by flash-column chromatography (petroleum ether 40–60/ethyl acetate, 2:1) to provide the diethylethylene-stapled cysteine 5 (0.54 g, 0.98 mmol, 98%) as white solid.}\]

\[
\text{1H NMR (500 MHz, CDCl3) } \delta 5.36 (d, J = 7.9 Hz, 2H), 4.84 (t, J = 1.1 Hz, 2H), 4.54 (q, J = 5.8 Hz, 2H), 3.76 (s, 6H), 2.98 (t, J = 4.6 Hz, 4H), 2.69 – 2.55 (m, 4H), 2.28 (ddt, J = 8.6, 7.6, 1.0 Hz, 4H), 1.45 (s, 18H); 13C NMR (125 MHz, CDCl3) \delta 171.55, 155.11, 145.41, 111.97, 80.17, 53.28, 52.58, 35.80, 34.66, 30.93, 28.44, 28.32; HRMS (ESI+) m/z: calcd. for C24H43N2O8S2 [M+H]+: 551.2461 found: 551.2453.}\\n\]
**Thiol-ene reaction-generated glucose-cysteine conjugate 6**

To a solution of stapled cysteine 5 (22.0 mg, 40 μmol, 1 equiv.) in dichloromethane (2 mL) was added DPAP (3.1 mg, 12 μmol, 0.3 equiv.) and 4AcGlcSH (43.7 mg, 120 μmol, 3 equiv.) at room temperature. The solution was degassed with argon, followed by UV irradiation. When the TLC indicated the disappearance of starting material, the UV light was turned off. The resulting mixture was concentrated under reduced pressure and purified by flash-column chromatography (petroleum ether 40–60/ethyl acetate, 2:1 to 1:1) to provide amide conjugate compound 6 (26.1 mg, 0.028 mmol, 71%) as a white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 5.37 (d, $J = 8.0$ Hz, 2H), 5.21 (t, $J = 9.4$ Hz, 1H), 5.07 (t, $J = 9.8$ Hz, 1H), 5.02 (t, $J = 9.7$ Hz, 1H), 4.51 (d, $J = 7.4$ Hz, 2H), 4.47 (d, $J = 10.0$ Hz, 1H), 4.25 (dd, $J = 12.4$, 4.7 Hz, 1H), 4.14 (dd, $J = 12.4$, 2.3 Hz, 1H), 3.76 (s, 6H), 3.72 (ddd, $J = 10.1$, 4.8, 2.3 Hz, 1H), 3.05 – 2.88 (m, 4H), 2.71 (dd, $J = 12.6$, 5.5 Hz, 1H), 2.63 (dd, $J = 12.7$, 6.2 Hz, 1H), 2.54 (dqd, $J = 12.2$, 6.0, 3.9 Hz, 4H), 2.09 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.94 – 1.85 (m, 1H), 1.74 – 1.53 (m, 4H), 1.44 (s, 18H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 171.57, 170.63, 170.14, 169.41, 155.14, 83.20, 80.16, 75.94, 73.83, 69.78, 68.23, 62.05, 53.23, 52.58, 35.59, 34.50, 34.41, 32.83, 32.38, 29.80, 29.69, 28.32, 20.79, 20.72, 20.61, 20.59; HRMS (ESI+) m/z: calcd. for C$_{38}$H$_{62}$N$_2$O$_{17}$S$_3$Na [M+Na]$^+$: 937.3103 found: 937.3073.
Diethylethylene stapling of octreotide

To a solution of 0.6 mM octreotide (2.7 mg, 2.6 μmol, 1 equiv.) in H₂O was added TCEP·HCl (1.1 mg, 3.75 μmol, 1.5 equiv.) and potassium carbonate (1.7 mg, 13.2 μmol, 5 equiv.). The mixture was stirred at room temperature for 1 h. A solution of 1,5-dibromo-3-methylenepentane (1.3 mg, 5.3 μmol, 2 equiv.) in DMF (0.5 mL) was added and stirred for 24 h at room temperature. A 10 μL aliquot was analysed directly by HPLC and LC-MS, and full conversion was observed. HPLC: Rt = 14.700 min (Grad: water 0.1% TFA/acetonitrile (73:27), 15 min, λ = 254 nm).
Figure 5.42 HPLC chromatogram at 254 nm of (a) octreotide and (b) diethylethylene-stapled octreotide.

Free Octreotide
SS_Free Oct_061116_1

b) [M+2H]^2+

Calc. Mass: 1020
Observed Mass: 1021

Figure 5.43 LC–MS data and processing of native octreotide. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to peptide ions (retention time range: 3.123–4.041 min) is combined and reported as m/z.
Figure 5.44 LC–MS data and processing of diethylethylenestapled octreotide. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to peptide ions (retention time range: 4.339–4.728 min) is combined and reported as m/z.

Thiol-ene conjugation of diethylethylene-stapled octreotide
To a solution of diethylethylene-stapled octrotide (0.95 mg, 0.87 μmol, 1 equiv.) in H$_2$O (1 mL) was added DPAP (26 μL, 0.01 M in DMF, 0.26 μmol, 0.1 equiv.) and 4AcGlcSH (26 μL, 0.1 M in DMF, 2.6 μmol, 3 equiv.) at room temperature. The solution was degassed with argon, followed by irradiated with UV light. After 15 min, a 10 μL aliquot was analysed directly by HPLC and LC-MS, and full conversion to thiol-ene products is observed. HPLC: Rt = 16.953 min (Grad: water 0.1% TFA/acetonitrile (73:27), 15 min, λ = 254 nm).

**Figure 5.45** HPLC chromatogram at 254 nm of the octreotide-glucose conjugate.

**Figure 5.46** LC–MS data and processing of octreotide-glucose conjugate. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to peptide ions (retention time range: 5.013–5.150 min) is combined and reported as m/z.
5.4.6. Largazole synthesis and decaging reaction

**Synthesis of largazole thiol and largazole**

*Ethyl 2-[N-(tert-butoxycarbonyl)aminomethyl]thiazole-4-carboxylate (C1)*

![Chemical structure of C1](image)

Ethyl bromopyruvate (0.84 mL, 6.7 mmol, 1.2 equiv.) and calcium carbonate (0.56 g, 5.6 mmol, 1.0 equiv.) were added in sequence to a solution of *tert*-butyl 2-amino-2-thioxoethylcarbamate C0 (1.06 g, 5.6 mmol, 1.0 equiv.) in ethanol (20 mL) at room temperature. The reaction mixture was stirred for 7 h at room temperature. The product mixture was concentrated, and the residue obtained was purified by flash column chromatography (petroleum ether 40–60/ethyl acetate, 8:1 to 2:1) to provide the thiazole C1 as a white solid (1.02 g, 3.6 mmol, 65%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.11 (s, 1H), 4.64 (d, $J = 6.3$ Hz, 2H), 4.41 (q, $J = 7.1$ Hz, 2H), 1.45 (s, 9H), 1.39 (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 170.09, 161.43, 155.73, 147.07, 128.03, 80.63, 61.67, 42.53, 28.45, 14.52. The preparation followed the reference and the characterisation was in agreement with those reported.$^{[268]}$

*2-[N-(tert-Butoxycarbonyl)aminomethyl]thiazole-4-carboxamide (C2)*

![Chemical structure of C2](image)

A solution of aqueous ammonia (28% w/v, 42 mL) was added to a solution of the thiazole C1 (1.01 g, 3.5 mmol, 1.0 equiv.) in anhydrous methanol (35 mL) at room temperature. The resulting mixture was stirred overnight at room temperature. The product mixture was concentrated, and the residue obtained was dried by azeotropic distillation from toluene (50 mL×2) to afford the product C2 as a yellow solid (0.87 g, 3.4 mmol, 96%). $^1$H NMR (400 MHz, DMSO) $\delta$ 8.09 (s, 1H), 7.11 (s, 1H), 5.74 (s, 1H), 5.27 (s, 1H), 4.60 (d, $J = 6.2$ Hz, 2H),
1.47 (s, 9H); $^{13}$C NMR (100 MHz, DMSO) δ 171.66, 162.42, 155.92, 150.07, 124.17, 78.88, 
42.08, 28.29. The preparation followed the reference and the characterisation was in agreement 
with those reported.$^{[268]}$

2-[N-(tert-Butoxycarbonyl)aminomethyl]thiazole-4-carbonitrile (C3)

Trifluoroacetic anhydride (0.51 mL, 3.6 mmol, 1.1 equiv.) was added dropwise over 20 min to 
a solution of the amide C1 (0.086 g, 3.3 mmol, 1.0 equiv.) and triethylamine (1.0 mL, 
7.26 mmol, 2.2 equiv.) in dichloromethane (50 mL) at 0 °C. The resulting mixture was stirred 
for 30 min at 0 °C. The reaction mixture was then allowed to warm over 30 min to room 
temperature. The reaction mixture was stirred for 2 h at room temperature. The product mixture 
was concentrated, and the residue obtained was purified by flash column chromatography 
(petroleum ether 40–60/ethyl acetate, 4:1 to 3:1) to furnish the nitrile C3 as a white solid (0.4 g, 
1.65 mmol, 50%). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.94 (s, 1H), 5.43 – 5.19 (m, 1H), 4.62 (d, $J$ 
= 6.3 Hz, 2H), 1.47 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 171.39, 155.61, 130.84, 126.49, 
113.79, 80.85, 42.33, 28.28. The preparation followed the reference and the characterisation 
was in agreement with those reported.$^{[268]}$

(R)-2-((tert-Butoxycarbonylamino)methyl)thiazol-4-yl)-4-methyl-4,5-dihydrothiazole-4-
carboxylic acid (C4)

Triethylamine (0.37 mL, 2.65 mmol, 1.6 equiv.) was added dropwise to a solution of the nitrile 
C3 (395.6 mg, 1.65 mmol, 1.0 equiv.) and 2-methyl-L-cysteine (454.0 mg, 2.65 mmol, 
1.6 equiv.) in methanol (25 mL) at room temperature. The reaction mixture was heated at reflux 
overnight. The product mixture was cooled to room temperature and the cooled product
mixture was concentrated. The residue obtained was dissolved in saturated aqueous sodium bicarbonate solution (40 mL) and the resulting solution was washed with ether (30 mL). The aqueous layer was acidified to pH 3–4 by the dropwise addition of 3.0 N aqueous hydrochloric acid solution. The resulting mixture was extracted with ethyl acetate (3×30 mL) and the organic layers were combined. The combined organic layers were dried over sodium sulfate and the dried solution was filtered. The filtrate was concentrated to provide the thiazole–thiazoline C4 as a white solid (590.9 mg, 1.65 mmol, 100%).

\[ ^1 \text{H NMR (400 MHz, CDCl}_3 \] \( \delta \) 7.95 (s, 1H), 5.32 (s, 1H), 4.63 (d, \( J = 6.3 \) Hz, 2H), 3.86 (d, \( J = 11.6 \) Hz, 1H), 3.36 (d, \( J = 11.6 \) Hz, 1H), 1.67 (s, 3H), 1.47 (s, 9H); \[ ^{13} \text{C NMR (100 MHz, CDCl}_3 \] \( \delta \) 174.98, 170.12, 164.57, 155.63, 147.93, 122.70, 84.51, 80.57, 42.29, 40.97, 28.32, 24.33.

The preparation followed the reference and the characterisation was in agreement with those reported.[267]

\((R)-1-(4\text{-Benzyl-2-thioxothiazolidin-3-yl})\text{ethanone (auxiliary)}\)

\[ \begin{align*}
\text{O} & \quad \text{N} \\
\text{Bn} & \quad \text{S} \\
\text{S} & \quad \text{S}
\end{align*} \]

\( \text{d-Phenylalaninol (2.5 g, 16.5 mmol, 1.0 equiv.)} \) was dissolved in aqueous potassium hydroxide (3 M, 35 mL). Carbon disulfide (5.0 mL, 82.7 mmol, 5.0 equiv.) was added and the solution was heated at reflux overnight. The solution was extracted with dichloromethane (3×200 mL), dried (\( \text{Na}_2\text{SO}_4 \)), filtered, and concentrated to afford \((R)-4\text{-benzylthiazolidine-2-thione, which was used without further purification. The auxiliary (16.5 mmol, 1.0 equiv.)}, 4\text{-dimethylaminopyridine (0.2 g, 1.65 mmol, 0.1 equiv.)}, \) and triethylamine (3.4 mL, 24.8 mmol, 1.5 equiv.) were dissolved in dry CH\(_2\)Cl\(_2\) (45 mL) and cooled to 0 °C. Acetyl chloride (1.8 mL, 24.8 mmol, 1.5 equiv.) was added dropwise and the reaction was allowed to reach room temperature and stirred overnight. Then, the reaction was quenched with saturated \( \text{NH}_4\text{Cl} \) (48 mL), diluted with Et\(_2\)O (45 mL), and the organic phase was washed with saturated \( \text{CuSO}_4 \) (3×20 mL), water (20 mL), and brine (20 mL), dried (MgSO\(_4\)), filtered, and concentrated to give the crude compound as a yellow solid. Recrystallisation from EtOH afforded the title auxiliary (4.0 g, 16.2 mmol, 98%) as yellow needles \(^1\text{H NMR (500 MHz, CDCl}_3 \] \( \delta \) 7.36 – 7.26 (m, 5H), 5.41 – 5.34 (m, 1H), 3.38 (ddd, \( J = 11.6, 7.3, 1.1 \) Hz, 1H), 3.21 (dd, \( J = 13.2, 3.8 \) Hz, 1H), 3.03 (dd, \( J = 13.2, 10.6 \) Hz, 1H), 2.88 (dd, \( J = 11.5, 0.7 \) Hz, 1H), 2.79 (s, 3H); \[ ^{13} \text{C NMR (125 MHz, CDCl}_3 \] \( \delta \) 201.71, 170.86, 136.65, 129.59, 129.05, 127.37, 68.36, 36.83, 31.96,
27.22. The preparation followed the reference and the characterisation was in agreement with those reported.\textsuperscript{[269]}

\textit{(2E)-5-[(Triphenylmethyl)thio]-2-pentenal (A2)}

![Chemical structure of A2]

To a solution of triphenylmethanethiol (2.7 g, 9.8 mmol, 2.1 equiv.) in dichloromethane (100 mL) was added acrolein A0 (0.8 g, 13.6 mmol, 2.9 equiv.) and triethylamine (1.4 g, 13.6 mmol, 2.9 equiv.). The resulting mixture was stirred for 1 h at room temperature and was concentrated to give the aldehyde A1 as a white solid, which was used in the next step without purification. A solution of the aldehyde A1 obtained above and (triphenylphosphoranylidene)acetaldehyde (1.4 g, 4.7 mmol, 1.0 equiv.) in dry benzene (58 mL) was refluxed overnight. The reaction mixture was concentrated and purified by flash column chromatography (petroleum ether 40–60/dichloromethane, 1:3 to 1:1) to afford aldehyde A2 (1.68 g, 4.7 mmol, 100%). \textit{^1H NMR} (400 MHz, CDCl\textsubscript{3}) \(\delta\) 9.43 (d, \(J = 7.8\) Hz, 1H), 7.45 (dd, \(J = 7.8, 1.8\) Hz, 6H), 7.30 (dd, \(J = 8.5, 6.7\) Hz, 6H), 7.26 – 7.20 (m, 3H), 6.63 (dt, \(J = 15.6, 6.4\) Hz, 1H), 5.99 (ddt, \(J = 15.7, 7.9, 1.4\) Hz, 1H), 2.34 (dd, \(J = 22.3, 6.5, 2.0\) Hz, 4H); \textit{^13C NMR} (100 MHz, CDCl\textsubscript{3}) \(\delta\) 193.82, 155.87, 144.63, 133.67, 129.59, 128.05, 128.00, 127.97, 126.86, 67.06, 31.79, 30.08. The preparation followed the reference and the characterisation was in agreement with those reported.\textsuperscript{[270]}  

\textit{3S-Hydroxy-1-(4R-benzyl-2-thioxothiazolidin-3-yl)-7-tritylsulfanylhept-4E-en-1-one (A3)}

![Chemical structure of A3]

To a solution of acetyl Nagao chiral auxiliary (2.07 g, 8.24 mmol, 1.7 equiv.) in dichloromethane (42 mL) at 0 °C was added TiCl\textsubscript{4} (1.0 mL, 9.04 mmol, 1.9 equiv.). After the reaction mixture was stirred for 5 min and cooled to 78 °C, DIPEA (1.6 mL, 9.06 mmol, 1.9 equiv.) was added. The mixture was stirred for 2 h at the same temperature, and to it was added the aldehyde A2 (1.74 g, 4.85 mmol, 1.0 equiv.) in dichloromethane (5 mL) dropwise. The reaction mixture was stirred for 1 h at 78 °C. It was removed from cooling bath, treated with water (15 mL), and diluted with dichloromethane (50 mL). The aqueous portion was
extracted with dichloromethane; the organic layer was washed with saturated NaCl (40 mL) and dried over anhydrous Na$_2$SO$_4$. It was concentrated under reduced vacuum and the residue was purified by flash column chromatography (petroleum ether 40–60/dichloromethane, 1:2 to 1:3) to obtain the compound A3 (1.79 g, 2.94 mmol, 60%) as a thick yellow oil. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.43 – 7.38 (m, 6H), 7.37 – 7.32 (m, 2H), 7.31 – 7.27 (m, 9H), 7.23 – 7.16 (m, 3H), 5.60 (dtd, $J = 15.5, 6.6, 1.2$ Hz, 1H), 5.48 (ddt, $J = 15.5, 6.0, 1.3$ Hz, 1H), 5.36 (ddd, $J = 10.7, 7.0, 3.9$ Hz, 1H), 4.63 – 4.55 (m, 1H), 3.56 (dd, $J = 17.6, 3.0$ Hz, 1H), 3.37 – 3.26 (m, 2H), 3.21 (dd, $J = 13.2, 3.9$ Hz, 1H), 3.03 (dd, $J = 13.1, 10.5$ Hz, 1H), 2.86 (d, $J = 11.6$ Hz, 1H), 2.69 (d, $J = 4.6$ Hz, 1H), 2.22 (dd, $J = 7.7, 6.2$ Hz, 2H), 2.10 (q, $J = 7.3$ Hz, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 201.29, 172.55, 144.85, 136.37, 131.90, 130.09, 129.63, 129.57, 129.43, 128.93, 127.85, 127.28, 126.58, 68.39, 68.33, 66.56, 45.58, 36.75, 32.07, 31.44, 31.35. The preparation followed the reference and the characterisation was in agreement with those reported.$^{[270]}$

$(3S,4E)$-3-Hydroxy-7-[(triphenylmethyl)thio]-4-heptenoic acid (2-trimethylsilyl)ethyl ester (A4)

To a solution of thiazoline-thione A3 (0.21 g, 0.34 mmol, 1.0 equiv.) was dissolved in 5 mL dichloromethane was added 2-trimethylsilyethanol (0.49 mL, 3.3 mmol, 10.0 equiv.), followed by imidazole (35 mg, 0.51 mmol, 1.5 equiv.). The resulting solution was stirred overnight, when TLC revealed complete disappearance of starting material A3. The reaction mixture was concentrated under reduced vacuum and submitted purified by flash column chromatography (petroleum ether 40–60/ethyl acetate, 8:1) to provide compound A4 (0.17 g, 0.32 mmol, 93%) as a clear oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.46 – 7.40 (m, 6H), 7.32 – 7.27 (m, 6H), 7.26 – 7.19 (m, 3H), 5.59 (dtd, $J = 14.4, 6.6, 1.2$ Hz, 1H), 5.43 (ddt, $J = 15.4, 6.2, 1.3$ Hz, 1H), 4.51 – 4.39 (m, 1H), 4.27 – 4.12 (m, 2H), 2.90 (d, $J = 4.0$ Hz, 1H), 2.49 (d, $J = 4.3$ Hz, 1H), 2.22 (dd, $J = 8.4, 7.1$ Hz, 2H), 2.09 (q, $J = 7.2$ Hz, 2H), 1.07 – 0.94 (m, 2H), 0.05 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 201.29, 172.55, 144.85, 136.37, 131.91, 130.16, 129.57, 127.85, 126.59, 68.58, 66.57, 63.08, 41.48, 31.43, 31.35, 17.31, –1.50. The preparation followed the reference and the characterisation was in agreement with those reported.$^{[267]}$
(S,E)-2-(Trimethylsilyl)ethyl-3-(((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanoyl)oxy)-7-(tritylthio)hept-4-enoate (AB1)

To a solution of compound A4 (1.0 g, 1.93 mmol, 1.0 equiv.) in dichloromethane (48 mL) at room temperature was added N-Fmoc-L-valine (3.3 g, 9.64 mmol, 5 equiv.), EDCI–HCl (2.2 g, 11.6 mmol, 6.0 equiv.), DMAP (23.6 mg, 0.19 mmol, 0.1 equiv.), and DIPEA (2.0 mL, 11.6 mmol, 6.0 equiv.). After stirring for 18 h, the reaction mixture was concentrated. The crude residue was purified by flash column chromatography (petroleum ether 40–60/ethyl acetate, 20:1 to 5:1) to provide compound AB1 (1.16 g, 1.38 mmol, 72%) as a clear oil. ^1H NMR (400 MHz, CDCl3) δ 7.77 (dq, J = 7.7, 1.1 Hz, 2H), 7.61 (dd, J = 7.5, 3.1 Hz, 2H), 7.45–7.15 (m, 19H), 5.81–5.52 (m, 2H), 5.48–5.26 (m, 2H), 4.53–4.32 (m, 2H), 4.31–4.15 (m, 4H), 2.68 (dd, J = 15.8, 7.9 Hz, 1H), 2.55 (dd, J = 15.9, 5.7 Hz, 1H), 2.20 (tdd, J = 9.8, 7.0, 1.7 Hz, 3H), 2.11–1.99 (m, 2H), 1.02–0.90 (m, 5H), 0.81 (d, J = 6.9 Hz, 3H), 0.04 (d, J = 9.4 Hz, 9H). The preparation followed the reference and the characterisation was in agreement with those reported.[271-272]

(3S,4E)-2-(Trimethylsilyl)ethyl-3-((S)-2-(((R)-2-[[(tert-butoxycarbonyl)methyl]thiazol-4-yl]-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyloxy)-7-(tritylthio)hept-4-enoate (ABC1)

To a solution of Fmoc-protected amine AB1 (1.16 g, 1.38 mmol, 1.0 equiv.) in acetonitrile (70 mL) at room temperature was added diethylamine (7.0 mL). After 2 h, the reaction mixture was concentrated under reduced vacuum, taken back up in ethyl acetate (35 mL), and concentrated again. In a separate flask, acid C4 (542 mg, 1.52 mmol, 1.1 equiv.), PyBOP
(1.45 g, 2.76 mmol, 2.0 equiv.), and DIPEA (0.72 mL, 4.14 mmol, 3.0 equiv.) was combined in dichloromethane (25 mL). The freshly deprotected amine was added via acetonitrile (12.5 mL) to the flask containing the activated acid at room temperature. After 3 h, the resulting mixture was concentrated under reduced pressure. The crude residue was purified by flash column chromatography (petroleum ether 40–60/ethyl acetate, 4:1 to 2:1) to provide amide ABC1 (1.13 g, 1.19 mmol, 86%) as a clear oil. 

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.90 (s, 1H), 7.40 – 7.36 (m, 6H), 7.30 – 7.15 (m, 9H), 5.74 – 5.56 (m, 2H), 5.37 (ddt, $J = 15.4, 7.6, 1.4$ Hz, 1H), 5.31 – 5.27 (m, 1H), 4.62 (d, $J = 6.5$ Hz, 2H), 4.48 (dd, $J = 9.0, 4.7$ Hz, 1H), 4.20 – 4.13 (m, 2H), 3.77 (d, $J = 11.4$ Hz, 1H), 3.32 (d, $J = 11.5$ Hz, 1H), 2.68 (dd, $J = 15.7, 7.8$ Hz, 1H), 2.55 (dd, $J = 15.7, 5.7$ Hz, 1H), 2.18 – 2.04 (m, 5H), 1.57 (s, 3H), 1.47 (s, 9H), 1.00 – 0.93 (m, 2H), 0.82 (d, $J = 6.8$ Hz, 3H), 0.74 (d, $J = 6.9$ Hz, 3H), 0.03 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 174.43, 170.37, 169.66, 155.63, 148.66, 144.82, 133.91, 129.55, 128.03, 127.86, 127.77, 127.68, 126.60, 121.40, 121.35, 85.16, 80.46, 71.77, 66.59, 63.13, 56.79, 42.33, 41.50, 39.71, 31.30, 31.21, 31.16, 31.07, 28.33, 24.75, 19.05, 17.46, 17.28, −1.49. The preparation followed the reference and the characterisation was in agreement with those reported.[267, 271]

(5R,8S,11S)-5-Methyl-8-(1-methylethyl)-11-[(1E)-4-[(triphenylmethyl)thio]-1-buten-1-yl]-10-oxa-3,17-dithia-7,14,19,20-tetraazatricyclo[14.2.1.1$^{2,5}$]eicosa-2(20),16(19),18-triene-6,9,13-trione (ABC3)

To a solution of linear precursor ABC1 (0.10 g 0.11 mmol, 1.0 equiv.) in dichloromethane (5 mL) at 0 °C was added TFA (1 mL). The reaction was allowed to warm to room temperature and stirred overnight. Solvents were evaporated, and the crude amino acid was taken up in toluene and concentrated a second time to remove residual TFA. The crude amino acid was then taken up in 5 mL dichloromethane and added dropwise to a stirred solution of DIPEA (0.122 mL, 0.70 mmol, 6.0 equiv.) in dry acetonitrile (100 mL). The resulting moderately opaque solution was stirred for 10 min, before HATU (84.0 mg, 0.22 mmol, 2.0 equiv.) and HOBr (30.0 mg, 0.22 mmol, 2.0 equiv.) were added dropwise in 5 mL acetonitrile. The reaction was allowed to stir for 16 h, then concentrated and submitted immediately to flash
column chromatography (petroleum ether 40–60/ethyl acetate, 9:1 to ethyl acetate) to afford the macrocycle **ABC3** (44.1 mg, 0.06 mmol, 55%) as a clear oil. **1H NMR** (500 MHz, CDCl$_3$) δ 7.74 (s, 1H), 7.42 – 7.36 (m, 6H), 7.31 – 7.23 (m, 6H), 7.23 – 7.16 (m, 4H), 6.54 (dd, $J$ = 9.2, 3.3 Hz, 1H), 5.72 (ddt, $J$ = 15.1, 6.8, 1.1 Hz, 1H), 5.67 – 5.59 (m, 1H), 5.41 (ddt, $J$ = 15.5, 6.5, 1.5 Hz, 1H), 5.20 (dd, $J$ = 17.5, 9.2 Hz, 1H), 4.56 (dd, $J$ = 9.4, 3.7 Hz, 1H), 5.67 – 5.59 (m, 1H), 5.41 (ddt, $J$ = 15.5, 6.5, 1.5 Hz, 1H), 4.56 (dd, $J$ = 9.4, 3.7 Hz, 1H), 4.12 (d, $J$ = 14.3 Hz, 1H), 4.03 (d, $J$ = 11.3 Hz, 1H), 3.27 (d, $J$ = 11.3 Hz, 1H), 2.79 (dd, $J$ = 16.2, 9.5 Hz, 1H), 2.66 (dd, $J$ = 16.2, 3.2 Hz, 1H), 2.29 – 2.15 (m, 2H), 2.13 – 1.97 (m, 3H), 1.84 (s, 3H), 0.69 (d, $J$ = 6.9 Hz, 3H); 13C NMR (125 MHz, CDCl$_3$) δ 173.49, 169.29, 168.73, 167.88, 164.43, 147.48, 144.76, 133.05, 129.55, 127.95, 127.88, 126.62, 124.03, 84.42, 71.76, 66.60, 57.84, 43.29, 40.99, 40.60, 34.00, 31.35, 31.21, 24.23, 18.84, 16.76. The preparation followed the reference and the characterisation was in agreement with those reported.[267]

(5R,8S,11S)-11-[(1E)-4-Mercapto-1-buten-1-yl]-5-methyl-8-(1-methylethyl)-10-oxa-3,17-dithia-7,14,19,20-tetraazatricyclo[14.2.1.1$^{2,5}$]eicosa-2(20),16(19),18-triene-6,9,13-trione (largazole thiol)

The S-trityl macrocycle **ABC3** (40.0 mg, 0.05 mmol, 1.0 equiv.) was dissolved in dry dichloromethane (7 mL) and cooled to 0 °C. The mixture was successively treated with iPr$_3$SiH (22.2 μL, 0.10 mmol, 2.0 equiv.) and TFA (0.27 mL, to 0.2 M in **ABC3**). The reaction mixture was allowed to warm to room temperature and stirred for 1 h, before being concentrated and purified by flash column chromatography (ethyl acetate) to provide **largazole thiol** (17.2 mg, 0.03 mmol, 64%) as a clear oil. **1H NMR** (400 MHz, CDCl$_3$) δ 7.76 (s, 1H), 7.18 (d, $J$ = 9.4 Hz, 1H), 6.50 (d, $J$ = 8.8 Hz, 1H), 5.83 (ddt, $J$ = 15.0, 6.9, 1.0 Hz, 1H), 5.72 – 5.64 (m, 1H), 5.54 (ddt, $J$ = 15.5, 6.8, 1.4 Hz, 1H), 5.27 (dd, $J$ = 17.6, 9.3 Hz, 1H), 4.60 (dd, $J$ = 9.5, 3.5 Hz, 1H), 4.28 (dd, $J$ = 17.6, 3.2 Hz, 1H), 4.04 (d, $J$ = 11.3 Hz, 1H), 3.28 (d, $J$ = 11.4 Hz, 1H), 2.86 (dd, $J$ = 16.3, 10.0 Hz, 1H), 2.71 (dd, $J$ = 16.3, 3.0 Hz, 1H), 2.61 – 2.51 (m, 2H), 2.41 – 2.31 (m, 2H), 2.15 – 2.05 (m, 1H), 1.86 (s, 3H), 0.70 (d, $J$ = 6.9 Hz, 3H), 0.53 (d, $J$ = 6.9 Hz, 3H); HRMS (ESI+) m/z: calcd. for C$_{21}$H$_{29}$N$_4$O$_4$S$_3$ [M+H]$^+$: 497.1351 found: 497.1331. The
preparation followed the reference and the characterisation was in agreement with those reported.\(^{267}\)

\(S-[(E)-4-[5R,8S,11S]-8-\text{Isopropyl-5-methyl-6,9,13-trioxo-10-oxa-3,17-dithia-7,14,19,20-tetraazatricyclo[14.2.1.1^{2,5}]eicosa-1(18),2(20),16(19)-trien-11-yl]but-3-en-1-yl}]\)octanethioate (largazole)

To a stirred solution of largazole thiol (14.9 mg, 0.03 mmol, 1.0 equiv.) in dichloromethane (4 mL) at 0 °C were added triethylamine (8.4 μL, 0.06 mmol, 2.0 equiv.) and octanoyl chloride (25.6 μL, 0.15 mmol, 5.0 equiv.). After the mixture was stirred for 3 h at room temperature, the reaction was quenched with methanol at 0 °C. Then the mixture was concentrated under reduced vacuum and purified by flash column chromatography (ethyl acetate) to provide amide largazole (8.5 mg, 0.01 mmol, 45%) as a clear oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.76 (s, 1H), 7.16 (d, \(J = 9.3\) Hz, 1H), 6.43 (d, \(J = 9.2\) Hz, 1H), 5.83 (dt, \(J = 14.2, 6.8\) Hz, 1H), 5.66 (t, \(J = 8.3\) Hz, 1H), 5.52 (dd, \(J = 15.5, 6.9\) Hz, 1H), 5.28 (dd, \(J = 17.6, 9.4\) Hz, 1H), 4.61 (dd, \(J = 9.4, 3.4\) Hz, 1H), 4.27 (dd, \(J = 17.6, 3.2\) Hz, 1H), 4.05 (d, \(J = 11.3\) Hz, 1H), 3.28 (d, \(J = 11.4\) Hz, 1H), 2.90 (t, \(J = 7.2\) Hz, 2H), 2.83 (d, \(J = 10.4\) Hz, 1H), 2.69 (dd, \(J = 16.3, 2.9\) Hz, 1H), 2.57 – 2.50 (m, 2H), 2.31 (q, \(J = 7.0\) Hz, 2H), 2.11 (ddt, \(J = 10.3, 6.9, 3.4\) Hz, 1H), 1.87 (s, 3H), 1.76 – 1.50 (m, 3H), 1.35 – 1.23 (m, 7H), 0.92 – 0.84 (m, 3H), 0.69 (d, \(J = 6.9\) Hz, 3H), 0.52 (d, \(J = 6.8\) Hz, 3H); HRMS (ESI+) m/z: calcd. for \(C_{29}H_{45}N_3O_5S_3\) [M+H]\(^+\): 623.2396 found: 623.2426. The preparation followed the reference and the characterisation was in agreement with those reported.\(^{273}\)
**Synthesis of stapled largazole**

[Largazole thiol](9.0 mg, 0.018 mmol, 2.0 equiv.) and potassium carbonate (6.3 mg, 0.045 mmol, 5.0 equiv.) were dissolved in DMF (1 mL). 3-Bromo-2-bromomethyl-1-propene 1 (0.52 µL, 0.005 mmol, 0.5 equiv.) was added to the stirred solution. After being stirred at room temperature for 2 h, the same amount of 3-bromo-2-bromomethyl-1-propene 1 was added and stirred for another 5 h. After this, the reaction was diluted with Et₂O (10 mL) and washed sequentially with a saturated solution of LiBr (10 mL) and brine (10 mL). The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (ethyl acetate) and HPLC to provide the **stapled largazole** (6.5 mg, 0.62 mmol, 69%) as white solid. HPLC: Rt = 24.285 min (Grad: water 0.1% TFA/acetonitrile (80:20) → (10:90), 30 min, λ = 254 nm). ¹H NMR (500 MHz, CDCl₃) δ 7.76 (s, 2H), 7.18 (d, J = 9.5 Hz, 2H), 6.57 (dd, J = 9.4, 3.3 Hz, 2H), 5.87 (dd, J = 14.6, 7.7 Hz, 2H), 5.73 – 5.63 (m, 2H), 5.27 (dd, J = 17.6, 9.3 Hz, 2H), 4.97 (s, 2H), 4.60 (dd, J = 9.4, 3.5 Hz, 2H), 4.25 (dd, J = 17.6, 3.3 Hz, 2H), 4.03 (d, J = 11.4 Hz, 2H), 3.32 – 3.15 (m, 6H), 2.86 (dd, J = 16.3, 10.0 Hz, 2H), 2.71 (dd, J = 16.3, 3.0 Hz, 2H), 2.45 (t, J = 7.2 Hz, 4H), 2.31 (q, J = 6.9 Hz, 4H), 2.10 (pd, J = 6.9, 3.6 Hz, 2H), 1.86 (s, 6H), 0.69 (d, J = 6.9 Hz, 6H), 0.52 (d, J = 6.8 Hz, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 173.71, 169.50, 169.03, 168.10, 164.69, 162.67, 147.63, 140.88, 133.39, 128.08, 124.27, 115.74, 84.58, 72.14, 57.94, 43.48, 41.26, 40.67, 36.63, 35.56, 34.29, 32.06, 31.59, 30.50, 29.85, 24.40, 19.06, 16.83; HRMS (ESI+) m/z: calcd. for C₄₆H₆₁N₅O₅S₆ [M+H]⁺: 1045.2931 found: 1045.2901.
Figure 5.47 HPLC chromatogram at 254 nm of stapled largazole.

**Unstapling of stapled largazole**

The **stapled largazole thiol** (4.0 mg, 3.8 µmol, 1.0 equiv.) was dissolved in DMF (1.5 mL) at room temperature. The DPAP (7 µL, 0.05 M in DMF, 0.38 µmol, 0.1 equiv.) and 4AcGlcSH (120 µL, 0.1 M in DMF, 11.5 µmol, 3.0 equiv.) was added to the solution. The mixture was degassed with argon, followed by irradiation with UV light for 30 min. After being stirred in air for 10 min, TCEP·HCl (5.2 mg, 20.9 µmol, 6.0 equiv.) was added to the solution. After 1 h, the solvent was removed under reduced pressure and purified by HPLC to afford the **largazole thiol** (3.0 mg, 3.0 µmol, 79%) as a white solid. HPLC: Rt = 17.931 min (Grad: water 0.1% TFA/acetonitrile (80:20) → (10:90), 30 min, λ = 254 nm). HRMS (ESI+) m/z: calcd. for C_{21}H_{29}N_{4}O_{4}S_{3} [M+H]^{+}: 497.1351 found: 497.1331.
Figure 5.48 HPLC chromatogram at 254 nm of unstacking reaction.

5.4.7. Cell culture

HCT-116 (ATCC® CCL-247™), kindly provided by Dr Marco Di Antonio (Department of Chemistry, University of Cambridge, UK), was maintained at 37 °C humidified air, 5% CO₂, and assayed in McCoy’s 5A (Modified) Medium (Gibco™, Thermo Fisher Scientific) supplemented with 10% FBS (Gibco™, Thermo Fisher Scientific), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco™, Thermo Fisher Scientific). The cells were split before reaching confluence with 0.05% Trypsin-EDTA (Gibco™, Thermo Fisher Scientific).

5.4.8. Cell viability assay

HCT-116 cells suspended in McCoy’s 5A medium were plated in 96-well plates (200 μL, 2500 cells/well), incubated (37 °C, 5% CO₂), and 24 h later treated with various concentrations of compounds or solvent control (0.25% DMSO). After another 48 h of incubation, by adding 20 μL CellTiter-Blue® solution to each well and incubating 6 h. Fluorescence was then measured (Ex/Em = 555 nm / 585 nm), using the SpectraMax® i3x Multi-Mode Microplate Reader. Cell viability was calculated according to the manufacturer’s instructions (Promega).

5.4.9. Photoactivation of stapled largazole in HCT-116 cells

Scheme 5.1 The photoactivation of stapled largazole in HCT-116 cells.
HCT-116 cells were plated in 200 μL of medium per well at a density of 2,500 cells/well and grown for 24 h in two sterile 96-well solid bottom plates. incubated (37 °C, 5% CO₂). After 24 h, the cells were treated with various compounds (largazole, largazole Thiol and stapled largazole at 150 nM, DPAP at 1.5 μM) or solvent control (0.25% DMSO). Then the UV group plate were irradiated with UVA light (365 nm, 80 W) for 15 min. After another 48 h of incubation, the cell viability was measured according to the general method above.

5.4.10. Fluorometric histone deacetylase (HDAC) activity assay

Following the measurement of the cell viability, the plates were washed with cold PBS for three times. Then, the whole-cell lysates were prepared on ice using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific™). The activity assay was carried out with the cell lysates following the manufacturer’s instruction (Abcam). Briefly, 20 μL ddH₂O, 5 μL HDAC Assay Buffer and 5 μL Substrate Peptide were added to 96-well plate. Then the reactions were initiated by adding 20 μL cell lysates or buffer for no enzyme control to each well and mixed thoroughly at 25 °C. After being incubated for 20 min, 20 μL of Stop Solution was added to each well and mixed thoroughly, followed by 5 μL of Developer solution. After being incubated for another 30 min at 25 °C, the fluorescence intensity was read at Ex/Em = 355 nm / 460 nm.

![Figure 5.49 RIPA buffer control with HDAC activity kit.](image-url)
5.4.11. Unstapling the isobutylene-grafted protein conjugates

*Installing the electrophilic handle on protein*

A 25 μL aliquot of a solution of Ub-K63C in 50 mM NaPi buffer pH 8.0 (100 μM, 2.5 nmol) was added to a 1.5 mL Eppendorf containing 68.8 μL of the same buffer. To this solution was added 6.25 μL of a solution of 3-bromo-2-bromomethyl-1-propene 1 (20 mM in DMF, 125 nmol, 50 equiv.). The reaction mixture was vortexed briefly, then incubated at 4 °C for 6 h. At the end of the reaction, a 10 μL aliquot was analysed by LC–MS. The major product formed was Ub-iso-Br (calculated mass: 8700; observed mass: 8700). Small molecules were removed from the reaction mixture by loading the sample onto a Zeba™ spin desalting column (7 kDa MWCO, Thermo Scientific) pre-equilibrated with 50 mM NaPi buffer pH 9.0. The sample was eluted by centrifugation (2 min, 1500 × g). (The Ub-K63C was expressed and purified by Dr Ester Jiménez-Moreno.)
**Figure 5.50** LC–MS data and processing of the native ubiquitin. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 5.037–5.625 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
**Figure 5.51** LC–MS data and processing of the Ub-iso-Br. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 6.965–8.643 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

A 50 μL aliquot of a solution of C2Am in 50 mM NaPi buffer pH 9.0 (20 μM, 1 nmol) was added to a 1.5 mL Eppendorf containing 49.5 μL of the same buffer. To this was added 0.5 μL of a solution of TCEP·HCl (100 mM in H2O, 50 nmol, 50 equiv.). The reaction mixture was vortexed briefly, then shaken at 25 °C for 45 min. Then, 2.5 μL of a solution of 3-bromo-2-bromomethyl-1-propene 1 (40 mM in DMF, 100 nmol, 100 equiv.) was then added and the resulting mixture was vortexed briefly. After 1 h of additional shaking at 25 °C, a 10 μL aliquot
was analysed by LC–MS. The expected conversion to C2Am-iso-Br was observed (calculated mass: 16356; observed mass: 16356). Small molecules were removed and buffer exchange into 50 mM NaPi buffer pH 9.0 using Vivaspin 500 protein concentrators (5 kDa MWCO, GE Healthcare). (The C2Am was expressed and purified by Dr André A. Neves.)

**Figure 5.52** LC–MS data and processing of the native C2Am. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 2.853–3.381 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Figure 5.53 LC–MS data and processing of the C2Am-iso-Br. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 6.651–7.351 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

Conjugating with nucleophiles

A 20 µL aliquot of a solution of C2Am-iso-Br (10 µM in 50 mM NaPi buffer, pH 9.0, 0.2 nmol) was added to a 0.5 mL Eppendorf. To this was added 1 µL of a solution of β-D-thioglucose sodium salt (20 mM in H₂O, 20 nmol, 100 equiv.). The reaction mixture was vortexed briefly, then shaken at 25 °C for 30 min. At the end of the reaction, a 10 µL aliquot was analysed by
LC-MS. The expected conversion to C2Am-iso-Glu was observed (calculated mass: 16471; observed mass: 16471). Small molecules were removed and buffer exchange into 1× PBS buffer pH 7.4 using Vivaspin 500 protein concentrators (5 kDa MWCO, GE Healthcare).

**Figure 5.54** LC–MS data and processing of the C2Am-iso-Glu. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 2.912–3.753 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
Unstapling the protein conjugates

To a solution of C2Am or ubiquitin conjugates in 1× PBS buffer (20 μL, 20 μM) were added lithium phenyl-2,4,6-trimethylbenzoylphosphinate in H₂O (2 μL, 20 mM, 100 equiv.) and GSH in H₂O (2 μL, 200 mM, 1000 equiv.). The reaction mixture was vortexed briefly, followed by irradiated with UV light for 15–30 min at room temperature. At the end of the reaction, a 10 μL aliquot was analysed by LC–MS.

Figure 5.55 LC–MS data and processing of the decaging of the Ub-iso-Br. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time Calcd. Mass: 8569, Observed Mass: 8569; c)
range: 5.121–6.716 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.

**Figure 5.56** LC–MS data and processing of the decaging of the C2Am-iso-Glu. a) Total ion count trace of liquid chromatography step; b) the peak corresponding to protein ions (retention time range: 6.968–9.319 min) is combined and reported as m/z; c) deconvolution of the m/z charge spectrum identifies the observed mass.
APPENDICES (NMR SPECTRA)

$^1$H NMR of 3

$^{13}$C NMR of 3
Appendices (NMR spectra)

COSY NMR of 3

$^1$H NMR of GlcN3
$^1$H NMR of NAGSH

$^1$H NMR of GlcN4
**1H NMR of 5**

![1H NMR spectrum of 5](image1)

**13C NMR of 5**

![13C NMR spectrum of 5](image2)
Appendices (NMR spectra)

COSY NMR of 5

HSQC NMR of 5
Appendices (NMR spectra)

HMBC NMR of 5

1H NMR of 6
$^{13}$C NMR of 6

COSY NMR of 6
Appendices (NMR spectra)

HSQC NMR of 6

HMBC NMR of 6
$^1$H NMR of C2

$^{13}$C NMR of C2
COSY NMR of C2

HSQC NMR of C2
$^1$H NMR of C3

$^{13}$C NMR of C3
COSY NMR of C3

1H NMR of C4
$^{13}$C NMR of C4

COSY NMR of C4
$^1$H NMR of **auxiliary**

$^{13}$C NMR of **auxiliary**
Appendices (NMR spectra)

$^1$H NMR of A2

![NMR spectrum of A2](Image)

$^{13}$C NMR of A2

![NMR spectrum of A2](Image)
\(^1\)H NMR of \(\text{A3}\)

\(^{13}\)C NMR of \(\text{A3}\)
Appendices (NMR spectra)

$^1$H NMR of A4

$^{13}$C NMR of A4
COSY NMR of A4

\[ \text{Diagram of COSY NMR of A4} \]

\[ \text{Diagram of } ^1H \text{ NMR of AB1} \]

Appendices (NMR spectra)
Appendices (NMR spectra)

$^1$H NMR of ABC1

![Image of $^1$H NMR spectrum of ABC1]

$^{13}$C NMR of ABC1

![Image of $^{13}$C NMR spectrum of ABC1]
COSY NMR of ABC1

1H NMR of ABC3
Appendices (NMR spectra)

$^{13}$C NMR of **ABC3**

![COSY NMR of ABC3](image)

COSY NMR of **ABC3**
1H NMR of largazole thiol

1H NMR of largazole
\(^1\)H NMR of stapled largazole

\[\text{Appendices (NMR spectra)}\]

\[^{13}\text{C NMR of stapled largazole}\]
Appendices (NMR spectra)

COSY NMR of **stapled largazole**

[Image: COSY NMR spectrum of stapled largazole]

HSQC NMR of **stapled largazole**

[Image: HSQC NMR spectrum of stapled largazole]
HMBC NMR of **stapled largazole**
REFERENCES


[9] M. D. A. C. Center, N. C. Institute, [https://ClinicalTrials.gov/show/NCT03725436](https://ClinicalTrials.gov/show/NCT03725436), **2019**.


References


