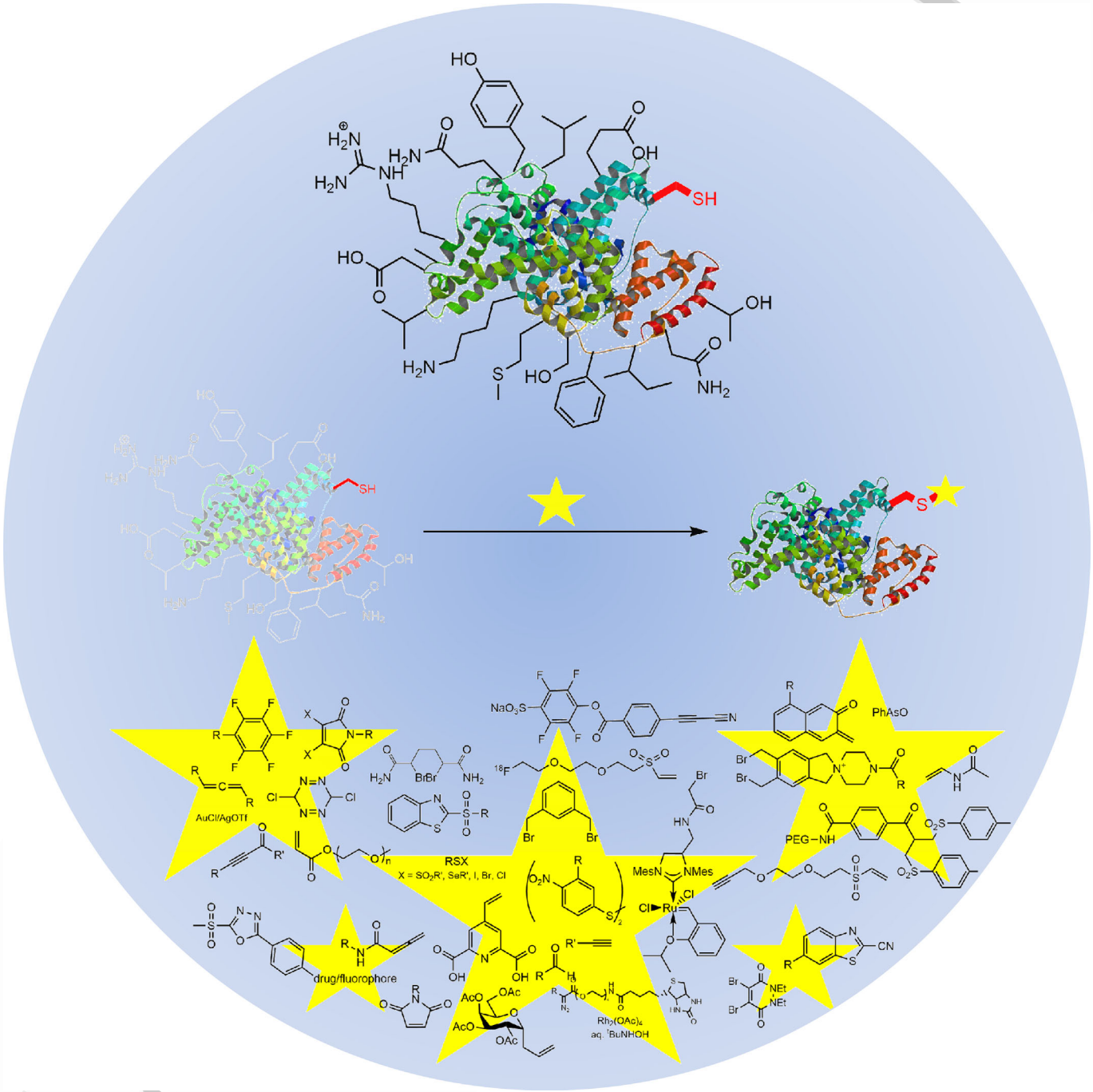
ChemPubSoc Europe
DOI: 10.1002/cbic.201500667
CHEMBIOCHEM Reviews

Chemical Protein Modification through Cysteine

Smita B. Gunnoo and Annemieke Madder*[a]



The modification of proteins with non-protein entities is important for a wealth of applications, and methods for chemically modifying proteins attract considerable attention. Generally, modification is desired at a single site to maintain homogeneity and to minimise loss of function. Though protein modification can be achieved by targeting some natural amino acid side chains, this often leads to ill-defined and randomly modified proteins. Amongst the natural amino acids, cysteine combines advantageous properties contributing to its suitability for site-selective modification, including a unique nucleophilicity, and a low natural abundance—both allowing chemo- and regioselectivity. Native cysteine residues can be targeted, or Cys can be introduced at a desired site in a protein by means of reliable genetic engineering techniques. This review on chemical protein modification through cysteine should appeal to those interested in modifying proteins for a range of applications.

1. Introduction

Our bodies are able and required to modify proteins post-translationally with a wealth of moieties both site-selectively and site-specifically for a myriad of essential processes including cell growth, repair and death. The ability to mimic these processes ■■outside of an in vivo environment *"ex vivo"?*■■ allows researchers to ■■recapitulate *"mimic"?*■■ nature, to explore biosynthetic pathways, to manipulate and to decipher natural protein function, and to attach affinity or fluorescent tags for imaging. Chemical protein modification contributes to the enhancement of therapeutic properties,[1,2] as well as in diagnostic applications in, for example, surface immobilisation for biosensor design.[3] It is important not to underestimate the potential involved in the marrying of two or more bio-macromolecular and/or small-molecule functionalities to form structurally and functionally diverse bioconjugates.

Proteins, as an example of a class of bio-macromolecules, are robust and powerful entities; however, their structure and function can be sensitive to general conditions employed in conventional organic syntheses. These include raised temperatures and the use of organic solvent, two factors likely to perturb protein structure. As well as ambient reaction conditions, high conversion rates and minimal generation of side products are desired, in order to avoid tedious purification steps. Modification at a single site is therefore generally preferred, to avoid heterogeneity.

Existing methods for chemical protein modification include the use of naturally occurring amino acids such as lysine, serine, histidine and tyrosine,[4–6] and also of unnatural amino acids (UAAs), incorporated by use of genetically engineered bacterial strains[7,8] and/or enzymatic methods.[9] These methods although useful, do have drawbacks.

The use of naturally occurring amino acids does allow the avoidance of demanding genetic engineering. Although lysine modification is commonly used and facile, the residue's high level of occurrence in proteins means that multiple sites can be modified in an unpredictable manner. This leads to heterogeneity and/or the blocking of functional sites by the newly introduced group. Site-specificity during endogenous amino acid modification can be promoted by exploiting the differential reactivity of the N terminus, for example,[10] or by the use of enzyme recognition sequences.[11] Overall, however, the use of most endogenous amino acids for modification purposes is difficult to apply in a general manner due to higher levels of occurrence, leading to modification of multiple sites.

A wealth of UAAs can be incorporated into proteins by use of the amber suppression methods pioneered by Schultz and Chin.[7,12,13] Such UAAs include photocaged cysteine residues.[14,15] The methodology, based on incorporating orthogonally reactive functionalities, is very elegant and a sure-fire way to ensure reactivity at a single site. Yet it can be more time-consuming than the use of endogenous amino acids. Alternatively, unnatural tags can be incorporated through chemoenzymatic methods.[9] Here, site-specific reactivity is guaranteed, but the use of enzymes is more resource-dependent.

It can be argued that modification through cysteine residues combines the advantages of the approaches described above. The presence of multiple accessible free cysteine residues within a protein is likely to result in multi-site modification, but cysteine has a low natural abundance, which means that selectivity on a general level is more likely. In addition, cysteine residues can be readily introduced by use of facile mutagenesis techniques prior to expression, allowing control over the site of modification. The ease of incorporation and associated expression yields are specifically dependent on the protein's structure and behaviour. Another characteristic of cysteine is that it has a unique nucleophilicity, in comparison with other reactive side chains. Under physiological conditions, it has a high propensity to form the nucleophilic thiolate ion with a general pK_a of 8.2. This, though, is highly dependent on its conformation and its environment in terms of buffer and neighbouring residues, and can be even lower.[16,17] As well as a unique reactivity, cysteine also has an incredibly diverse reactivity profile, as exemplified by the abundance of post-translational modifications (PTMs) it undergoes.[18] It is able to interconvert between multiple oxidation states, often negates the need for co-factors in vivo, and is essential in cellular redox regulation. Cysteine is able to partake in exchange, radical and atom-, electron- and hydride-transfer reactions, and also to bind to metals.[19] Its evolutionary diversity undoubtedly provides inspiration for the protein chemist developing cysteine-based methodology at the bench.

This review focuses on methodologies involving cysteine as a precursor for chemical modification of proteins since 2009,

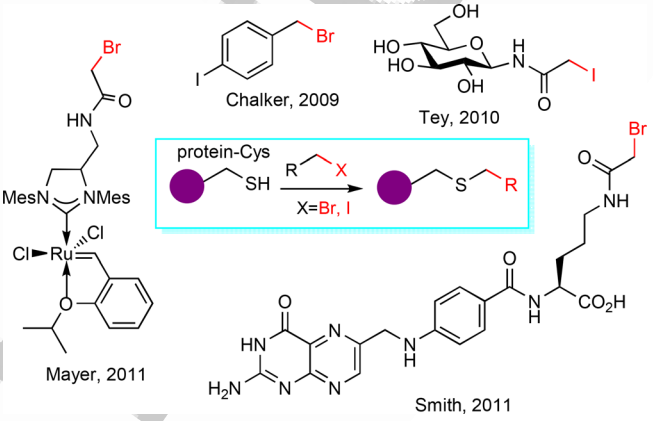
[a] *Dr. S. B. Gunnoo, Prof. Dr. A. Madder*
Organic & Biomimetic Chemistry Research Group
Department of Organic and Macromolecular Chemistry, Ghent University
Krijgslaan 281, 9000, Gent (Belgium)
E-mail: annemieke.madder@ugent.be

when Chalker et al. reviewed the same topic.[20] Since then, there has been exponential growth both in new cysteine-based chemistries and in more applied reports based on existing cysteine methodologies. Native chemical ligation (NCL)[21] is not discussed in great detail, but has been reviewed elsewhere.[22,23] Although NCL is an elegant method for the total synthesis of proteins (ligating peptide fragments together) and has seen many applications, our interests lie in conjugating non-protein moieties to proteins through cysteine residues. We have opted for a systematic review of new developments, classified according to the specific sulfur-based chemistry used.

2. Modification by Substitution

2.1. Alkylation through S_N2 reaction with haloalkyl reagents

One of the earliest methods used for modifying cysteine residues within proteins is by substitution with haloalkyl substrates bearing groups of interest. Iodide and bromide groups in particular are commonly used.[20] Indeed, substitution with iodoacetamide is routinely used to "cap" cysteine residues in protein digestion prior to analysis. Drawbacks associated with haloalkyl substitution are potential crossreactivity with other nucleophilic side chains such as lysine and histidine, and susceptibility of reagents to hydrolysis; hence the need to develop alternative methodologies as outlined in the remainder of this review. However, despite these drawbacks, there are many examples of the use of haloalkyl substituents in alkylating or arylating cysteine residues within proteins over the last few years (Scheme 1). In many cases the common ease and reliability of these reactions means that the need for modification optimisation is potentially minimised, and an application can be realised in a quicker timeframe. One example is the incorporation of the nitrile group into proteins by using benzyl compounds with either bromo or nitrile substituents for the development of infrared probes.[24]



Scheme 1. Examples of reagents used for protein modification through nucleophilic substitution on Cys.

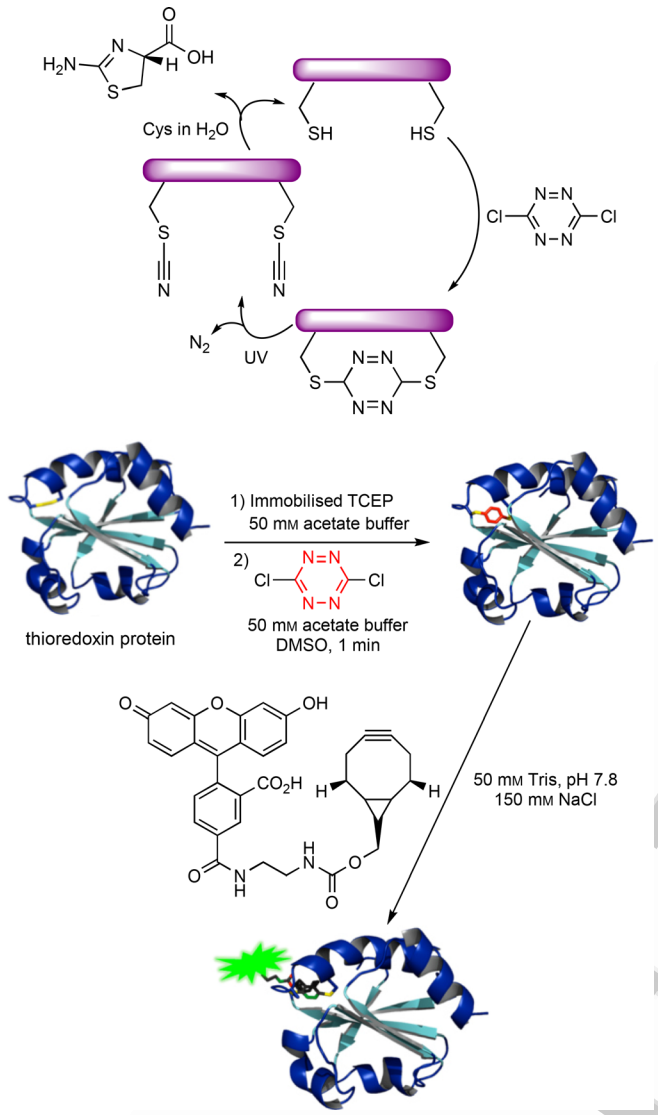
Chalker et al. used cysteine as one component of a precursor incorporating a thioether-linked *para*-iodophenyl moiety as a coupling partner for Suzuki–Miyaura coupling,[25] whereas Mayer et al. modified the Grubbs catalyst with a bromoacetamide group for conjugation to a protein to form a metalloenzyme capable of catalysing a ring-closing metathesis, although further optimisation is required.[26]

The use of folic acid as a targeting moiety in therapeutics is widely known,[27] and a folic acid derivative bearing a bromoalkyl group was conjugated to an engineered cysteine residue in RNase A, leading to a homogenous conjugate capable of targeting cancerous cells without loss of activity.[28] Substitution in this way can also be used as a method for forming homogenous glycoproteins.[29–31]

"Stapling" of residues within peptide chains is known to induce secondary structure, aiding the interaction ability of the peptide with, for instance, protein binding pockets or DNA. So called peptide stapling can be carried out on two proximal cysteine residues with a structurally rigid ■■di-bromoalkyl *"di-bromoalkyl"? "bis(bromoalkyl)"?*■■ linker for protein–protein interaction (PPI) mimicry.[32–34] Stapling can also be applied to proteins, as reported recently with the use of tetrazines bearing chloride groups for cysteine conjugation (Scheme 2). A novel feature of this method is that, post-stapling, further functionalisation can be carried out through inverse-electron-

Smita Gunnoo is a postdoctoral researcher at Ghent University in the laboratory of Prof. Annemieke Madder. During her DPhil (Oxford) she worked on site-specific antibody modification through cysteine modulation under the guidance of Prof. Ben Davis. Prior to this, she carried out her Master's project on the chemical modification of self-assembling peptide fibres under Prof. Dek Woolfson (Bristol). Her research interests now lie in the development and application of protein modification methodologies for therapeutic purposes.

Annemieke Madder studied chemistry at Ghent University, receiving her Ph.D. in 1997 on the development of non-enzymatic hydrolases. After postdoctoral work with Prof. Cesare Gennari (Milan) on synthesising ligands for β-turn mimetics and Prof. Roger Strömberg (Stockholm) on synthesising modified nucleosides targeting RNA bulges, she returned to Ghent, where she was appointed in 2002. She currently leads a group researching the development of methods for crosslinking, labelling and conjugation of bio-macromolecules, design and synthesis of new catalytic systems based on modified oligonucleotide duplexes and construction of conformationally defined multipodal peptide architectures as peptide vaccines, DNA-binding ligands and receptor mimics.



Scheme 2. Peptide stapling by use of tetrazine chloride reagents, together with functionalisation of thioredoxin with a fluorophore post stapling. Thioredoxin (PDB ID: 1XOB).

demand Diels–Alder cycloaddition with a strained cyclooctyne, as demonstrated on thioredoxin protein (Scheme 2). Alternatively, unstapling can be performed photochemically, although this is not the most generally applicable method.[35] Alkylation of maltose-binding protein (MBP) with a ■■chloroacetamide-appended *OK?*■■ fluorophore was achieved by Sunbul et al., followed by proximity-induced binding to a fused fluorophore-binding peptide. The methodology was found to achieve selective labelling of bacterial cells overexpressing the MBP-fluorophore binding peptide fusion.[36]

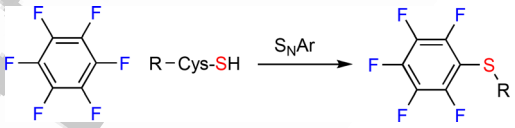
Hamamoto et al. reported a method for spontaneous yet controlled cyclisation, in which an unnatural amino acid bearing a haloalkyl moiety, as well as a suitably positioned cysteine residue, is genetically incorporated into a protein. Hence, post-expression, cyclisation occurs through reaction between the two appropriately placed amino acids.[37]

An impressive display of cysteine's ability to react not only selectively, but also in a plethora of different ways, is shown in the synthesis of diubiquitin probes. Two ubiquitin moieties were conjugated through haloalkyl substitution, through which a Michael addition acceptor (in this case an α,β-unsaturated moiety) was ■■attached for probing purposes to the active-site cysteine residue in deubiquitinases *OK?*■■.[38] DARPins (designed ankyrin repeat proteins) can be dually modified at carefully positioned cysteine residues, as shown by Moody et al., who functionalised these binding proteins by sequential substitution and maleimide coupling.[39]

An example of cysteine alkylation through non-halogen-mediated nucleophilic substitution is the ring opening of aziridines, such as methylthiocarbonyl-aziridine. A series of histone PTM mimics were synthesised and found to be recognised by antibodies raised against the naturally occurring PTMs.[40]

2.2. Perfluoroarylation

Pentelute's laboratory reported nucleophilic aromatic substitution (S_NAr) between cysteine residues in peptides and proteins and perfluoroaromatic molecules (Scheme 3). They stapled a range of peptides, each bearing two cysteine residues, ■■inferring *"conferring"?*■■ increased structural stability, as



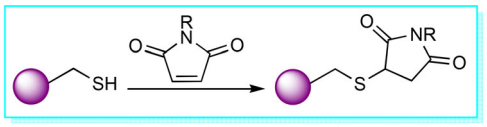
Scheme 3. S_NAr reactions between perfluoroaromatic molecules and cysteine residues in peptides and proteins.

well as a trihelical affibody.[41] This led to enhanced binding ability, cell permeability and overall stability.[42] Although a clear application was achieved in terms of enhancement of the affibody's properties, yields were low, due to poor solubility and reactivity of the perfluoroaromatic reagents. As an alternative, glutathione transferase (GST) was used for catalysing the reaction with a protein containing an N-terminal glutathione motif, allowing site-selectivity in the presence of other cysteine residues.[43] Although some genetic engineering is required for applying the procedure on proteins, the reaction is complete within a matter of seconds. The methodology has also been applied extensively to peptidic systems, particularly in the context of performing macrocyclisations rapidly.[44,45]

3. Maleimides

3.1. ■■C-Unsubstituted■■ maleimides

Maleimides are Michael acceptors, and reaction between a maleimide and a cysteine thiolate forms a thiosuccinimide bond (Scheme 4). The first conjugation reaction between a maleimide and a cysteine residue within a protein was described by Moore and Ward; bis-maleimides were used to cross-link cysteine-containing proteins.[46] Tsao and Bailey also carried out maleimide–thiol coupling in a method for the extraction and



R = fluorescent tags, polymers, radio labels, albumin-binding moieties, metalloenzymes, lipids, drugs

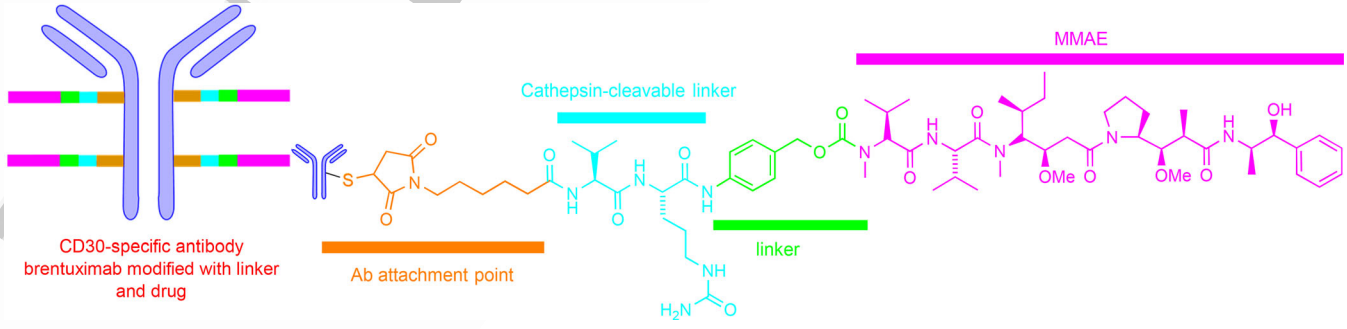
Scheme 4. Cysteine-mediated protein modification with maleimide reagents.

purification of actin and subsequent identification of sulfhydryl content by treatment with *N*-ethylmaleimide (NEM, titration of protein with NEM displacing sodium nitroprusside).[47] They were inspired by Friedmann's investigations into the antimitotic activity of maleimide compounds, which involved treatment of maleimides with glutathione (GSH).[48] The impact that maleimide–thiol coupling would have in the future was understandably not fully appreciated or recognised at this point. Over the next few years after their initial applications, maleimide reagents were developed as blocking reagents for sulfhydryl groups within proteins for analytical purposes, for mapping cysteine residues in proteins with the aid of fluorescent maleimides[49,50] and, years later, for modification of proteins with maleimides for various applications. Maleimide–thiol conjugation became well-established, and therefore more application-based papers appeared, despite degradation worries that are discussed below.

To date, maleimide–thiol coupling still remains the biochemist's preferred method for the scalable chemical modification of proteins through cysteine residues. The reliability of maleimide–thiol conjugation means it can be used as a handle for multi-step functionalisations, to introduce, for example, an alkene functionality, which is then subjected to further reaction.[51] Antibody-containing therapeutics based on conjugates formed by maleimide–thiol coupling are already on the market, exemplifying the applicability of this chemistry, and further research is ongoing.[52] Cimzia, a PEGylated anti-TNF construct developed by UCB, for example, is FDA-approved for the treatment of ■■Crohn's *OK?*■■ disease and rheumatoid arthritis.[53] Antibody–drug conjugates (ADCs) comprise an area of research worth billions of dollars, utilising the cytotoxic effect of small-molecule drugs and the unmatched specificity of antibodies, resulting in targeted therapies.[1,2] Brentuximab vedotin—consisting of an anti-CD30 antibody conjugated to monomethyl aurostatin E (MMAE) through a protease-cleavable linker—is the first ADC to be approved for the treatment of Hodgkin's lymphoma and systemic ■■anaplastic large cell lymphoma (ALCL *OK?*■■, Scheme 5).[55] MMAE is too cytotoxic to be administered alone; hence the importance of conjugation to the antibody. In order for drugs to maintain their cytotoxic effects when conjugated to antibodies, more than one drug molecule usually needs to be attached to a single antibody (efficient drug/antibody ratio, DAR). In this respect, multiple sites need to be modified but residue specificity is necessary. This can be achieved by the incorporation of cysteine residues for modification at predicted sites, where they will not perturb antibody function or disrupt folding. This approach (coined THIOMAB technology), developed by researchers at Genentech, can be advantageous over the use of native lysine residues, for which DAR is less predictable.[52,56]

The use of maleimides in chemical protein modification is advantageous for a number of reasons, including their high selectivity towards thiol groups to form succinimide thioethers, a lack of side products, and the ability to carry out transformations in aqueous media in the absence of catalysts, often without any heating. In addition, the maleimide moiety can be functionalised through the nitrogen atom in the ring with a wide range of conjugation partners such as fluorophores, polymer moieties and lipids. The maleimide moiety can be introduced into a target compound for conjugation by use of straightforward chemistry, such as by the addition of maleic anhydride to amines, and many maleimide compounds are now commercially available. Thiosuccinimide bond formation was previously considered to be irreversible; however, observations that retro-Michael addition takes place as well as hydrolysis of the ring have led to uncertainties and further explorations into the integrity of the bond. This is discussed after a brief overview of recent applications of maleimide use in chemical protein modification through cysteine.

3.1.1. Recent examples of cysteine modification involving maleimide chemistry: In 2008, Dumelin et al. discovered a series of low-molecular-weight noncovalent albumin-binding entities based on the lead structure 4-(*p*-iodophenyl)butyric acid, identified from a DNA-encoded chemical library.[57] A maleimide-containing version of the parent compound—(2-(3-maleimidopropanamido-)-6(4-(4-iodophenyl)butanamido hexanoate)—



Scheme 5. The structure of the ADC Brentuximab vedotin.

was then synthesised in a small number of steps by simple *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling chemistry, and then conjugated to an antibody fragment specific for a tumour-associated antigen and containing a C-terminal cysteine residue. A dramatic increase in tumour uptake in rodents was observed; this demonstrated that success had been achieved in increasing the half-life of the antibody in vivo through binding to albumin, and also that the conjugate formed was sufficiently stable in blood plasma.[58] The simple yet effective strategy has the potential to be quite general. Simon et al. coupled two biorthogonal chemistries (one of them maleimide–cysteine thiol coupling) to assemble stable DARPin conjugates each bearing a cytotoxic drug, as well as albumin, for half-life extension.[59]

For Cheng et al., the thiosuccinimide bond again proved stable in another in vivo application, in which conjugation of an affibody to a maleimide-functionalised chelator allowed radiolabelling with ^{64}Cu and subsequent positron emission tomography (PET) imaging in mice, to establish biodistribution patterns.[60] Tavare et al. reported a slightly different method for radiolabelling, in which a C-terminal sequence consisting of a cysteine, spacer and hexahistidine motif was engineered into a phosphatidyl-binding domain of rat synaptotatgmin I; this might be useful for mapping apoptosis through the resulting exposure of phosphatidyl serine on apoptotic cell surfaces. The hexahistidine tag was used for the attachment of $[99mTc(CO)_3(OH_2)_3]^+$, whereas fluorescein-maleimide was conjugated to the free cysteine for imaging purposes.[61]

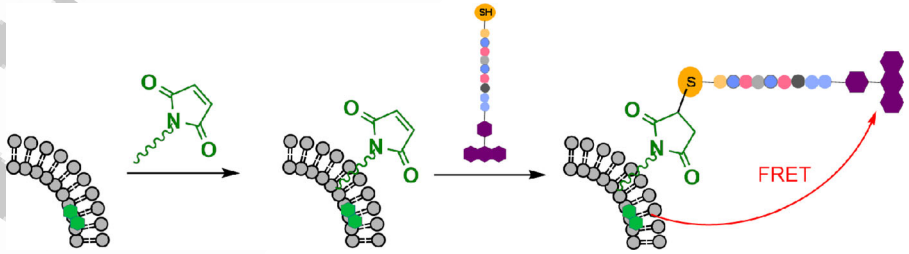
Protein-based artificial metalloenzymes might help in ■■inferring *"achieving"?*■■ enantioselectivity; their development involves introducing synthetic catalytic moieties into proteins.[62,63] Laan et al. successfully synthesised a ruthenium complex containing a maleimide unit and were able to couple it to photoactive yellow protein (PYP).[64] Its catalytic activity is yet to be tested, and might be affected by a need for organic co-solvents, likely to cause protein denaturation, as well as by a potential need for high pressures and temperatures and long reaction times.

Site-selective glycosylation of bovine haemoglobin was demonstrated by Zhang et al., who conjugated a maleimide-derivatised lactose unit onto a free cysteine residue. It was demonstrated that the glycoconjugate had a high affinity for oxygen.[65] A conjugate of this sort might help with stabilisation of the protein in vivo, and hence in the development of a strategy for oxygen delivery after haemorrhagic shock.

A very recent example of the use of unsubstituted maleimides has been described by Haralampiev et al. They incorporated a palmitic acid moiety functionalised with a maleimide unit into model unilamellar vesicle systems, as well as into a biological membrane (on macrophages); this could be further used to recruit a cysteine-containing rhodamine-labelled peptide for potential detection and monitoring of certain membrane processes (Scheme 6).[66] However, cell-surface thiols (exofacial thiols) have been proposed as a potential recruitment strategy for drugs, and so crossreactivity is possible. Recent work by Li and Takeoka has exploited these thiols, using liposomes incorporating maleimide moieties for specific targeting of exofacial thiols. Enhanced uptake of these liposomes (encapsulating doxorubicin) was observed both in vitro and in vivo. The reactions between thiols and maleimides are remarkably selective, as demonstrated in this case, in which the reactive groups are not only surrounded by other proteinogenic functionalities, but also by the plethora of chemical groups present on a cell surface.[67,68]

Egenberger et al. carried out multiple glycine-to-cysteine mutations within the substrate-binding domain of OCT1 (organic cation transporter 1). Facile fluorescence labelling with the bulky tetramethylrhodamine-6-maleimide led to substrate blocking, identified by fluorescence measurements, and the importance of this position could be elucidated as a result.[69] The generality of maleimide–thiol coupling is demonstrated by the fact that many maleimide-derivatised fluorophores are commercially available (as well as other maleimide-containing compounds), which promotes their use in a routine manner, especially by non-chemists. Fluorescent probes have been used in investigating protein folding mechanisms and pathways,[70,71] as well as for characterisation and mapping of cysteine residues in antibody variants.[72] Song et al. reported the synthesis of a small-molecule fluorescent probe containing a coumarin and maleimide unit, which was used for antibody labelling.[73]

SMCC [succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate], a heterobifunctional linker containing a maleimide and an NHS ester unit] was recently used to conjugate a polyhistidine peptide to DNA capable of cross-linking to cisplatin in order to perform "pull-down" experiments to identify modification states of proteins binding to cisplatin.[74] Maleimides have also been used to investigate the role of cysteine oxidation in cell signalling,[75] for elucidating roles of endogenous cysteine residues in various protein systems,[76,77] and for



Scheme 6. Recruitment of maleimide-functionalised lipids to biological membranes.

identifying and isolating epidermal growth factor receptor (EGFR)-binding peptides generated by phage display.[78]

Maleimide–thiol coupling has also been used extensively for the conjugation of biomolecules (proteins and peptides) to polymers[79] in, for example, the synthesis of nanoparticle drug-delivery systems,[80] conjugates able to cross the blood–brain barrier,[81] self-assembling fibril systems,[82] antibody–polymer conjugates[83] and multimeric polymer–protein conjugates.[84] De et al. reported a synthesis of a temperature-responsive polymer–protein conjugate in which polymerisation is carried out after conjugation of BSA to an initiating group.[85]

Hydrogels have many uses, including as scaffolds in tissue engineering and as controlled-release drug-delivery systems.[86] Ito et al. used maleimide-terminated four-armed PEG derivative for covalent attachment to a peptide capable of binding to the PDZ domain (a common structural domain in signalling proteins). This domain in turn mediated certain protein–protein interactions, which drove hydrogel self-assembly.[87] More recently, Wang et al. used the same PEG scaffold, but hydrogel formation was driven by reaction with accessible cysteine residues on a ubiquitin-like domain (ULD) tetramer.[88] A method for improved ultrasound targeted imaging based on conjugation between lipid-based maleimide-containing "bubbles" and cysteine residues on gelatine might lead to improved detection and monitoring of thrombus, inflammation and tumour angiogenesis, among other illnesses.[89]

Finally, the maleimide–thiol methodology has been used in protein immobilisation techniques: in, for instance, the immobilisation of RGD-containing peptides[90] onto a maleimide-functionalised poly-*p*-xylylene coating[91] and the capture of anti-EGFR antibodies to quantum dots coated with protein G.[92]

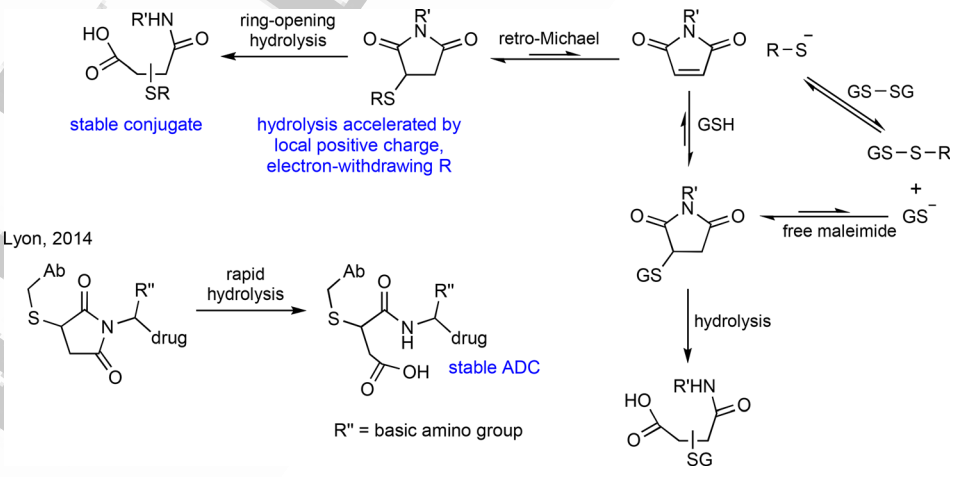
The above examples by no means constitute an exhaustive list of maleimide–cysteine coupling over the last few years, but they do demonstrate the high potential maleimide chemistry offers many research groups, leading to a broad spectrum of applications.

3.1.2. Concerns over the integrity of the thiosuccinimide bond: The integrity of the thiosuccinimide bond has been the subject of many recent studies, especially in a therapeutic context. Depending on the application, the stability of this bond is a rather subjective topic because in some cases a cleavable linker is desirable—with, for example, the release of a drug forming part of an antibody–drug conjugate (ADC) at the site of action—but not in others, such as when a bioconjugate is synthesised for in vivo tracking or imaging.[93]

In 1998, Lewis et al. observed that bis-maleimide-linked radiometal immunoconjugates were unstable in serum, detecting radiometal–albumin conjugates, indicative of free maleimide being present and reactive.[94] Ten years later, a team from Seattle Genetics also observed conjugation between their maleimide-derivatised drug and albumin in plasma.[95] Replacing the maleimidocaproyl conjugation partner with a bromoacetamidecaproyl ■■variant *OK?*■■ led to overall higher intratumoural drug exposure and a slight increase in in vivo stability.

In general, succinimide thioethers are considered to be stable, but remain sensitive in reducing environments under near-physiological conditions. Retro-Michael addition (Scheme 7) leads to free maleimide in solution and hence to the potential formation of albumin conjugates. Thiol exchange can also occur with glutathione, present in blood plasma (Scheme 7). The equilibrium between maleimide–thiol coupling and retroaddition generally favours thiosuccinimide bond formation, but this can be dependent on the acidity of the protons adjacent to the carbonyl groups in the ring. In addition, the rate and extent of thiol exchange can be modulated by modifying the Michael donor's reactivity.[96] Substituents on the ring can affect the pK_a values of protons adjacent to the carbonyl groups, which has led to the development of next-generation maleimides, with tuneable bond-forming properties (discussed in Section 3.2).

Another side reaction that is known to occur is hydrolysis and subsequent ring opening (Scheme 7). This does not necessarily represent a problem, because it leads to a stable product, insusceptible to thiol exchange. Therefore, promoting hydrolysis of maleimide–thiol adducts is popular at the moment, in particular in the field of ADCs.[97] In contrast, thiosuccinimide bond breaking is undesirable because it can lead to off-target



Scheme 7. Ring opening of maleimide–thiol conjugates and thiol-exchange routes with GSH. Ab = antibody.

cytotoxic effects, due to thiol exchange resulting in the free drug entering the circulation. The exact location of the cysteine moiety is important, because differences in solvent accessibility and local charge might affect rates of potential thiol exchange and/or retro-Michael reaction. Shen et al. found that for ADCs, a partially accessible site with a positive local charge promoted post-conjugation hydrolysis between antibody and drug, and this method should be generally applicable to other systems.[98]

Alternatively, inductive effects due to electron-withdrawing substituents on nitrogen can accelerate ring opening rates,[99] as can an ethylene glycol spacer, a hexamer form of which was found to enhance hydrolysis over time in neutral buffer.[100] It was hypothesised that this proceeded with the aid of a water molecule coordinated to a proximal PEG oxygen atom, with correct geometry for subsequent attack on the carbonyl group of the ring to occur. Lyon et al. developed a self-hydrolysing conjugate, incorporating a basic amino group adjacent to the maleimide unit such that, after conjugation to the thiolate-containing antibody, the basic amino group can catalyse ring hydrolysis under physiological conditions.[101]

3.2. ■■C-Substituted■■ maleimides

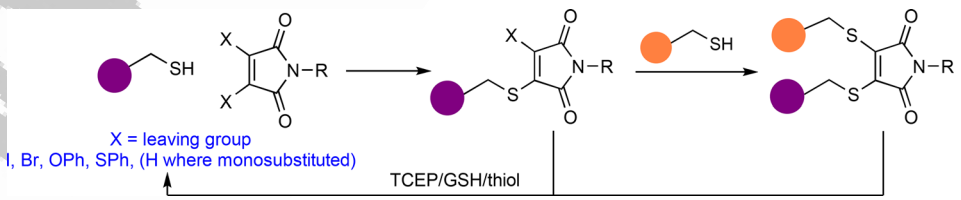
In 2009, the groups of Baker and Caddick presented next-generation maleimides (NGMs), demonstrating their bio-orthogonality and reactivity towards cysteine on an amino acid, peptide and protein level (Scheme 8).[102,103] At a minimum, experiments at an amino acid level reveal whether reactivity is possible, reaction behaviour with peptides reveals chemoselectivity, and reaction behaviour at a protein level tells whether or not the conjugation conditions required are compatible with the tertiary structure of the protein.

Since then, much work on both applications and exploration of NGMs has been published. An NGM contains one or two leaving groups ■■adjacent *"attached"?*■■ to the double bond of the maleimide, and coupling to a thiolate therefore occurs by way of an addition elimination sequence to form a thiomaleimide bond (Scheme 8). This is in contrast to the saturated succinimide that forms in the absence of a leaving group. When two leaving groups (a C-■■disubstituted maleimide) are present, the protein can be further functionalised by addition of another thiol.[103,104] The range of applications demonstrated over the last few years is quite extraordinary, and includes disulfide bridging for protein stabilisation, reversible addition for drug delivery systems and fluorescence tagging. Moreover, a single NGM can be synthesised to effect a combination of these applications.

NGM syntheses appear to be straightforward and non-problematic.[105] In the majority of cases, a little over 1 equiv of reagent is required, along with mild conditions compatible with protein environments and short reaction times for protein modification to occur. The thiomaleimide conjugate resulting from reaction with a bromomaleimide is reversibly reactive, and can be cleaved with tris(2-carboxyethyl)phosphine (TCEP; 1 equiv) or a large excess of thiol without any racemisation at the cysteine residue.[102,103] Cleavage was also observed under simulated intracellular cytoplasmic conditions, and then under actual cellular conditions by incubation of rhodamine–green fluorescent protein conjugates in human cells and testing for cleavage by monitoring differences in fluorescence resonance energy transfer (FRET) intensities.[106] This intracellular cleavage is likely due to an elevated level of GSH in cells relative to plasma, thus implying that the methodology could be useful in a drug-release strategy.[107]

3.2.1. Structural features of substituted■■C-substituted■■ maleimides and demonstrated applications: Halogen leaving groups were compared for reactivities, and it was found that the rate-determining step for conjugation was likely to be influenced by leaving group ability, because the iodide group reacted more rapidly than bromide and chloride groups.[108]

■■Dithiophenylmaleimide *"Bis(thiophenyl)maleimide"?*■■ was also found to be very reactive with cysteine residues in proteins, and was applied in disulfide bridging applications. Disulfide bridging involves the replacement of native disulfide bonds with structural mimics, with the aim of mimicking the disulfide's ability to stabilise the protein structure, whilst rendering the structure impervious to reduction. A challenge in disulfide bridging is the tendency for disulfides to reform and perhaps to scramble, and so Schumacher et al. used an in situ approach, with TCEP present to prevent this. They observed rapid conversion with a dithiophenylmaleimide■■, as well as no TCEP-mediated cleavage, whereas TCEP–bromomaleimide adducts were observed on treatment with dibromomaleimides. In addition, bridging was successful with an N-functionalised PEG dithiophenylmaleimide■■ on the 14-residue peptide hormone somatostatin, resulting in a dual-purpose modification with a single reagent. The disulfide bridge in the hormone is essential because it maintains a β-turn motif that mediates binding interactions to ■■G protein-coupled receptors *? (GPCR, not GCPR)*■■, but in its native form suffers from proteolytic degradation and a short half-life in vivo. Therefore, PE-

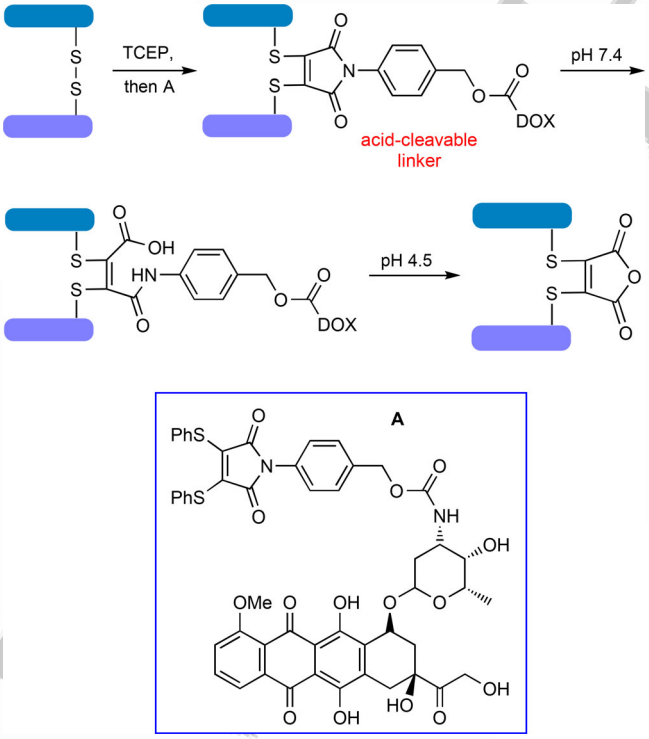


Scheme 8. Protein modification with the aid of mono- or di-C-substituted■■ maleimides.

Gylation is useful for half-life extension, whereas disulfide bridging ■■infers *"confers"?*■■ structural integrity.

Oxytocin is a peptide hormone known to help prevent postpartum haemorrhaging after childbirth, but has a low solubility and is prone to degradation, particularly at its disulfide bond. This was replaced with a succinimide bridge in a process in which the starting maleimide was functionalised with a polymer; the combination aided in providing improved solubility and increased stability at elevated temperatures.[109] A further example of disulfide bridging in combination with polymer attachment was demonstrated on salmon calcitonin, a 32-amino-acid hormone, with a dithiophenylmaleimide■■ polymer, synthesised in a facile way in the absence of any protecting groups.[110,111] The disulfide bridging strategy has been used to synthesise antibody conjugates incorporating trastuzumab, with other relevant moieties. Schumacher et al. prepared ADCs containing trastuzumab and the cytotoxic drug doxorubicin, targeting multiple interchain disulfide bonds,[112] and the methodology has most recently been applied to MMAE (structure shown in Scheme 5), with the resulting conjugate being further hydrolysed to produce a serum-stable conjugate.[112]

Castañeda et al. also prepared trastuzumab–doxorubicin conjugates, but included a benzyloxycarbonyl spacer between the two components (Scheme 9).[113] This spacer is stable at physiological pH, but not at acidic pH values, so drug release at selected sites is possible. This might have application in drug-release systems, for endosomal escape after ADC internalisation. The elegant concept was demonstrated by simply lowering the pH of the conjugate solution, and it remains to be seen if it will be successful in cells and in vivo.



Scheme 9. Disulfide rebridging of trastuzumab and functionalisation with doxorubicin and an acid-cleavable linker.

Bryden et al. reported the conjugation of trastuzumab with a water-soluble porphyrin for photodynamic therapy (replacing the disulfide bond linking the C_L and C_{H1} chains).[114] Hull et al. illustrated the assembly of two different antibody fragments by using a bis-dibromomaleimide linker, forming a homogeneous and bispecific construct, still capable of binding antigen.[115] Schumacher et al. conjugated an scFv fragment to a C-■■disubstituted maleimide containing a spin label.[116] As well as conferring■■ increased structural rigidity, the presence of the spin label also allowed EPR sensing of antibody–antigen interaction, which could be useful in diagnostics and therapeutics.

In an attempt to improve the half-life and stability of growth hormone (GH) in circulation, researchers from Pfizer conjugated GH to aldolase antibody Covx.[117] Dual coupling has been used to prepare ubiquitin–ubiquitin and ubiquitin–GFP conjugates; this emphasises the control possible by minimising the number of equivalents used.[118]

Interestingly, Marculescu et al. have demonstrated the use of aryloxymaleimides as improved reagents for disulfide bridging. Their attenuated reactivity means that less monoaddition is encountered, and as an alternative application, a bromomaleimide with an alternative functionality at the nitrogen can be added after monoaddition, providing a facile method for dual modification.[104]

Robin et al. made the rather interesting discovery that dithiomaleimides have emissive properties in certain solvents, and these fluorescent agents can be conjugated to protein partners in the presence of other useful groups, such as PEG, thus giving another example of providing a dual function with a single modification.[119]

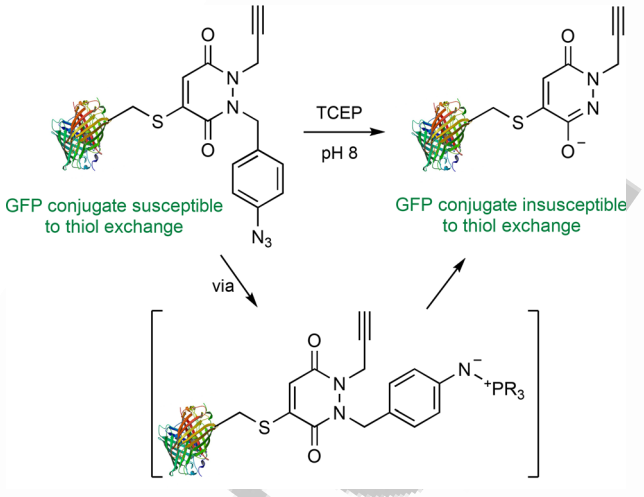
As exemplified by the above examples, functionalisation of maleimides at the N-position provides access to a range of interesting groups, which also includes metallocarbonyl complexes.[120]

The issue of reagent and conjugate hydrolysis is important (Section 3.1.2). Hydrolysis leading to ring opening of the maleimide (applicable to both C-unsubstituted and C-substituted■■) renders the reagent unreactive, whereas ring-opening hydrolysis after conjugation affords stable adducts. Ring opening after conjugation might be preferred, particularly in therapeutic applications, because the ring-opened form is not susceptible to GSH-mediated thiol exchange (Scheme 7). Ryan et al. found that when *N*-phenyl-substituted maleimides were used, hydrolysis could be induced post-conjugation by extended incubation at pH 8 at varying temperatures. This is likely due to the electron-withdrawing effect of the aryl moiety on the ring.[121] This observation was further corroborated by another study in which electron-donating N substituents (such as alkyl groups) were found to minimise hydrolysis.[122] A further disadvantage associated with the use of maleimides is the retro-Michael reaction. Positively, conjugation with use of bromomaleimides is advantageous over that with use of unsubstituted maleimides because this is not mechanistically possible.[112,123]

3.3. Analogous structures

The same groups responsible for the development of the bromomaleimide platform reported on the reactions between cysteine and monobromo- and dibromo-1,2-dihydropyridazine-3,6-diones (Scheme 10). The nitrogen atoms within the ring were ethylated, because the presence of the protons led to there being no reaction with cysteine, probably due to the protons being too acidic at pH 8. The conjugates obtained were found to be stable to hydrolysis, but susceptible to thiol exchange and therefore cleavable under intracellular conditions.[124]

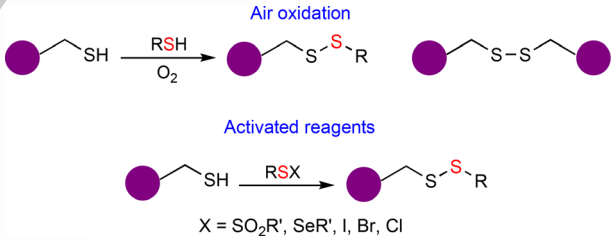
A further development of this work involves a strategy for rendering the conjugates resistant to thiol exchange.[125] On the basis of the previous observation that reagents with unsubstituted nitrogen atoms within the ring were not reactive at higher than physiological pH, Maruani et al. postulated that the structure probably exists as its enol tautomer. They set about devising a method for generating the enol tautomer post-conjugation. This was achieved by attaching a *para*-azidobenzyl group to a nitrogen atom within the ring susceptible to thiol exchange. Quite simply, removal of this group (after conjugation to a protein) was carried out by treatment with TCEP at pH 8 (Scheme 11). Cleavage of the *para*-azidobenzyl group rendered the resulting conjugate insusceptible to thiol exchange with glutathione, which could be useful in applications in which robust conjugates are required, such as in vivo imaging. The fact that the nitrogen atoms need to be substituted is a great advantage and allows a route to further decoration of a protein with groups of interest. For example, the methodology was applied to the functionalisation of the therapeutically relevant Fab-Her2. Using a dibromopyridazine scaffold that was functionalised through its nitrogen atoms with both alkyne and cyclooctyne handles, ■■Maruani et al. *OK?*■■ prepared antibody conjugates ■■that contained various combinations of a drug, a fluorescent marker and PEG, whilst bridging *unclear, please rephrase*■■.[126] The methodology provides a rather elegant method for what can be regarded as a ■■triple *"quadruple-"?*■■-purpose modification—disulfide bridging providing increased stability of the antibody, functionalisation with a drug for cytotoxic purposes, a fluorescent marker for detection during cellular assays, or PEGylation for increased half-life.



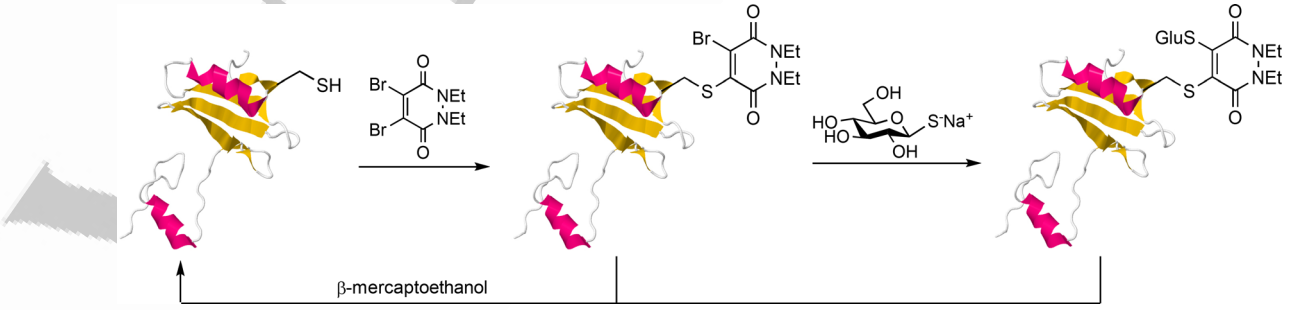
Scheme 11. TCEP treatment of GFP conjugate (wild-type: PDB ID: 1GFL) rendering it insusceptible to thiol exchange.

4. Disulfides

Two appropriately positioned cysteine residues within a protein can oxidise to form an intramolecular disulfide bond—a modification that is prevalent in the vast majority of proteins, playing key roles in protein folding and structural integrity.[127] Crossed disulfide bond formation has also been exploited as a chemical modification method of choice for conjugation of proteins bearing singly reactive cysteine residues to other thiol-containing moieties. Air oxidation is the simplest way to promote the formation of disulfide bonds; however, this method does have drawbacks including slow reaction times and an increased chance of forming protein dimers (Scheme 12). This depends



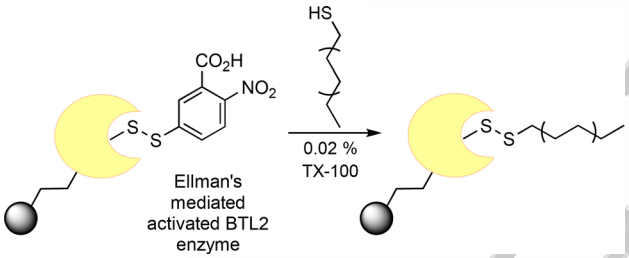
Scheme 12. Common methods for disulfide formation.



Scheme 10. Modification of Grb-SH2 domain L111C with dibromo-1,2-dihydropyridazine-3,6-diones. Wild-type: PDB ID: 1JYU.

on the accessibility of the cysteine residue in question and is difficult to control because the residue has to be somewhat accessible in order to be able to react with the desired reagent.

The use of activated reagents has proven paramount in the continued use of disulfide bond formation, because they are able to direct crossed disulfide formation.[128,129] In addition, the use of activated reagents means that these reagents themselves are unable to form homodimeric structures. The use of the Ellman reagent—5,5′-dithiobis(2-nitrobenzoic acid)—as an indication of sulfhydryl content is a good example of the reliability of certain activated agents because it is commonly used for the activation of cysteine thiols on proteins for disulfide bond formation.[130] The disulfide bonds formed by treatment with the Ellman reagent have recently been used for the construction of antibody–drug conjugates[131,132] and in an enzyme-immobilisation strategy (Scheme 13).[133] In this example, site-specifically grafting a dodecane chain onto BCL2 in the presence of detergent resulted in an increase in enzymatic activity due to mimicry of lipase interfacial activation exogenously triggered by detergents. Similarly, 2,2′-dithiobis(5-nitropyridine) (DTNP) can also be used as an activating agent in, for instance, the ubiquitination of histones.[134]



Scheme 13. Crossed disulfide formation mediated by Ellman's reagent. ■ ■*no number next to brackets–?*■■

Methanethiosulfonate reagents are well-established thiol-coupling partners for disulfide bond formation, and are used extensively in protein modification through cysteine (Scheme 12). They are synthetically accessible and react selectively with cysteine to yield disulfide bonds. The number of reports in which methanethiosulfonate reagents have been utilised is vast, and we do not provide an exhaustive list of studies carried out here. Some recent applications of the modification, however, include the elucidation of protein function and pathways,[135–139] EPR studies,[140] pull down/purification assays,[29,141] the synthesis of polymer–protein conjugates for improved therapeutic performance[142] and the construction of protein conjugates with altered biological activities.[143]

Elaborate and efficient protecting group strategies for disulfide formation on peptides exist, and can allow good control, but many are not applicable to proteins due to use of organic solvents and sometimes microwave irradiation.[144]

Disulfide bonds are sensitive to reduction; this can be either problematic or highly advantageous, depending on the desired application. For applications in which a stable, irreducible bond is required, multiple disulfide bridging techniques have been developed (Section 3.2). Strategies for disulfide contraction, giving rise to irreducible thioether bonds, also exist.[20,128,129]

Alternatively, the redox-sensitive nature of the disulfide bond is useful in drug-delivery systems,[145–147] particularly when a drug needs to be delivered into the cell. If a conjugate consists of a cell uptake portion (e.g., a cell-penetrating peptide)[148] and a cytotoxic portion (e.g., a small-molecule drug or a peptide therapeutic) linked by a disulfide bond, this becomes susceptible to reduction within the cell (containing elevated levels of GSH). This can lead to drug release from lysosomal compartments.

The site-specific sulfonation of cysteine was shown to result in a functional redox-sensitive phosphoserine mimic, but the protocol, albeit simple (addition of sodium tetrathionate and sodium sulfite), would not be applicable to proteins with accessible disulfide bonds.[149]

5. Cysteine as a Precursor to Dehydroalanine

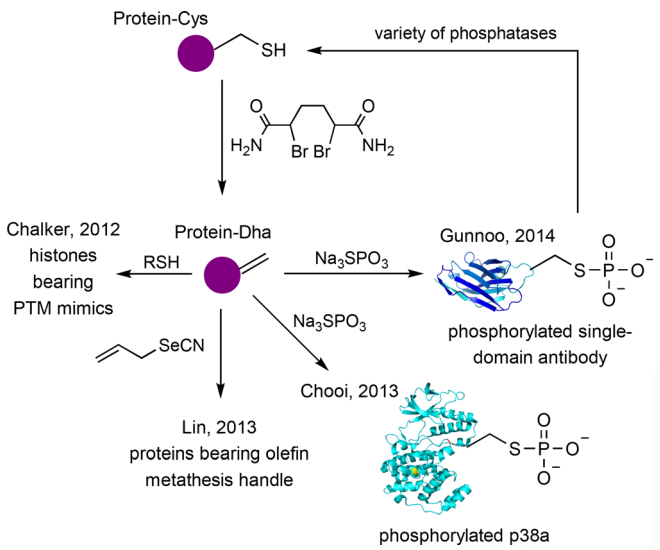
Dehydroalanine (Dha) is a naturally occurring amino acid present in many proteins as a PTM at serine,[150] less commonly at cysteine[151] and also in some peptidic antibiotic structures.[152–154] Dha is known to have a rigidifying effect on a peptide chain, inducing or stabilising secondary structure and increasing proteolytic stability. Chemically, it also provides a unique electrophilicity that can be exploited by addition of nucleophiles, in particular thiol groups, as extensively discussed below.

There are ample methods for incorporating dehydroalanine into peptides and proteins by using serine as a precursor,[155] but its use does have drawbacks. Serine has a higher natural occurrence than cysteine, so site-selectivity is increasingly difficult to control. In addition, the potentially harsh conditions required to dehydrate serine—the use of H_2O_2, high pH values etc.—might not be applicable to a wide range of proteins.

Cysteine as a precursor to Dha is less well-known than serine on a natural level, yet its utility in chemical protein modification is of high importance. Post-translationally introduced Dha in ■■microcystins? a microcystin?■■ was reported to "bind" to cysteine on a protein phosphatase, inhibiting its activity, yet demonstrating the ability of the thiol group of cysteine to react with Dha.[152] Despite the fact that various reports involve the use of Dha in peptide and protein modification, its potential (as a precursor to further selective protein modification) has only really been realised in recent years, particularly since the Davis group reported the use of a generally applicable reagent for forming Dha from a cysteine precursor (Scheme 14).[155] This method is more facile and economical and less time-consuming than the use of serine as a precursor.

5.1. ■■Transformation of cysteine residues into Dha residues *OK?*■■

A study on different methods for converting cysteine into dehydroalanine was carried out by the Davis group (Scheme 14).[155] Methods for this transformation that had been



Scheme 14. Dehydroalanine formation followed by further protein functionalisation. Single-domain antibody cAb-Lys3: PDB ID: 1MEL. p38α: PDB ID: 1R3C.

reported earlier involved the use of conditions incompatible with many proteins, such as high temperatures[156] and high proportions of organic co-solvents.[157] DuMond et al. observed unintentional Dha formation from cysteine on using HNO donors.[158] Tedaldi et al. reported that Dha could be formed at an amino acid level through an addition/elimination sequence of a bromomaleimide with the thiolate ion and subsequent base-mediated elimination of the resulting thiomaleimide bond.[102] This methodology was, however, not successful in aqueous medium and has not been applied to proteins since.

The aim of the study carried out by the Davis group was to find a reagent for the modification that could be applied to a wide range of proteins ■■without problems of stability or use of organic co-solvent, operating at ambient temperatures and pH, and providing chemoselectivity *OK?*■■. Although Cys-to-Dha conversion in a selective manner was previously found to be possible in the case of a single cysteine mutant of the robust subtilisin *Bacillus lentus* (SBL-156C) in DMF with use of *O*-mesitylenesulfonylhydroxylamine (MSH),[159] it was found to be unsuccessful with other proteins.[155,160] In the SBL-156C case, however, Dha produced by treatment with MSH has been used as an orthogonal handle in the first example of cross-metathesis on a protein,[161,162] radiolabelling with ^{18}F-containing sugars[163] and the synthesis of homogenous glycoconjugates.[164,165]

One alternative method for forming Dha from cysteine was by a ■■dialkylation/elimination method with the diamide *OK?*■■ based on a 1,4-dibromobutane core (2,5-dibromohexanediamide■■2,5-dibromohexanedioic acid diamide?■■), through which conversion into Dha could be carried out on three proteins, including an antibody (Scheme 14). The reaction was selective at the desired cysteine residue despite others being present (albeit in disulfide bonds), and the antibody was found to retain secondary structure. Although the reagent is only partially soluble in water, the reaction could be carried out in the absence of organic solvent. It proceeds through dialkylation/elimination■■, with the pre-elimination intermediate (cyclic or linear) identifiable by LC/MS. A slightly elevated ■■pH (8) *OK?*■■ is required for the reaction, but this is not detrimental to the vast majority of proteins, and an increment of temperature to 37 °C is sufficient for elimination to form Dha. Also, because the pK_a of the thiol proton varies from cysteine residue to cysteine residue, depending on its microenvironment,[16] reaction at a lower pH can be possible. Over the last few years, the reaction has been applied to a range of proteins, followed by Michael-type addition of thiol moieties, both by the Davis group and by others.[155,166–169]

There are a few perceived issues associated with the use of Dha as a precursor.[170] The introduction of an sp^2-hybridised carbon causes the chiral centre to be lost, and so diastereomeric products can be obtained after addition. The stereochemical outcome can be difficult to predict unless protein crystal structures are available, to provide a 3D view of which direction addition would be less hindered. Aydillo et al. developed a synthesis of a chiral Dha amino acid derivative from a cysteine precursor based on a chiral auxiliary approach, and then allowed this to react with thiosugars, achieving high diastereoselectivity.[171] These derivatised amino acid building blocks can be used in solid-phase peptide synthesis, or perhaps even combined with an NCL strategy for protein synthesis, thus avoiding the creation of diastereomers. However, at a protein level, this might not be required, because neighbouring and ■■proximate *"proximal"?*■■ residues might themselves act as auxiliaries, promoting attack from one face. A wealth of functionality is possible, with introduction of both natural and unnatural functionalities, as well as mimics of functionality, all via the same orthogonally reactive handle—Dha.[155,172] Moreover, Dha is prone to hydrolysis, but because the transformation from Cys into Dha takes a few hours at most for the majority of tested substrates, further reaction can then be carried out, affording stable modified protein within a day. This method of chemical post-expression installation under ambient conditions is advantageous over genetic installation in this respect, because no manipulation of cell machinery is required.

5.2. Application of modifications via Dha

Once incorporated in a protein, a Dha residue is susceptible to nucleophilic attack by a thiol moiety. This has been exploited to form PTM mimics, in which a conventional O-link is replaced by an S-link. Chalker et al., for example, synthesised methylated and acetylated lysine mimics on histone H3 by use of Dha, detectable by western blot with antibodies raised against the natural O-linked modification,[166] and Chooi et al. successfully switched protein kinase to an active state by incorporating phosphocysteine (pCys) via Dha (Scheme 14).[167] The presence of this mimic led to phosphorylation of the natural substrate ATF2, thus chemically recapitulating triggering of the MAPK-signalling pathway in vitro. Blagg's lab have used the same methodology to elucidate the necessity of two different phosphorylation sites on protein kinase aurora-A.[173] As well as for

the formation of PTM mimics, Dha has been used as a handle on various proteins to incorporate both sulfur- and selenium-containing groups suitable for subsequent metathesis (Scheme 14).[168] Additionally, Timms et al. tested the effect of thialysine in place of native lysine on the activity of the protein *N*-acetylneuraminic acid lyase.[174]

Back in the Davis group, Gunnoo et al. synthesised an AND-gated antibody of the single-domain antibody cAb-Lys3, in which antibody function was dependent not only on antigen but also on a PTM-processing enzyme, a phosphatase. By incorporation of pCys via Dha within the CDR3 loop of cAb-Lys3, antigen-binding ability was turned off. This effect on the addition of various phosphatases was reversible, and the antibody was able to bind again (Scheme 14). The AND gate was demonstrated both in vitro and in mammalian tissue. Phosphorylated antibody was able to bind selectively to cells expressing cell-surface antigen only when phosphatase (either exogenous or endogenous) was present.[169] This strategy of reversibly inhibiting antibody function by a combination of chemical and enzymatic modification might be applicable in antibody therapeutics, reducing side effects caused by antibody binding to cell-surface antigen in healthy tissue.[175]

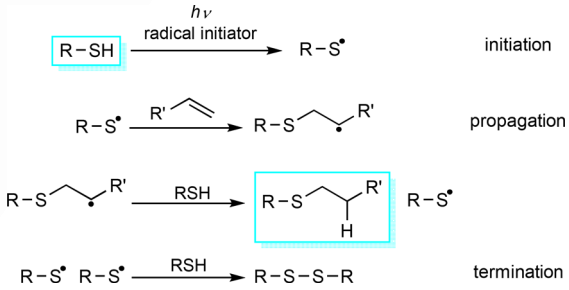
In terms of PTM mimics, S-linked PTMs are naturally occurring, and so thiol addition to Dha will not always result in a structural mimic.[18] The chemical introduction of S-linked PTMs allows a facile method for exploration of the modification. In addition, S-linked glycoconjugates are in fact desirable in drug strategies because they are known to be resistant to enzymatic processing—by glycosidases, for example—whereas thiophosphate can be processed by the more promiscuous phosphatases.[169]

There are examples of the use of Michael addition of thiols to Dha in which the aim is not to introduce PTM mimics. Haj-Yahya et al., for instance, synthesised a diubiquitin-based probe to study the activity of deubiquitinases through the chemical installation of Dha.[176] The dibromo-mediated conversion of cysteine into Dha in soluble proteins and membrane proteins was monitored by Branigan et al., to test the efficiency of a cysteine quantification protocol involving thermally induced protein folding and conjugation to a fluorogenic compound through alkylation.[177] It was observed that elimination to yield Dha could be slow, leaving the sulfonium intermediate (Scheme 14), as detected by LC/MS.

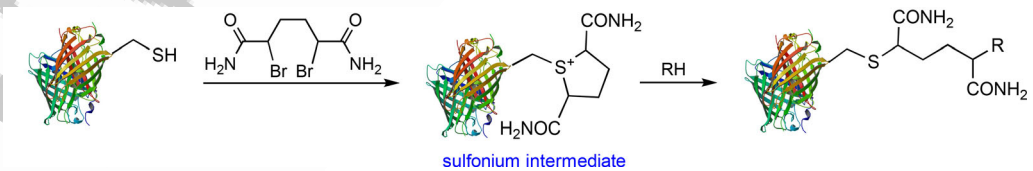
This was used by Nathani et al. as a positive point for further or dual functionalisation of a protein (Scheme 15).[178] With GFP, the cyclic sulfonium intermediate was found to be stable and to persist for 4 h at 37 °C or for 24 h at 21 °C. Immediate functionalisation by ring opening mediated by a nucleophile was therefore required to maximise modified protein recovery. A range of nucleophiles were effective, and included other bioorthogonal handles such as an azide. The stability of the sulfonium ring could probably be attributed to the environment surrounding the site of modification. This was extended further in a dual-labelling strategy, taking advantage of the differential reactivity of two cysteine residues within GFP. One cysteine residue was converted into Dha by use of the dibromo reagent (and modified further with thiol nucleophiles), whereas the sulfonium adduct formed from the other cysteine residue was further functionalised with an azide nucleophile and then with an ■■alkyne-appended fluorophore *OK?*■■.[179] In this strategy, in which two cysteine residues were essentially treated as different amino acids, the challenge lay in having and identifying two cysteine residues that exhibited essentially orthogonal reactivity within a protein, thereby avoiding the creation of mixtures of modified products. Although thiol nucleophiles are very good partners for Michael addition to Dha in terms of ease of reaction, resulting conjugate stability and function, the ability to apply other reactions, perhaps forming carbon–carbon bonds, would be desirable.[170]

6. Thiol-Ene and Thiol-Yne Reactions

The widely used thiol-ene reaction is a reaction between a thiol and alkene, and is commonly radical-mediated, thus requiring irradiation■■*please confirm*■■. Photoinitiation generates the thiyl radical, and propagation then consists of alkene addition and protonation. The process is terminated by disulfide formation between two thiyl radicals, this can be reversed by the addition of a reducing agent (Scheme 16).[180–182] The reaction, which meets many requirements of a "click" reaction, has been exploited extensively in polymer chemistry.[183] It is orthogonal, does not require organic solvent in order to proceed and is rapid in most cases, criteria that make it an attractive choice for site-selective biomolecule modification. Further-



Scheme 16. Thiol-ene reaction mechanism.



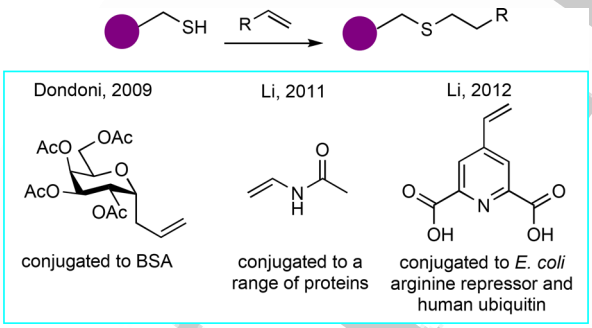
Scheme 15. Trapping of an intermediate on treatment of cysteine with 2,5-dibromohexanediamide and a nucleophile. GFP: PDB ID: 1GFL.

more, it is fully compatible with water and oxygen, a highly attractive feature for protein modification. However, some proteins are unstable to the UV irradiation conditions required for radical generation, despite it being a relatively low-energy (365 nm) process. This has not precluded its application to protein modification, and, depending on the protein substrate, UV-mediated modification can result in a functional conjugate. Some proteins' lack of tolerance towards irradiation does mean that it would be difficult to present the thiol-ene reaction as a generally applicable strategy.[184]

Recent protein modification work utilising the thiol-ene reaction tends to follow one of two routes—either the alkene is incorporated pre- or post-translationally[185] and then treated with a thiol-derivatised group of interest, or an alkene-derivatised group of interest is conjugated to a cysteine within the protein. Because this review concerns modification through cysteine, we focus more on studies in which cysteine has been employed as an orthogonal handle for the thiol-ene reaction, and discuss why researchers would choose to incorporate an unnatural alkene instead of using the natural cysteine handle, in discussion of examples in which proteins have been employed.

6.1. Thiol-ene reaction with proteins

The photodegradation of proteins by UV irradiation is widely documented.[184,186] The excitation of aromatic residues promotes electron transfer to proximal disulfide bonds, thus causing cleavage. Dondoni et al. glycosylated BSA (bovine serum albumin) containing one disulfide bond and one free cysteine residue through a thiol-ene reaction at 365 nm, and found that after a short while all three cysteine residues were glycosylated (Scheme 17). They clarified that the protein was intact by



Scheme 17. Alkene-containing partners for thiol-ene reactions with cysteine residues in proteins.

MALDI analysis, but site-specificity was not achieved.[186] This observation reduces the generality of the thiol-ene reaction, because intact disulfide bonds are not necessarily tolerant to irradiation conditions. However, it is important to note that finding chemical modification strategies applicable to a multitude of proteins is very challenging.

An example of thiol-ene protein modification through an alkene unit within the protein is the glycosylation of the virus-like nanoparticle ssβG with the aid of genetically incorporated homoallylglycine (Hag) residues. The thiol-ene reaction itself was carried out at pH 4–6, and so might have potential in sequential dual-modification strategies, but both cysteine and methionine residues were mutated out of the protein, thus reducing its utility as a site-specific and selective modification.[187] Weinrich et al. incorporated a farnesyl group into ■■a protein/proteins and used the alkene group to immobilise this protein/these proteins■■ onto surfaces coated with thiol groups.[188] Another example of the thiol-ene reaction on proteins incorporating an alkene tag is provided in work by Li and co-workers.[185] They genetically incorporated alkene-bearing pyrrolysine analogues into the proteins HdeA and Asn^{II} in *Escherichia coli* by use of a mutant PylRS-tRNA pair.[7] They allowed these to react with bi-dansyl-cystamine and PEG-bearing thiols, with varying yields, with the aid of the radical initiator Vaso44 and irradiation at 365 nm. The bioactivity of the proteins after conjugation was tested and was found to be retained. Asn^{II} contains three free cysteine residues, and although the authors could see, by means of a fluorescent gel, that the fluorophore had conjugated, mass spectrometry and enzymatic digests were able to establish with certainty that there is no crossreaction with cysteine residues. Li et al. argue that if more than one cysteine is present in a protein, the thiol-ene reaction cannot be carried out. This is not necessarily true, and strongly depends on the microenvironment and accessibility of the cysteine residues, factors that need to be determined with each protein.

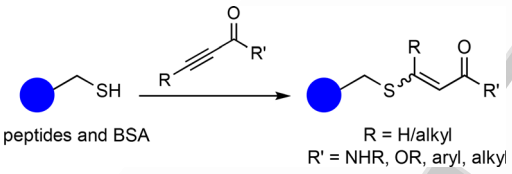
Further examples of thiol-ene conjugation based on natural cysteine residues do exist. Li et al. allowed cysteine residues on ubiquitin and histones H3 and H4 to react with *N*-vinylacetamide, forming functional post-translational mimics (Scheme 17),[189] whereas Valkevich et al. used the reaction to form ubiquitin trimers in order to gain insight into ubiquitin's biological function.[190] A paramagnetic lanthanide ligation partner was synthesised and ligated to an *E. coli* arginine repressor and ubiquitin through cysteine (Scheme 17). The conjugates could be useful in NMR analysis. Interestingly, no radical initiator or light was used and around 80% conversion was achievable in 24 h. Although the reaction time is quite long, the lack of irradiation is an impressive feature of the reaction, which probably proceeds through Michael addition with the aid of the adjacent conjugated system in the compound.[191] Ma et al. have recently carried out kinetic studies in relation to substrate choice for the thiol-ene reaction. When vinyl-substituted pyridine derivatives are used, the protonation of the pyridine N is of importance in terms of rate acceleration. Similarly, α,β-unsaturated alkene systems are useful in potentially alleviating the need for radical formation.[192]

A further interesting example of the use of the thiol-ene reaction is the incorporation of photoswitchable amino acids bearing alkene groups that are able to react with cysteine when appropriately positioned in peptides.[193] ■■A related approach, but utilising S_N2 substitution of a photoswitchable group with cysteine (rather than the thiol-ene reaction as had been carried out on peptides), was found to be suitable for inducing changes in protein conformation *OK?*■■.[194]

6.3. Reactions between cysteine residues and alkynes

The reactions between thiols and alkynes have long been known, but in more recent years their applicability to protein environments through cysteine has been explored. Most researchers are aware of the famous copper-catalysed azide–alkyne cycloaddition (CuAAC),[183] but not that alkynes are also reactive towards thiols. Crossreactivity with cysteine thiols when carrying out CuAAC is not often observed (although it has been reported and/or speculated on and is dependent on the inherent reactivity of the alkyne).[195–197]

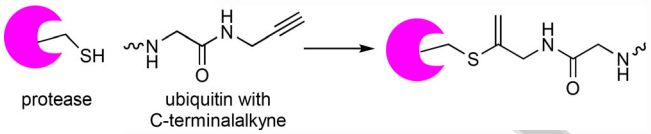
Alkynes and thiols can be conjugated with the aid of light irradiation (Section 6.4), but as Shiu et al. have described extensively, this is not necessary despite the weakly electrophilic nature of the alkyne functionality. They have described the addition of electron-deficient alkynes (alkynoic amides, esters and alkynones) to unprotected peptides and BSA in aqueous solutions (albeit with 10% CH_3CN) to target cysteine residues, resulting in vinylsulfide linkages (Scheme 18).[198] The selectivity and efficiency of the conjugation was demonstrated by the enrichment of cysteine-containing peptides in a solution containing peptides with and without cysteine residues. Previously, the corresponding reactions of electron-deficient alkynes had been carried out with "simple" thiols (namely cysteine and glutathione) in the presence of organic solvent.[199,200]



Scheme 18. Reactions between cysteine residues and electron-deficient alkynes.

Depending on the alkyne used, the vinylsulfide linkage can be cleaved by the addition of a thiol. This observation was used to develop FRET-based fluorescent[201] and luminescent probes.[202] In the latter example, the alkyne groups are attached to an iridium(III) complex. In a more recent example, the same group has demonstrated the attachment of phosphorescent Ir^{III} complexes appended to an alkynoic amide to peptides and proteins. The resulting conjugates display long emission lifetimes and large Stokes shifts, and might therefore be useful in cell-imaging studies.[203]

Ekkebus et al. incorporated a propargylamide group onto the C terminus of ubiquitin as a cycloaddition handle,[204] and unexpectedly observed irreversible inhibition of corresponding deubiquitinases—which, incidentally, were cysteine proteases—at stoichiometric amounts (Scheme 19). Cysteine was found to be the site of attachment for the alkyne, as supported by several pieces of experimental evidence, such as by competition experiments with *N*-ethylmaleimide, and also by solving of the crystal structure of the enzyme·substrate complex. The crystal structure revealed that the terminal alkyne can react with cysteine within the protease active site, which is

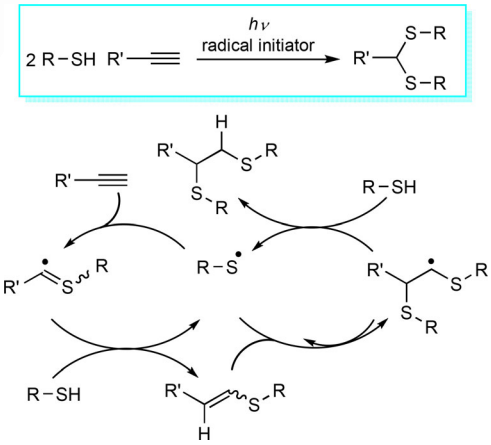


Scheme 19. Cysteine proteases react with alkynes.

lined with multiple partial positive charges of hydrogen-bond-donor groups, thus creating an environment favouring the reaction transition state, leading to the formation of a quaternary vinyl thioether (Scheme 19). The reaction is completely dependent on the presence of the enzyme, because the C-terminal alkyne is unreactive towards thiols in excess and towards other cysteine residues within proteins. Sommer et al. made similar observations in a report published soon after, with SUMO (a ubiquitin-like protein) and ubiquitin.[205] They reported the high specificity of the reaction, and deduced that it was driven by the close proximity and alignment of the alkyne and the thiolate ion in the enzyme oxoanion hole, hence driving the reaction forward. This remarkable discovery of irreversible inhibition of cysteine proteases in such a selective manner might find use in activity-based protein probes, potentially for many protease–substrate partners.[206,207]

6.4. Radical-mediated thiol-yne coupling

The photoinitiated thiol-yne reaction, which can be considered the "sister" reaction of the thiol-ene reaction, ordinarily results in two thiol groups adding across a single alkyne, which leads to functionalisation in a multivalent fashion (Scheme 20). After irradiation between 254–470 nm (UV/Vis range), the thiyl radical adds to the triple bond to give an intermediate vinyl thioether that then undergoes a second (faster) thiyl radical addition (formally a thiol-ene reaction) to yield a dithioether with exclusive 1,2-addition (Scheme 20). Once again, the reaction is prevalent in polymer synthesis, and its multifunctional nature has led to applications in supramolecular chemistry, dendrimer synthesis and the immobilisation of various materials.[208–211]



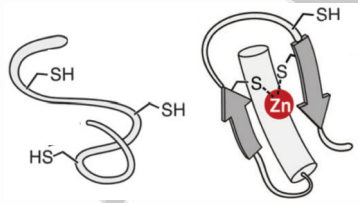
Scheme 20. Thiol-yne reaction mechanism. ■■*please explain back-facing arrow bottom right*■■

The requirements for a radical initiator and/or irradiation have minimised its application to protein chemistry, due to worries about protein stability under such conditions.

In the context of proteins, BSA has been dually functionalised through its cysteine residues[196] (including through a native disulfide bond as a result of irradiation, as observed previously[186]), and Li et al. opted to incorporate an alkyne handle genetically for reaction with bi-dansyl-cystamine in an analogous fashion to an alkene handle described previously.[212] The thiol-yne reaction has been applied less than other methods to proteins through cysteine, but further understanding of it by, for example, the publication of studies into optimisation of conditions and reaction outcomes[213] might lead to wider uses as a facile method for dual functionalisation of a protein.

7. Use of Metals in Cysteine Modification

Metal-mediated conversions are widespread in organic chemistry; ■■certain *OK?*■■ metals' high functional group tolerance means that they can be employed in synthetic steps involving substrates containing multiple functionalities. Historically, the use of metals in chemical protein modification has been quite uncommon, and there are worries about perceived toxic effects of certain metals in vivo, meaning that intra- or extracellular modifications might not be feasible.[214,215] However, protein modification strategies are useful outside of the body too, such as in the construction of antibody–drug conjugates[2] or for radiolabelling prior to patient administration.[216] In fact, selective reactions at cysteine are not best suited for in vivo transformations, due to the presence of exofacial and plasma thiols.[217] Previously, metal-mediated reactions activating otherwise inert endogenous amino acids had been developed, resulting in the addition of reactions to the protein modification "toolbox".[218–220] Sulfur atoms within cysteine residues are capable of binding to a wide range of metal ions, including iron, zinc and cadmium (namely soft metal centres).[19,221] Protein immobilisation onto gold plates through cysteine residues for surface plasmon resonance (SPR) is well established.[222] Sulfur's affinity towards metals has contributed to cysteine being used in thiol protection strategies and metal-mediated modifications.



Scheme 21. Protection and folding with the aid of Zn^{2+}. Taken from Rodriguez et al.[225]

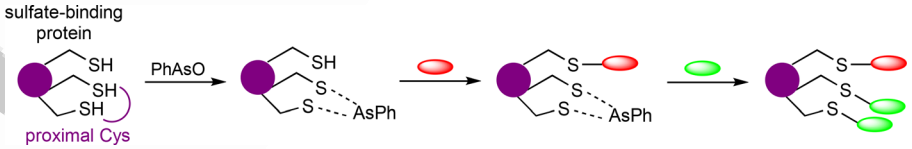
7.1. Metal protection

Many have addressed the need for multiple modifications within a single protein by use of unnatural amino acids,[223] but Smith et al. took a different approach—independently labelling multiple thiols by using sufficiently differentially reactive cysteine residues. The differential reactivity was achieved by reversible protection of cysteine residues through metal coordination. Maltose-binding protein (MBP) containing a fused Cys_2His_2 zinc finger domain together with a single additional cysteine residue was functionalised with two different maleimide-containing fluorophores. Zinc was added, temporarily blocking the reactivity of the cysteine residues within the zinc finger domain whilst the free cysteine was functionalised, and then the zinc was removed, and the remaining cysteine residues were functionalised.[224] ■■Smith et al. *OK?*■■ also used the technique to immobilise glucose-binding protein (GBP) onto glass slides. A recent publication reports the use of the coordination of Zn^{2+} ions to the Cys_2His_2 motif to alkylate another free cysteine for the synthesis of DNA-binding zinc finger conjugates.[225] In this study, the purpose of Zn coordination was twofold—it blocked the cysteine residues' reactivity, but also behaved as a folding agent, assembling the DNA-binding portion (Scheme 21).

Kuiper et al. used a similar strategy but protected two vicinal thiol groups (spaced 6.3–7.3 Å apart) with phenyl arsenic oxide, whilst engaging a free cysteine in reaction in sulfate-binding protein from *Salmonella typhimurium*.[226] Two thiolate groups are able to form a high-affinity ring structure with phenyl arsenic oxide, which is removable by DTT (Scheme 22). Significant engineering to find optimal conditions for forming the complex was required in the study, but these might also be applicable to other protein substrates to which the method might be applied, with the exception of those containing solvent-exposed disulfide bonds. Puljung et al. have also used metals in a protective way.[227,228] They found that the microenvironment of the cysteine is what defines its affinity towards metals. It is increased by incorporation of cysteine into minimal binding sites in existing secondary structural motifs, so reactivity can be tweaked.

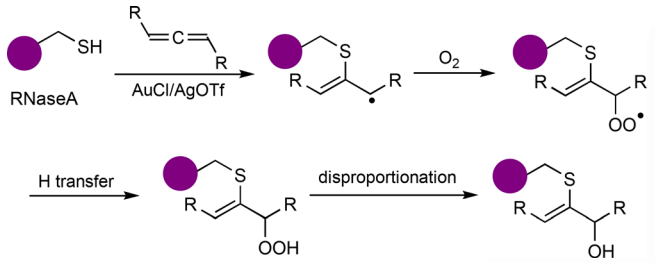
7.2. Mediation by metals

An emerging field involves the use of metals as mediators for selective modifications at cysteine. Chan et al. demonstrated



Scheme 22. Thiol protection with phenyl arsenic oxide, leading to modification through cysteine with two different groups.

gold-mediated oxidative allene–thiol coupling on proteins under mild reaction conditions, forming hydroxy vinyl thioethers (Scheme 23).[229] The gold activates the π system of the allene. The proposed mechanism is the formation of a thiolene radical intermediate that is generated by the reaction of the gold compound, the allene and the thiol. The seemingly anomalous hydroxy group is probably obtained through capture of molecular oxygen, hydrogen atom transfer and disproportionation (Scheme 23).

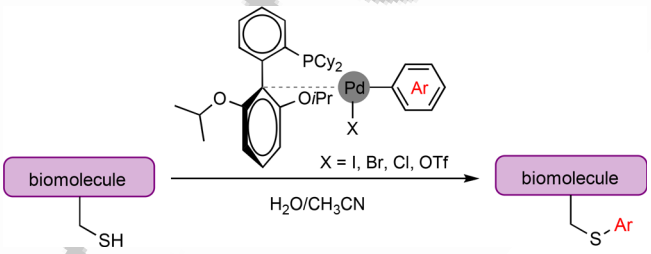


Scheme 23. Gold-mediated coupling of proteins with allene compounds.

Gold has also been used for selective modification at cysteine through well-defined complexation. Cysteine is reported to interact with cyclometallated gold(III) complexes in cancer cell lines,[230] and Kung et al. used this observation to carry out ligand-controlled carbon–sulfur bond formation by use of cyclometallated gold(III) complexes with bidentate C,N-donor ligands and ancillary ligands.[231] After complexation, reductive elimination yielded the alkylated cysteine. Although impressive work, the compatibility of the gold complexes with bio-macromolecular systems is questionable. The large and hydrophobic gold substrates are probably insoluble in aqueous systems; hence the use of 90% DMSO solution for the modifications of BSA and human serum albumin (HSA). Improvements to the aqueous solubility of the modification reagent might help make the procedure more general.

Rhodium-catalysed transformation at cysteine by its alkylation through S–H insertion of a metallocarbene intermediate (formed by reaction between a rhodium complex and a diazo reagent, Scheme 24) is possible. The thioether linkage formed on the protein cystic fibrosis transmembrane conductance regulator-associated ligand and its PDZ domain (CALP) was found to be more stable than thiosuccinimide linkages (formed by reactions with maleimides, Section 3) in human plasma serum, indicating potential applicability in a therapeutic context. However, the method's generality is restricted by crossreaction with solvent-exposed tryptophan, which can perhaps be alleviated by mutagenesis to an amino acid that is inert under the reaction conditions.[232]

Most recently, Vinogradova et al. have described the arylation of cysteine residues for peptide stapling and in the construction of ADCs with the aid of palladium(II) complexes (Scheme 25).[233] Pd and thiol reagents had previously been considered incompatible, owing to observations of Pd-catalysed cross-couplings being inhibited by thiols and of Pd^{II} complexes exhibiting protease-like behaviour. The researchers alleviated these factors by careful choice of ligand, and reported rapid conversions (< 30 min) into arylated biomolecules under mild conditions with peptides, proteins (antibody mimetics) and the therapeutically relevant antibody trastuzumab.



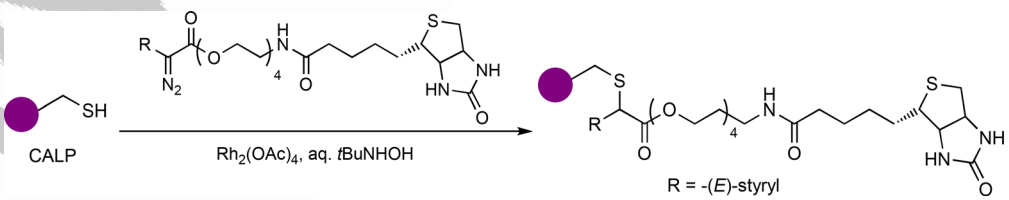
Scheme 25. Pd-mediated cysteine arylation.

The use of metals in cysteine-based protein modification is both underdeveloped and exciting. The use of metal-mediated "protection" still allows a cysteine residue to be used as a uniquely reactive handle, despite the presence of other cysteine residues. It remains to be seen whether metal-mediated reactions, newly applied to proteins, will be useful for further applications, largely due to solubility issues.

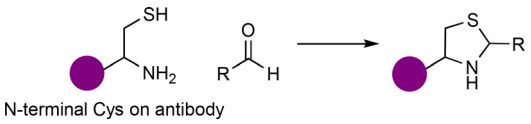
8. Miscellaneous

8.1. Thiazolidine formation

A thiazolidine is formed by condensation between amine and thiol on the same entity with an aldehyde or ketone. A cysteine residue at the N terminus of a protein is itself a 1,2-aminothiol, and can be conjugated to an aldehyde-bearing moiety, as demonstrated by Casi et al. with the clinical stage human antibody F8 and the cytotoxic drug cematodin (Scheme 26).[234,235] The reaction is a clever demonstration of orthogonality with an endogenous amino acid. Because an



Scheme 24. Rh-mediated cysteine modification.



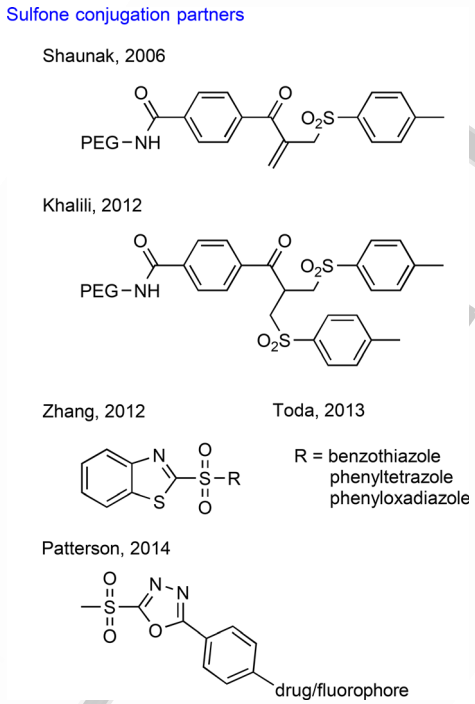
Scheme 26. Antibody modification based on use of an N-terminal cysteine residue and an aldehyde.

amino group is required for reaction, only an N-terminal cysteine is reactive, thus making mutation of other cysteine residues within the sequence (if present) unnecessary. Because methionine is the first amino acid incorporated during protein biosynthesis, incorporation of an appropriate upstream cleavable amino acid sequence is required. The obtained ADC exhibited favourable properties as a releasable, and therefore separable, conjugate in vitro, regenerating the cysteine and aldehyde, thus leading to drug release. However, there is precedence that intracellular oxidation of aldehydes to their corresponding carboxylic acids might have a cytotoxic effect, specifically in cancer stem cells.[234]

As an alternative conjugation strategy, the authors proposed that a C-terminal cysteine can be induced to react with a heterobifunctional maleimide-thiazolidine reagent and that the thiazolidine can be deprotected with methoxyamine and then induced to react with an aldehyde-bearing moiety.[234] This strategy is also relevant to internal cysteine residues. This second strategy requires more steps, which is disadvantageous, especially in view of the wealth of maleimide-modified conjugation partners available. However, maleimide-functionalised reagents are prone to hydrolysis (converting them to an unreactive state), whereas aldehydes are not, yet the second strategy depends on maleimide–thiol coupling. One could argue that use of the aldehyde portion is unnecessary if coupling is to be carried out through a maleimide–thiol reaction, and that a maleimide-modified drug would suffice. For applications in which a stable linkage is required—for in vivo imaging or diagnostics, for example—thiazolidine formation would be unsuitable, due to the instability of the ring.[236]

8.2. Reactions with aryl sulfones and ■■similar reagents *OK?*■■

Cysteine residues within proteins can also be induced to react selectively with functionalised sulfone reagents through Michael addition for the purpose of improving therapeutic efficacy through, for example, PEGylation. Shaunak et al. used the α,β-unsaturated β-monosulfone with PEG to bridge disulfides and to PEGylate proteins simultaneously.[237] The reaction is a sequential addition/elimination process and is initiated by thiol addition to the double bond, followed by sulfinic acid elimination, and then by addition by another thiol. The same dual-purpose functionalisation can also be carried out with a latently reactive bis-sulfone, in which elimination of the sulfinic acid occurs in situ, generating a reagent suitable for nucleophilic attack by a cysteine thiol (Scheme 27).[238] Pfisterer et al. synthesised bis-sulfones also containing iodine or ethynyl moieties and conjugated these to the disulfide bridge of somatostatin.[239] Wang et al. used the same bis-sulfone scaffold with the R group being a drug or fluorophore to bridge the disulfide bond of somatostin. The resulting bridged conjugate was intact when incubated with low concentrations of GSH, but reducible at higher levels, thus showing potential for release in intracellular tumour delivery systems.[240] An impressive example exhibiting the versatility and tuneability of cysteine conjugation was demonstrated by the same group,[241] who synthesised a thiol-reactive cross-conjugation partner containing a maleimide group at one end and a bis-sulfone at the other. At a lower pH, the bis-sulfone remains intact and reaction proceeds between the first bio-macromolecule and maleimide. Then, at pH 8, the bis-sulfone is reactive, allowing conjugation to the second bio-macromolecule. A recent publication on bis-sulfones as disulfide "intercalators" further emphasises the broad applicability of this approach.[242] Badescu et al. further developed a monosulfone conjugation partner in which the conjugate has increased stability. After conjugation to a single cysteine residue, the carbonyl group adjacent to an aryl group can be reduced ■■with a hydride to afford an alcohol that is not susceptible *OK?*■■ to elimination at a neutral pH.[243]



Scheme 27. Sulfone conjugation partners.

Zhang et al. reported on MSBT (2-(methanesulfonyl)benzothiazole) as a cysteine-blocking reagent for analytical purposes.[244] They proposed that the ring portion of MSBT would be susceptible to nucleophilic attack in the presence of a suitable adjacent leaving group (Scheme 27). After tests at a small-molecule level, they tested reactivity on a protein, and found by western blot detection after a biotin-switch assay that cysteine(s) had been blocked. Although no further clear experimental evidence was delivered, Toda et al. were inspired by this strategy, and showed that a range of heteroaromatic rings could be employed for efficient and selective cysteine modifi-

cation (Scheme 27). They used the methodology to modify HSA with PEG and fluorophore moieties.[245] Patterson et al. treated a phenyloxadiazole sulfone drug with the antibody trastuzumab, resulting in an ADC with improved plasma stability relative to the maleimide-linked drug (Scheme 27).[246] Finally, a fluorescein-derivatised phenyloxadiazole sulfone has recently been employed in a dual modification strategy of antibodies.[247]

8.3. Reactions with vinylsulfones

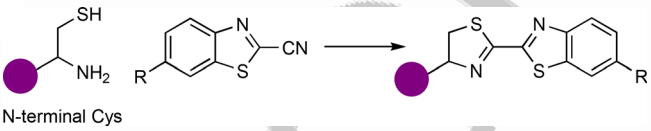
Masri and Friedman described Michael addition between cysteine and aliphatic vinylsulfones on BSA and wool in 1988.[248] They found that crossreactivity with nucleophilic histidine and lysine amine groups could be avoided by lowering the reaction pH. In addition, a smaller amount of equivalents of reagents minimises crossreactivity with lysine and histidine residues, which is beneficial from an economic point of view.[249] The conjugation has potential wide applicability as a general method for selectively modifying proteins because it can be carried out under ambient conditions and does not require any catalysts or produce by-products. The thioether bond formed is stable, and the vinylsulfone itself is more stable in storage than the hydrolysis-prone maleimide.

Morpurgo et al. tested the potential of the reaction for applied research by PEGylating ribonuclease with a vinylsulfone-functionalised PEG moiety.[250] Vinylsulfones derivatised with relevant and varied groups are synthetically accessible, and in recent years this reaction has been described for a wealth of applications, covalent protein immobilisation in particular.[251,252] In addition, more sophisticated compounds containing two orthogonally reactive groups can be prepared, allowing dual modification.[253] Ortega-Muñoz et al. described the synthesis of vinylsulfone-functionalised silica and subsequent immobilisation of cysteine-containing enzymes.[254] The hydroxy groups of the silica support could be functionalised with vinylsulfone groups in two steps, ready for reaction with cysteine-containing biomolecules. The authors state that as well as cysteine, lysine and histidine residues are also reactive; however, their immobilisation procedure is carried out at pH 7.5, and no direct determination of exactly by which residues the enzymes are immobilised was carried out. For the purposes of this application, control of site- and residue-specificity is paramount in order to avoid loss of biological activity on immobilisation. Despite this, in a later study, plant thioredoxins were covalently immobilised onto vinylsulfone-functionalised silica resin prior to successful investigation of their functions.[255] Furthermore, proteins can be immobilised onto super-paramagnetic nanoparticles functionalised with vinylsulfone groups.[256]

Stanley et al. have recently described the use of a tosyl-substituted sulfone bearing an adjacent electron-withdrawing group. Initial reaction with a cysteine results in an activity-based probe bearing a vinylsulfide linkage, which can then undergo further reaction with a proximal thiol in an irreversible fashion. This methodology can be used to profile transthiolation activity.[257]

8.4. Reactions with 2-cyanobenzothiazoles

Rao's group reported on the condensation reaction between an N-terminal cysteine residue and a 2-cyanobenzothiazole (2-CBT) to form a luciferin core linkage in 2009 (Scheme 28). The reaction is rapid in nature and proceeds under mild conditions suitable for chemical protein modification.[258] Site-specificity

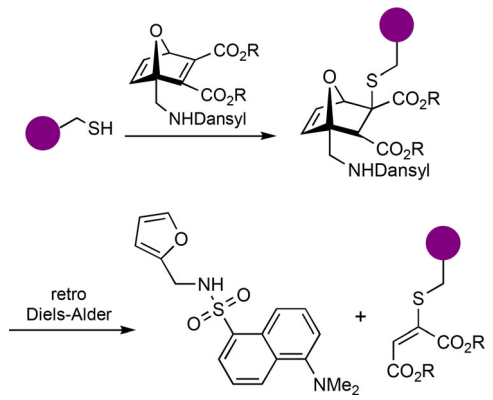


Scheme 28. N-terminal cysteine modification with 2-cyanobenzothiazoles.

was further confirmed by specific labelling of proteins ■■containing N-terminal cysteine residues expressed on cell surfaces *OK?*■■.[259] The fact that the reaction only works with an N-terminal cysteine residue is both an advantage and disadvantage. On one hand, it allows selectivity even in the presence of native cysteine residues, yet the N-terminal cysteine residue needs to be engineered in because methionine residues are the normal N-terminal residues. This can be carried out by expressed protein ligation (EPL) through fusion to an intein and subsequent pH-induced cleavage,[260] or by expression with a protease cleavage site such as in tobacco etch virus (TEV) protease.[259,261] The reaction has proven to be quite versatile and has been used for the covalent immobilisation of peptides and proteins on 2-CBT-functionalised microarrays[260,261] and for the labelling of proteins with ^{18}F for tumour imaging by PET,[262] amongst others. In a xenograft mouse model, a good tumour-to-background contrast was obtained, with the exception of high accumulation in the liver; this can be attributed to the lipophilicity of the obtained luciferin linkage and can probably be modified by chemical manipulation of the 2-CBT reagent. For more details and applications of this reaction to proteins, readers are referred to a recent review.[263] Most recently, the reaction has been employed alongside other orthogonal chemistries for the bridging of cells.[264]

8.5. 7-Oxanobornadiene dicarboxylates

The Finn laboratory have described the use of 7-oxanobornadiene dicarboxylate (OND) frameworks as conjugation partners for cysteine in BSA (also reporting crossreactivity with amine groups).[265] ONDs are potent Michael acceptors, the reactivities of which can be tailored by varying the functionalities adjacent to the reactive double bond. OND frameworks can be functionalised to a high degree, including with fluorophores, usually in three to five steps. It was found that attachment of a dansyl moiety to the ring portion resulted in a quenching of fluorescence and that conjugation regenerated this. As well as this fluorogenic quality, it was also found that a retro Diels–Alder can take place, releasing a furan moiety and a thioether (Scheme 29). This was found to be dependent on substituents,

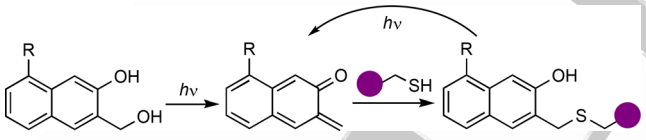


Scheme 29. Oxanorbornadiene dicarboxylates as conjugation partners for cysteine.

thus allowing release behaviour to be adjusted according to the desired application.[266]

8.6. Photochemical alkylation

A method for rapid photoinduced dehydrative modification at cysteine was presented by Arumugam et al. They photochemically generated a diverse range of ■■3-(hydroxymethyl)naphthalen-2-ol *OK?*■■ derivatives from 2-naphthoquinone-3-methides, which were able to react with cysteine within BSA to form thioether linkages under brief irradiation at 350 nm; these linkages remained intact under ambient conditions.[267] The reaction was found to be reversible under further irradiation under dilute conditions, and it might have application in light-directed drug delivery involving caged substrates



Scheme 30. Reversible photochemical modification of cysteine.

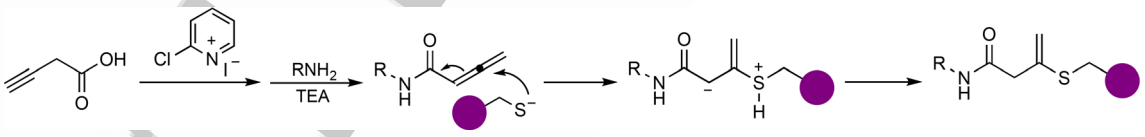
(Scheme 30). Similarly to the thiol-ene and -yne reactions, generality is dependent on the stability of the protein to irradiation (Section 6).

8.7. Allenamides

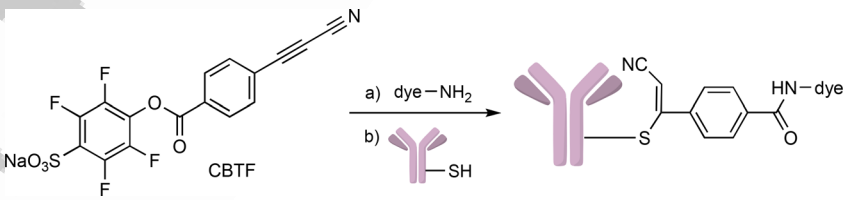
A wide range of synthetically accessible C-substituted allenamide reagents were able to react with cysteine residues in peptides and proteins.[268] These reagents showed exceptional stabilities, with no signs of hydrolysis or polymerisation. The reactions proceed by Michael addition, resulting in vinylsulfide linkages (Scheme 31). Conjugates were found to be stable, and should have application in settings in which a stable and irreversible bond is desired, such as for imaging purposes.

8.8. Arylpropionitriles

The Wagner lab have recently shown that 3-arylpropionitrile reagents are suitable as selective coupling partners for cysteine.[269] These electron-deficient reagents, which are functionalisable, undergo efficient and selective reaction with cysteine residues. The conjugates formed were tested for stability in serum, and were found to exhibit stabilities superior to those of conjugates formed by maleimide–thiol coupling. The reaction scope has been further developed for syntheses of heterobifunctional linkers for use in therapeutic conjugations such as in antibody–drug conjugate synthesis. Sodium 4-((4-(cyanoethynyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (CBTF) was synthesised and presented as an alternative to the commonly used SMCC, containing an arylpropionitrile system at one terminus for conjugation to cysteine on an antibody and an ester linkage for amide formation with an amine-containing moiety on the other terminus, in this case dyes for analytical purposes (Scheme 32).[54] The conjugates were found to be highly stable in plasma, and the methodology should find application in drug conjugation strategies, as well as in other fields. Indeed, a range of heterobifunctional linkers containing arylpropionitrile groups have recently become commercially available, including a compound containing a maleimide and an arylpropionitrile moiety for thiol–thiol conjugation.[54]



Scheme 31. Reaction between cysteine and C-substituted allenamides.



Scheme 32. Formation of antibody–dye conjugates by use of arylpropionitriles.

9. Summary and Outlook

This review has focused on the importance of cysteine as a tool for site-selective and specific protein modification, as exemplified by numerous application-based reports, and the emergence of new methodologies in just the last few years alone. The introduction of cysteine into a protein is in general much more facile and straightforward than unnatural amino acid incorporation techniques involving manipulation of biosynthetic machinery. In addition, selectivity superior to that achievable with alternative reactive endogenous amino acids can be obtained.

Protein modification involving cysteine is more industrially feasible, as shown by the emergence of ADCs based on cysteine chemistry already available on the market. As well as single site modification, dual modifications can be achieved, thanks to differential reactivities of varying cysteine residues (pK_a is highly dependent on environment) as well as cysteine's wide reactivity profile. We hypothesise that cysteine-based chemistry will continue to expand in the years to come and, in particular, look forward to seeing further development of these exciting and varied methodologies in various applications.

Note added in proof: Hocek's group demonstrated DNA–protein crosslinking between a DNA-binding portion of the tumour suppressor protein p53, and the binding DNA itself, in which one nucleobase was modified with a vinylsulfonamide group for Michael addition with a cysteine. Positively, out of two cysteine residues present in p53, only the one within the DNA-binding region was reactive.[270] In further work, a bifunctional conjugation moiety containing both an azide and a vinylsulfonamide was presented as a bioconjugation reagent.[271]

Acknowledgements

We would like to thank Dr. Emily Lumley for proofreading and IWT (Agentschap voor Innovatie door Wetenschap en Technologie) for funding.

Keywords: chemical protein modification · cysteine · proteins · site-selectivity · sulfur

[1] R. V. J. Chari, M. L. Miller, W. C. Widdison, *Angew. Chem. Int. Ed.* **2014**, *53*, 3796 – 3827; *Angew. Chem.* **2014**, *126*, 3872 – 3904.
[2] S. Panowski, S. Bhakta, H. Raab, P. Polakis, J. R. Junutula, *mAbs* **2014**, *6*, 34 – 45.
[3] B. K. Raliski, C. A. Howard, D. D. Young, *Bioconjugate Chem.* **2014**. ■ ■ *details?* ■ ■
[4] C. S. McKay, M. G. Finn, *Chem. Biol.* **2014**, *21*, 1075 – 1101.
[5] E. C. Hett, H. Xu, K. F. Geoghegan, A. Gopalsamy, E. Kyne, C. A. Menard, A. Narayanan, M. D. Parikh, L. Roberts, R. P. Robinson, M. A. Tones, L. H. Jones, *ACS Chem. Biol.* **2015**, *10*, 1094 – 1098.
[6] O. Koniev, A. Wagner, *Chem. Soc. Rev.* **2015**, *44*, 5495 – 5551.
[7] K. Lang, J. W. Chin, *Chem. Rev.* **2014**, *114*, 4764 – 4806.
[8] A. Dumas, L. Lercher, C. D. Spicer, B. G. Davis, *Chem. Sci.* **2015**, *6*, 50 – 69.
[9] M. Rashidian, J. K. Dozier, M. D. Distefano, *Bioconjugate Chem.* **2013**, *24*, 1277 – 1294.
[10] P. M. Levine, T. W. Craven, R. Bonneau, K. Kirshenbaum, *Chem. Commun.* **2014**, *50*, 6909 – 6912.
[11] J. J. Bellucci, J. Bhattacharyya, A. Chilkoti, *Angew. Chem. Int. Ed.* **2014**, *54*, 441 – 445.
[12] D. R. Liu, T. J. Magliery, M. Pastrnak, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10092 – 10097.
[13] C. H. Kim, J. Y. Axup, P. G. Schultz, *Curr. Opin. Chem. Biol.* **2013**, *17*, 412 – 419.
[14] R. Uprety, J. Luo, J. Liu, Y. Naro, S. Samanta, A. Deiters, *ChemBioChem* **2014**, *15*, 1793 – 1799.
[15] D. P. Nguyen, M. Mahesh, S. J. Elsässer, S. M. Hancock, C. Uttamapinant, J. W. Chin, *J. Am. Chem. Soc.* **2014**, *136*, 2240 – 2243.
[16] G. Bulaj, T. Kortemme, D. P. Goldenberg, *Biochemistry* **1998**, *37*, 8965 – 8972.
[17] N. M. Giles, A. B. Watts, G. I. Giles, F. H. Fry, J. A. Littlechild, C. Jacob, *Chem. Biol.* **2003**, *10*, 677 – 693.
[18] D. A. Shannon, E. Weerapana, *Biopolymers* **2014**, *101*, 156 – 164.
[19] C. Jacob, G. I. Giles, N. M. Giles, H. Sies, *Angew. Chem. Int. Ed.* **2003**, *42*, 4742 – 4758; *Angew. Chem.* **2003**, *115*, 4890 – 4907.
[20] J. M. Chalker, G. J. L. Bernardes, Y. A. Lin, B. G. Davis, *Chem. Asian J.* **2009**, *4*, 630 – 640.
[21] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776 – 779.
[22] L. R. Malins, R. J. Payne, *Curr. Opin. Chem. Biol.* **2014**, *22*, 70 – 78.
[23] M. Chen, P. Heimer, D. Imhof, *Amino Acids* **2015**, *47*, 1283 – 1299.
[24] H. Jo, R. M. Culik, I. V. Korendovych, W. F. Degrado, F. Gai, *Biochemistry* **2010**, *49*, 10354 – 10356.
[25] J. M. Chalker, C. S. C. Wood, B. G. Davis, *J. Am. Chem. Soc.* **2009**, *131*, 16346 – 16347.
[26] C. Mayer, D. G. Gillingham, T. R. Ward, D. Hilvert, *Chem. Commun.* **2011**, *47*, 12068 – 12070.
[27] W. Xia, P. S. Low, *J. Med. Chem.* **2010**, *53*, 6811 – 6824.
[28] B. D. Smith, J. J. Higgin, R. T. Raines, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5029 – 5032.
[29] L. H. Tey, E. J. Loveridge, R. S. Swanwick, S. L. Flitsch, R. K. Allemann, *FEBS J.* **2010**, *277*, 2171 – 2179.
[30] C. Vala, F. Chrétien, E. Balentova, S. Lamandé-Langle, Y. Chapleur, *Tetrahedron Lett.* **2011**, *52*, 17 – 20.
[31] S. Lamandé-Langle, C. Collet, R. Hensienne, C. Vala, F. Chrétien, Y. Chapleur, A. Mohamadi, P. Lacolley, V. Regnault, *Bioorg. Med. Chem.* **2014**, *22*, 6672 – 6683.
[32] H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. Degrado, D. C. Greenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 17704 – 17713.
[33] L. E. J. Smeenk, N. Dailly, H. Hiemstra, J. H. van Maarseveen, P. Timmerman, *Org. Lett.* **2012**, *14*, 1194 – 1197.
[34] A. Iyer, D. Van Lysebetten, Y. R. García, B. Louage, B. G. De Geest, A. Madder, *Org. Biomol. Chem.* **2015**, *13*, 3856 – 3862.
[35] S. P. Brown, A. B. Smith, *J. Am. Chem. Soc.* **2015**, *137*, 4034 – 4037.
[36] M. Sunbul, L. Nacheva, A. Jäschke, *Bioconjugate Chem.* **2015**, *26*, 1466 – 1469.
[37] T. Hamamoto, M. Sisido, T. Ohtsuki, M. Taki, *Chem. Commun.* **2011**, *47*, 9116 – 9118.
[38] G. Li, Q. Liang, P. Gong, A. H. Tencer, Z. Zhuang, *Chem. Commun.* **2014**, *50*, 216 – 218.
[39] P. Moody, V. Chudasama, R. I. Nathani, A. Maruani, S. Martin, M. E. B. Smith, S. Caddick, *Chem. Commun.* **2014**, *50*, 4898 – 4900.
[40] R. Huang, M. A. Holbert, M. K. Tarrant, S. Curtet, D. R. Colquhoun, B. M. Dancy, B. C. Dancy, Y. Hwang, Y. Tang, K. Meeth, R. Marmorstein, R. N. Cole, S. Khochbin, P. A. Cole, *J. Am. Chem. Soc.* **2010**, *132*, 9986 – 9987.
[41] J. Löfblom, J. Feldwisch, V. Tolmachev, J. Carlsson, S. Ståhl, F. Y. Frejd, *FEBS Lett.* **2010**, *584*, 2670 – 2680.
[42] A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946 – 5949.
[43] C. Zhang, A. M. Spokoyny, Y. Zou, M. D. Simon, B. L. Pentelute, *Angew. Chem. Int. Ed.* **2013**, *52*, 14001 – 14005; *Angew. Chem.* **2013**, *125*, 14251 – 14255.
[44] C. Zhang, P. Dai, A. M. Spokoyny, B. L. Pentelute, *Org. Lett.* **2014**, *16*, 3652 – 3655.
[45] Y. Zou, A. M. Spokoyny, C. Zhang, M. D. Simon, H. Yu, Y.-S. Lin, B. L. Pentelute, *Org. Biomol. Chem.* **2014**, *12*, 566 – 573.
[46] J. E. Moore, W. H. Ward, *J. Am. Chem. Soc.* **1956**, *78*, 2414 – 2418.
[47] T. C. Tsao, K. Bailey, *Biochim. Biophys. Acta* **1953**, *11*, 102 – 113.

[48] E. Friedmann, D. H. Marrian, I. Simonreuss, *Br. J. Pharmacol. Chemother.* **1949**, *4*, 105 – 108.
[49] A. Witter, H. Tuppy, *Biochim. Biophys. Acta* **1960**, *45*, 429 – 442.
[50] C. W. Wu, L. R. Yarbrough, *Biochemistry* **1976**, *15*, 2863 – 2868.
[51] M. E. Ourailidou, J. Y. van der Meer, B. J. Baas, M. Jeronimus-Stratingh, A. L. Gottumukkala, G. J. Poelarends, A. J. Minnaard, F. J. Dekker, *ChemBioChem* **2014**, *15*, 209 – 212.
[52] J. R. Junutula, K. M. Flagella, R. A. Graham, K. L. Parsons, E. Ha, H. Raab, S. Bhakta, T. Nguyen, D. L. Dugger, G. Li, E. Mai, G. D. L. Phillips, H. Hiraragi, R. N. Fuji, J. Tibbitts, R. Vandlen, S. D. Spencer, R. H. Scheller, P. Polakis, M. X. Sliwkowski, *Clin. Cancer Res.* **2010**, *16*, 4769 – 4778.
[53] N. Goel, S. Stephens, *mAbs* **2010**, *2*, 137 – 147.
[54] O. Koniev, S. Kolodych, Z. Baatarkhuu, J. Stojko, J. Eberova, J.-Y. Bonnefoy, S. Cianférani, A. Van Dorsselaer, A. Wagner, *Bioconjugate Chem.* **2015**, *26*, 197 – 200. ■■*ACS website gives authors as: S. Kolodych, O. Koniev, Z. Baatarkhuu, J.-Y. Bonnefoy, F. Debaene, S. Cianférani, A. Van Dorsselaer, A. Wagner – please check (i.e. are your original [270] and [271] the same reference?)*■■
[55] A. Younes, U. Yasothan, P. Kirkpatrick, *Nat. Rev. Drug Discovery* **2012**, *11*, 19 – 20.
[56] J. R. Junutula, H. Raab, S. Clark, S. Bhakta, D. D. Leipold, S. Weir, Y. Chen, M. Simpson, S. P. Tsai, M. S. Dennis, Y. Lu, Y. G. Meng, C. Ng, J. Yang, C. C. Lee, E. Duenas, J. Gorrell, V. Katta, A. Kim, K. McDorman, et al., *Nat. Biotechnol.* **2008**, *26*, 925 – 932.
[57] C. E. Dumelin, S. Trüssel, F. Buller, E. Trachsel, F. Bootz, Y. Zhang, L. Mannocci, S. C. Beck, M. Drumea-Mirancea, M. W. Seeliger, C. Baltes, T. Müggler, F. Kranz, M. Rudin, S. Melkko, J. Scheuermann, D. Neri, *Angew. Chem. Int. Ed.* **2008**, *47*, 3196 – 3201; *Angew. Chem.* **2008**, *120*, 3240 – 3245.
[58] S. Trüssel, C. Dumelin, K. Frey, A. Villa, F. Buller, D. Neri, *Bioconjugate Chem.* **2009**, *20*, 2286 – 2292.
[59] M. Simon, R. Frey, U. Zangemeister-Wittke, A. Plückthun, *Bioconjugate Chem.* **2013**, *24*, 1955 – 1966.
[60] Z. Cheng, O. Padilla De Jesus, D. J. Kramer, A. De, J. M. Webster, O. Gheysens, J. Levi, M. Namavari, S. Wang, J. M. Park, R. Zhang, B. Lee, F. A. Syud, S. S. Gambhir, *Mol. Imaging Biol.* **2010**, *12*, 316 – 324.
[61] R. Tavaré, R. T. M. de Rosales, P. J. Blower, G. E. D. Mullen, *Bioconjugate Chem.* **2009**, *20*, 2071 – 2081.
[62] A. Díaz-Rodríguez, B. G. Davis, *Curr. Opin. Chem. Biol.* **2011**, *15*, 211 – 219.
[63] P. J. Deuss, R. Denheeten, W. Laan, P. C. J. Kamer, *Chem. Eur. J.* **2011**, *17*, 4680 – 4698.
[64] W. Laan, B. K. Muñoz, R. den Heeten, P. C. J. Kamer, *ChemBioChem* **2010**, *11*, 1236 – 1239.
[65] Y. Zhang, V. S. Bhatt, G. Sun, P. G. Wang, A. F. Palmer, *Bioconjugate Chem.* **2008**, *19*, 2221 – 2230.
[66] I. Haralampiev, M. Mertens, R. Schwarzer, A. Herrmann, R. Volkmer, P. Wessig, P. Müller, *Angew. Chem. Int. Ed.* **2015**, *54*, 323 – 326; *Angew. Chem.* **2015**, *127*, 328 – 332.
[67] T. Li, S. Takeoka, *Int. J. Nanomed.* **2013**, *8*, 3855 – 3866.
[68] T. Li, S. Takeoka, *Int. J. Nanomed.* **2014**, *9*, 2849 – 2861.
[69] B. Egenberger, V. Gorboulev, T. Keller, D. Gorbunov, N. Gottlieb, D. Geiger, T. D. Mueller, H. Koepsell, *J. Biol. Chem.* **2012**, *287*, 31561 – 31573.
[70] H. Neuweiler, W. Banachewicz, A. R. Fersht, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22106 – 22110.
[71] C. A. Lemmon, T. Ohashi, H. P. Erickson, *J. Biol. Chem.* **2011**, *286*, 26375 – 26382.
[72] V. Voynov, N. Chennamsetty, V. Kayser, H. J. Wallny, B. Helk, B. L. Trout, *Bioconjugate Chem.* **2010**, *21*, 385 – 392.
[73] H. Y. Song, M. H. Ngai, Z. Y. Song, P. A. MacAry, J. Hobley, M. J. Lear, *Org. Biomol. Chem.* **2009**, *7*, 3400 – 3406.
[74] Y. He, Y. Ding, D. Wang, W. Zhang, W. Chen, X. Liu, W. Qin, X. Qian, H. Chen, Z. Guo, *Chem. Sci.* **2015**, *6*, 2074 – 2078.
[75] A. Izquierdo-Álvarez, A. Martínez-Ruiz, *J. Proteomics* **2011**, *75*, 329 – 338.
[76] H. Chuang, S. Lin, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 20097 – 20102.
[77] J. Fraser, I. Boo, P. Poumbourios, H. E. Drummer, *J. Biol. Chem.* **2011**, *286*, 31984 – 31992.
[78] B. Santoso, S. Lam, B. W. Murray, G. Chen, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 5680 – 5683.
[79] M. H. Stenzel, *ACS Macro Lett.* **2013**, *2*, 14 – 18.
[80] L. Milane, Z. Duan, M. Amiji, *Mol. Pharmaceutics* **2011**, *8*, 185 – 203.
[81] J. V. Georgieva, R. P. Brinkhuis, K. Stojanov, C. A. G. M. Weijers, H. Zuilhof, F. P. J. T. Rutjes, D. Hoekstra, J. C. M. van Hest, I. S. Zuhorn, *Angew. Chem. Int. Ed.* **2012**, *51*, 8339 – 8342; *Angew. Chem.* **2012**, *124*, 8464 – 8467.
[82] L. C. Radu, J. Yang, J. Kopecek, *Macromol. Biosci.* **2009**, *9*, 36 – 44.
[83] R. N. Johnson, P. Kopečková, J. Kopeček, *Bio-macromolecules* **2012**, *13*, 727 – 735.
[84] L. Tao, C. S. Kaddis, R. R. Q. Loo, G. N. Grover, J. A. Loo, H. D. Maynard, *Macromolecules* **2009**, *42*, 8028 – 8033.
[85] P. De, M. Li, S. R. Gondi, B. S. Sumerlin, *J. Am. Chem. Soc.* **2008**, *130*, 11288 – 11289.
[86] E. M. Ahmed, *J. Adv. Res.* **2015**, *6*, 105 – 121. ■■*correct year?*■■
[87] F. Ito, K. Usui, D. Kawahara, A. Suenaga, T. Maki, S. Kidoaki, H. Suzuki, M. Taiji, M. Itoh, Y. Hayashizaki, T. Matsuda, *Biomaterials* **2010**, *31*, 58 – 66.
[88] H. Wang, A. Han, Y. Cai, Y. Xie, H. Zhou, J. Long, Z. Yang, *Chem. Commun.* **2013**, *49*, 7448 – 7450.
[89] S. T. Kang, C. K. Yeh, *Ultrason. Sonochem.* **2011**, *18*, 327 – 333.
[90] F. Danhier, A. Le Breton, V. Préat, *Mol. Pharmaceutics* **2012**, *9*, 2961 – 2973.
[91] M.-Y. Tsai, C.-Y. Lin, C.-H. Huang, J.-A. Gu, S.-T. Huang, J. Yu, H.-Y. Chen, *Chem. Commun.* **2012**, *48*, 10969 – 10971.
[92] N. Kotagiri, Z. Li, X. Xu, S. Mondal, A. Nehorai, S. Achilefu, *Bioconjugate Chem.* **2014**, *25*, 1272 – 1281.
[93] J. D. Rodwell, V. L. Alvarez, C. Lee, A. D. Lopes, J. W. Goers, H. D. King, H. J. Powsner, T. J. McKearn, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 2632 – 2636.
[94] M. R. Lewis, J. E. Shively, *Bioconjugate Chem.* **1998**, *9*, 72 – 86.
[95] S. C. Alley, D. R. Benjamin, S. C. Jeffrey, N. M. Okeley, D. L. Meyer, R. J. Sanderson, P. D. Senter, *Bioconjugate Chem.* **2008**, *19*, 759 – 765.
[96] A. D. Baldwin, K. L. Kiick, *Bioconjugate Chem.* **2011**, *22*, 1946 – 1953.
[97] S. C. Alley, N. M. Okeley, P. D. Senter, *Curr. Opin. Chem. Biol.* **2010**, *14*, 529 – 537.
[98] B.-Q. Shen, K. Xu, L. Liu, H. Raab, S. Bhakta, M. Kenrick, K. L. Parsons-Reponte, J. Tien, S.-F. Yu, E. Mai, D. Li, J. Tibbitts, J. Baudys, O. M. Saad, S. J. Scales, P. J. McDonald, P. E. Haas, C. Eigenbrot. T. Nguyen, W. A. Solis, et al., *Nat. Biotechnol.* **2012**, *30*, 184 – 189.
[99] S. D. Fontaine, R. Reid, L. Robinson, G. W. Ashley, D. V. Santi, *Bioconjugate Chem.* **2015**, *26*, 145 – 152.
[100] A. D. Baldwin, K. L. Kiick, *Polym. Chem.* **2012**, 133 – 143. ■■*volume 3?*■■
[101] R. P. Lyon, J. R. Setter, T. D. Bovee, S. O. Doronina, J. H. Hunter, M. E. Anderson, C. L. Balasubramanian, S. M. Duniho, C. I. Leiske, F. Li, P. D. Senter, *Nat. Biotechnol.* **2014**, *32*, 1059 – 1062.
[102] L. M. Tedaldi, M. E. B. Smith, R. I. Nathani, J. R. Baker, *Chem. Commun.* **2009**, 6583 – 6585.
[103] M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick, J. R. Baker, *J. Am. Chem. Soc.* **2010**, *132*, 1960 – 1965.
[104] C. Marculescu, H. Kossen, R. E. Morgan, P. Mayer, S. A. Fletcher, B. Tolner, K. A. Chester, L. H. Jones, J. R. Baker, *Chem. Commun.* **2014**, *50*, 7139 – 7142.
[105] L. Castañeda, Z. V. F. Wright, C. Marculescu, T. M. Tran, V. Chudasama, A. Maruani, E. A. Hull, J. P. M. Nunes, R. J. Fitzmaurice, M. E. B. Smith, L. H. Jones, S. Caddick, J. R. Baker, *Tetrahedron Lett.* **2013**, *54*, 3493 – 3495.
[106] P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy, S. Caddick, *ChemBioChem* **2012**, *13*, 39 – 41.
[107] G. Saito, J. A. Swanson, K. D. Lee, *Adv. Drug Delivery Rev.* **2003**, *55*, 199 – 215.
[108] F. F. Schumacher, M. Nobles, C. P. Ryan, M. E. B. Smith, A. Tinker, S. Caddick, J. R. Baker, *Bioconjugate Chem.* **2011**, *22*, 132 – 136.
[109] J. Collins, J. Tanaka, P. Wilson, K. Kempe, T. P. Davis, M. P. McIntosh, M. R. Whittaker, D. M. Haddleton, *Bioconjugate Chem.* **2015**, *26*, 633 – 638.
[110] M. W. Jones, R. A. Strickland, F. F. Schumacher, S. Caddick, J. R. Baker, M. I. Gibson, D. M. Haddleton, *J. Am. Chem. Soc.* **2012**, *134*, 1847 – 1852.
[111] M. W. Jones, R. A. Strickland, F. F. Schumacher, S. Caddick, J. R. Baker, M. I. Gibson, D. M. Haddleton, *Chem. Commun.* **2012**, *48*, 4064 – 4066.

[112] F. F. Schumacher, J. P. M. Nunes, A. Maruani, V. Chudasama, M. E. B. Smith, K. A. Chester, J. R. Baker, S. Caddick, *Org. Biomol. Chem.* **2014**, *12*, 7261 – 7269.
[113] L. Castañeda, A. Maruani, F. F. Schumacher, E. Miranda, V. Chudasama, K. A. Chester, J. R. Baker, M. E. B. Smith, S. Caddick, *Chem. Commun.* **2013**, *49*, 8187 – 8189.
[114] F. Bryden, A. Maruani, H. Savoie, V. Chudasama, M. E. B. Smith, S. Caddick, R. W. Boyle, *Bioconjugate Chem.* **2014**, *25*, 611 – 617.
[115] E. A. Hull, M. Livanos, E. Miranda, M. E. B. Smith, K. A. Chester, J. R. Baker, *Bioconjugate Chem.* **2014**, *25*, 1395 – 1401.
[116] F. F. Schumacher, V. A. Sanchania, B. Tolner, Z. V. F. Wright, C. P. Ryan, M. E. B. Smith, J. M. Ward, S. Caddick, C. W. M. Kay, G. Aeppli, K. A. Chester, J. R. Baker, *Sci. Rep.* **2013**, *3*, 1525.
[117] M. S. S. Palanki, A. Bhat, B. Bolanos, F. Brunel, J. Del Rosario, D. Dettling, M. Horn, R. Lappe, R. Preston, A. Sievers, N. Stankovic, G. Woodnut, G. Chen, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 402 – 406.
[118] J. Youziel, A. R. Akhbar, Q. Aziz, M. E. B. Smith, S. Caddick, A. Tinker, J. R. Baker, *Org. Biomol. Chem.* **2014**, *12*, 557 – 560.
[119] M. P. Robin, P. Wilson, A. B. Mabire, J. K. Kiviaho, J. E. Raymond, D. M. Haddleton, R. K. O'Reilly, *J. Am. Chem. Soc.* **2013**, *135*, 2875 – 2878.
[120] B. Rudolf, M. Salmain, E. Fornal, A. Rybarczyk-Pirek, *Appl. Organomet. Chem.* **2012**, *26*, 80 – 85.
[121] C. P. Ryan, M. E. B. Smith, F. F. Schumacher, D. Grohmann, D. Papaioannou, G. Waksman, F. Werner, J. R. Baker, S. Caddick, *Chem. Commun.* **2011**, *47*, 5452 – 5454.
[122] R. I. Nathani, V. Chudasama, C. P. Ryan, P. R. Moody, R. E. Morgan, R. J. Fitzmaurice, M. E. B. Smith, J. R. Baker, S. Caddick, *Org. Biomol. Chem.* **2013**, *11*, 2408 – 2411.
[123] M. E. B. Smith, M. B. Caspersen, E. Robinson, M. Morais, A. Maruani, J. P. M. Nunes, K. Nicholls, M. J. Saxton, S. Caddick, J. R. Baker, V. Chudasama, *Org. Biomol. Chem.* **2015**, *13*, 7946 – 7949.
[124] V. Chudasama, M. E. B. Smith, F. F. Schumacher, D. Papaioannou, G. Waksman, J. R. Baker, S. Caddick, *Chem. Commun.* **2011**, *47*, 8781 – 8783.
[125] A. Maruani, S. Alom, P. Canavelli, M. T. W. Lee, R. E. Morgan, V. Chudasama, S. Caddick, *Chem. Commun.* **2015**, *51*, 5279 – 5282.
[126] A. Maruani, M. E. B. Smith, E. Miranda, K. A. Chester, V. Chudasama, S. Caddick, *Nat. Commun.* **2015**, *6*, 6645.
[127] G. Bulaj, *Biotechnol. Adv.* **2005**, *23*, 87 – 92.
[128] J. M. Chalker, G. J. L. Bernardes, B. G. Davis, *Acc. Chem. Res.* **2011**, *44*, 730 – 741.
[129] S. van Kasteren, *Biochem. Soc. Trans.* **2012**, *40*, 929 – 944.
[130] G. L. Ellman, *Arch. Biochem. Biophys.* **1959**, *82*, 70 – 77.
[131] G. J. L. Bernardes, G. Casi, S. Trüssel, I. Hartmann, K. Schwager, J. Scheuermann, D. Neri, *Angew. Chem. Int. Ed.* **2012**, *51*, 941 – 944; *Angew. Chem.* **2012**, *124*, 965 – 968.
[132] T. List, G. Casi, D. Neri, *Mol. Cancer Ther.* **2014**, *13*, 2641 – 2652.
[133] F. López-Gallego, O. Abian, J. M. Guisán, *Biochemistry* **2012**, *51*, 7028 – 7036.
[134] C. Chatterjee, R. K. McGinty, B. Fierz, T. W. Muir, *Nat. Chem. Biol.* **2010**, *6*, 267 – 269.
[135] J. Hu, Z. Zhang, W. J. Shen, A. Nomoto, S. Azhar, *Biochemistry* **2011**, *50*, 10860 – 10875.
[136] C. M. Borghese, J. A. Hicks, D. J. Lapid, J. R. Trudell, R. A. Harris, *J. Neurochem.* **2014**, *128*, 363 – 375.
[137] X. Sainsily, J. Cabana, P. E. Boulais, B. J. Holleran, E. Escher, P. Lavigne, R. Leduc, *Biochem. Pharmacol.* **2013**, *86*, 1584 – 1593.
[138] M. Xu, C. T. Smothers, J. J. Woodward, *J. Pharmacol. Exp. Ther.* **2015**, *353*, 91 – 101.
[139] X. Rong, X. Zhang, S. Qu, *Int. J. Biochem. Cell Biol.* **2015**, *60*, 1 – 7.
[140] J. D. Carter, J. D. Mathias, E. F. Gomez, Y. Ran, L. Galiano, N. Q. Tran, P. W. D'Amore, D. K. Chakravorty, G. E. Fanucci, *J. Phys. Chem. B* **2014**, *118*, 10607 – 10617.
[141] M. R. Wilson, Z. Hou, L. H. Matherly, *J. Biol. Chem.* **2014**, *289*, 25287 – 25295.
[142] Y. J. Zhao, Y. Q. Zhai, Z. G. Su, G. H. Ma, *Polym. Adv. Technol.* **2010**, *21*, 867 – 873.
[143] S. I. van Kasteren, D. Keane, H. Ovaa, C. Watts, *ACS Chem. Biol.* **2011**, *6*, 1198 – 1204.
[144] T. M. Postma, F. Albericio, *Eur. J. Org. Chem.* **2014**, 3519 – 3530.
[145] P. A. Wender, E. A. Goun, L. R. Jones, T. H. Pillow, J. B. Rothbard, R. Shinde, C. H. Contag, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 10340 – 10345.
[146] S. Ganta, H. Devalapally, A. Shahiwala, M. Amiji, *J. Controlled Release* **2008**, *126*, 187 – 204.
[147] A. M. Sauer, A. Schlossbauer, N. Ruthardt, V. Cauda, T. Bein, C. Bräuchle, *Nano Lett.* **2010**, *10*, 3684 – 3691.
[148] U. Langel, *Cell-Penetrating Peptides: Methods and Protocols*, ■■*publisher, city?*■■ **2011**.
[149] T. L. Lamoureaux, D. H. Lee, *Chem. Commun.* **2011**, *47*, 8623 – 8625.
[150] B. Schuster, J. Rétey, *FEBS Lett.* **1994**, *349*, 252 – 254.
[151] R. Bar-Or, L. T. Rael, D. Bar-Or, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3567 – 3577.
[152] R. W. MacKintosh, K. N. Dalby, D. G. Campbell, P. T. W. Cohen, P. Cohen, C. MacKintosh, *FEBS Lett.* **1995**, *371*, 236 – 240.
[153] C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk, *Chem. Rev.* **2005**, *105*, 633 – 683.
[154] M. C. Bagley, J. W. Dale, E. A. Merritt, X. Xiong, *Chem. Rev.* **2005**, *105*, 685 – 714.
[155] J. M. Chalker, S. B. Gunnoo, O. Boutureira, S. C. Gerstberger, M. Fernández-González, G. J. L. Bernardes, L. Griffin, H. Hailu, C. J. Schofield, B. G. Davis, *Chem. Sci.* **2011**, *2*, 1666 – 1676.
[156] D. Rich, J. Tam, C. Mathiaparanam, P. Grant, J. A. Mabuni, *J. Chem. Soc. Chem. Commun.* **1974**, 897 – 898.
[157] G. Holmes, T. J. Lawton, Jr., R. R. Lawton, *J. Am. Chem. Soc.* **1977**, *99*, 1984 – 1986.
[158] J. F. DuMond, M. W. Wright, S. B. King, *J. Inorg. Biochem.* **2013**, *118*, 140 – 147.
[159] G. J. L. Bernardes, J. M. Chalker, J. C. Errey, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 5052 – 5053.
[160] Z. U. Wang, Y. S. Wang, P. J. Pai, W. K. Russell, D. H. Russell, W. R. Liu, *Biochemistry* **2012**, *51*, 5232 – 5234.
[161] J. M. Chalker, Y. A. Lin, O. Boutureira, B. G. Davis, *Chem. Commun.* **2009**, 3714 – 3716.
[162] Y. A. Lin, J. M. Chalker, B. G. Davis, *ChemBioChem* **2009**, *10*, 959 – 969.
[163] O. Boutureira, G. J. L. Bernardes, F. D'Hooge, B. G. Davis, *Chem. Commun.* **2011**, *47*, 10010 – 10012.
[164] M. Fernández-González, O. Boutureira, G. J. L. Bernardes, J. M. Chalker, M. A. Young, J. C. Errey, B. G. Davis, *Chem. Sci.* **2010**, *1*, 709 – 715.
[165] E. J. Grayson, G. J. L. Bernardes, J. M. Chalker, O. Boutureira, J. R. Koeppe, B. G. Davis, *Angew. Chem. Int. Ed.* **2011**, *50*, 4127 – 4132; *Angew. Chem.* **2011**, *123*, 4213 – 4218.
[166] J. M. Chalker, L. Lercher, N. R. Rose, C. J. Schofield, B. G. Davis, *Angew. Chem. Int. Ed.* **2012**, *51*, 1835 – 1839; *Angew. Chem.* **2012**, *124*, 1871 – 1875.
[167] K. P. Chooi, S. R. G. Galan, R. Raj, J. McCullagh, S. Mohammed, L. H. Jones, B. G. Davis, *J. Am. Chem. Soc.* **2014**, *136*, 1698 – 1701.
[168] Y. A. Lin, O. Boutureira, L. Lercher, B. Bhushan, R. S. Paton, B. G. Davis, *J. Am. Chem. Soc.* **2013**, *135*, 12156 – 12159.
[169] S. B. Gunnoo, H. M. Finney, T. S. Baker, A. D. Lawson, D. C. Anthony, B. G. Davis, *Nat. Commun.* **2014**, *5*, 4388.
[170] J. M. Chalker, B. G. Davis, *Curr. Opin. Chem. Biol.* **2010**, *14*, 781 – 789.
[171] C. Aydillo, I. Compañón, A. Avenoza, J. H. Busto, F. Corzana, J. M. Peregrina, M. M. Zurbano, *J. Am. Chem. Soc.* **2014**, *136*, 789 – 800.
[172] F. Rowan, M. Richards, M. Widya, R. Bayliss, J. Blagg, *PLoS One* **2014**, *9*, e103935.
[173] F. C. Rowan, M. Richards, R. A. Bibby, A. Thompson, R. Bayliss, J. Blagg, *ACS Chem. Biol.* **2013**, *8*, 2184 – 2191.
[174] N. Timms, C. L. Windle, A. Polyakova, J. R. Ault, C. H. Trinh, A. R. Pearson, A. Nelson, A. Berry, *ChemBioChem* **2013**, *14*, 474 – 481.
[175] J. M. Donaldson, C. Kari, R. C. Fragoso, U. Rodeck, J. C. Williams, *Cancer Biol. Ther.* **2009**, *8*, 2147 – 2152.
[176] N. Haj-Yahya, H. P. Hemantha, R. Meledin, S. Bondalapati, M. Seenaiah, A. Brik, *Org. Lett.* **2014**, *16*, 540 – 543.
[177] E. Branigan, C. Pliotas, G. Hagelueken, J. H. Naismith, *Nat. Protoc.* **2013**, *8*, 2090 – 2097.
[178] R. Nathani, P. Moody, M. E. B. Smith, R. J. Fitzmaurice, S. Caddick, *ChemBioChem* **2012**, *13*, 1283 – 1285.
[179] R. I. Nathani, P. Moody, V. Chudasama, M. E. B. Smith, R. J. Fitzmaurice, S. Caddick, *Chem. Sci.* **2013**, *4*, 3455 – 3458.

These are not the final page numbers!

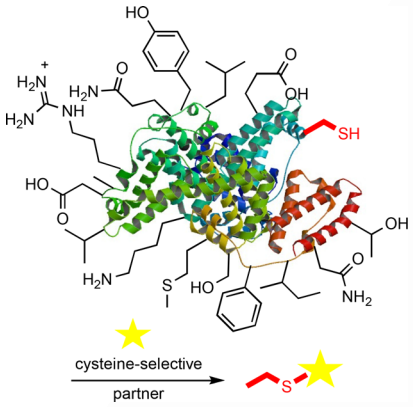
[180] C. E. Hoyle, C. N. Bowman, *Angew. Chem. Int. Ed.* **2010**, *49*, 1540 – 1573; *Angew. Chem.* **2010**, *122*, 1584 – 1617.
[181] C. E. Hoyle, A. B. Lowe, C. N. Bowman, *Chem. Soc. Rev.* **2010**, *39*, 1355 – 1387.
[182] Y. Chen, G. Triola, H. Waldmann, *Acc. Chem. Res.* **2011**, *44*, 762 – 773.
[183] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021; *Angew. Chem.* **2001**, *113*, 2056 – 2075.
[184] A. Dondoni, *Angew. Chem. Int. Ed.* **2008**, *47*, 8995 – 8997; *Angew. Chem.* **2008**, *120*, 9133 – 9135.
[185] Y. Li, M. Yang, Y. Huang, X. Song, L. Liu, P. R. Chen, *Chem. Sci.* **2012**, *3*, 2766.
[186] A. Dondoni, A. Massi, P. Nanni, A. Roda, *Chem. Eur. J.* **2009**, *15*, 11444 – 11449.
[187] N. Floyd, B. Vijayakrishnan, J. R. Koeppe, B. G. Davis, *Angew. Chem. Int. Ed.* **2009**, *48*, 7798 – 7802; *Angew. Chem.* **2009**, *121*, 7938 – 7942.
[188] D. Weinrich, P.-C. Lin, P. Jonkheijm, U. T. T. Nguyen, H. Schröder, C. M. Niemeyer, K. Alexandrov, R. Goody, H. Waldmann, *Angew. Chem. Int. Ed.* **2010**, *49*, 1252 – 1257; *Angew. Chem.* **2010**, *122*, 1274 – 1279.
[189] F. Li, A. Allahverdi, R. Yang, G. B. J. Lua, X. Zhang, Y. Cao, N. Korolev, L. Nordenskiöld, C. F. Liu, *Angew. Chem. Int. Ed.* **2011**, *50*, 9611 – 9614; *Angew. Chem.* **2011**, *123*, 9785 – 9788.
[190] E. M. Valkevich, R. G. Guenette, N. a. Sanchez, Y. C. Chen, Y. Ge, E. R. Strieter, *J. Am. Chem. Soc.* **2012**, *134*, 6916 – 6919.
[191] Q.-F. Li, Y. Yang, A. Maleckis, G. Otting, X.-C. Su, *Chem. Commun.* **2012**, *48*, 2704 – 2706.
[192] F. H. Ma, J. L. Chen, Q. F. Li, H. H. Zuo, F. Huang, X. C. Su, *Chem. Asian J.* **2014**, *9*, 1808 – 1816.
[193] C. Hoppmann, R. Kühne, M. Beyermann, *Beilstein J. Org. Chem.* **2012**, *8*, 884 – 889.
[194] C. Hoppmann, V. K. Lacey, G. V. Louie, J. Wei, J. P. Noel, L. Wang, *Angew. Chem. Int. Ed.* **2014**, *53*, 3932 – 3936; *Angew. Chem.* **2014**, *126*, 4013 – 4017.
[195] P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1821 – 1826.
[196] M. Lo Conte, S. Staderini, A. Marra, M. Sanchez-Navarro, B. G. Davis, A. Dondoni, *Chem. Commun.* **2011**, *47*, 11086.
[197] R. van Geel, G. J. M. Pruijn, F. L. van Delft, W. C. Boelens, *Bioconjugate Chem.* **2012**, *23*, 392 – 398.
[198] H.-Y. Shiu, T.-C. Chan, C.-M. Ho, Y. Liu, M.-K. Wong, C.-M. Che, *Chem. Eur. J.* **2009**, *15*, 3839 – 3850.
[199] H.-R. Tsou, N. Mamuya, B. D. Johnson, M. F. Reich, B. C. Gruber, F. Ye, R. Nilakantan, R. Shen, C. Discafani, R. DeBlanc, R. Davis, F. E. Koehn, L. M. Greenberger, Y.-F. Wang, A. Wissner, *J. Med. Chem.* **2001**, *44*, 2719 – 2734.
[200] O. Arjona, R. Medel, J. Rojas, A. M. Costa, J. Vilarrasa, *Tetrahedron Lett.* **2003**, *44*, 6369 – 6373.
[201] H. Y. Shiu, H. C. Chong, Y. C. Leung, M. K. Wong, C. M. Che, *Chem. Eur. J.* **2010**, *16*, 3308 – 3313.
[202] H.-Y. Shiu, M.-K. Wong, C.-M. Che, *Chem. Commun.* **2011**, *47*, 4367 – 4369.
[203] H.-Y. Shiu, H.-C. Chong, Y.-C. Leung, T. Zou, C.-M. Che, *Chem. Commun.* **2014**, *50*, 4375 – 4378.
[204] R. Ekkebus, S. I. van Kasteren, Y. Kulathu, A. Scholten, I. Berlin, P. P. Geurink, A. de Jong, S. Goerdayal, J. Neefjes, A. J. R. Heck, D. Komander, H. Ovaa, *J. Am. Chem. Soc.* **2013**, *135*, 2867 – 2870.
[205] S. Sommer, N. D. Weikart, U. Linne, H. D. Mootz, *Bioorg. Med. Chem.* **2013**, *21*, 2511 – 2517.
[206] C. Arkona, J. Rademann, *Angew. Chem. Int. Ed.* **2013**, *52*, 8210 – 8212; *Angew. Chem.* **2013**, *125*, 8368 – 8370.
[207] P. M. S. D. Cal, G. J. L. Bernardes, P. M. P. Gois, *Angew. Chem. Int. Ed.* **2014**, *53*, 10585 – 10587; *Angew. Chem.* **2014**, *126*, 10758 – 10760.
[208] A. B. Lowe, C. E. Hoyle, C. N. Bowman, *J. Mater. Chem.* **2010**, *20*, 4745 – 4750.
[209] R. Hoogenboom, *Angew. Chem. Int. Ed.* **2010**, *49*, 3415 – 3417; *Angew. Chem.* **2010**, *122*, 3489 – 3491.
[210] A. Massi, D. Nanni, *Org. Biomol. Chem.* **2012**, *10*, 3791 – 3807.
[211] A. Dondoni, A. Marra, *Eur. J. Org. Chem.* **2014**, 3955 – 3969.
[212] Y. Li, M. Pan, Y. Li, Y. Huang, Q. Guo, *Org. Biomol. Chem.* **2013**, *11*, 2624 – 2629.
[213] M. Minozzi, A. Monesi, D. Nanni, P. Spagnolo, N. Marchetti, A. Massi, *J. Org. Chem.* **2011**, *76*, 450 – 459.
[214] J. E. Hudak, R. M. Barfield, G. W. de Hart, P. Grob, E. Nogales, C. R. Bertozzi, D. Rabuka, *Angew. Chem. Int. Ed.* **2012**, *51*, 4161 – 4165; *Angew. Chem.* **2012**, *124*, 4237 – 4241.
[215] C. D. Spicer, T. Triemer, B. G. Davis, *J. Am. Chem. Soc.* **2012**, *134*, 800 – 803.
[216] N. Iznaga-Escobar, *Appl. Radiat. Isot.* **2001**, *54*, 399 – 406.
[217] A. G. Torres, M. J. Gait, *Trends Biotechnol.* **2012**, *30*, 185 – 189.
[218] J. M. Antos, M. B. Francis, *Curr. Opin. Chem. Biol.* **2006**, *10*, 253 – 262.
[219] Z. T. Ball, *Acc. Chem. Res.* **2013**, *46*, 560 – 570.
[220] Z. T. Ball, *Curr. Opin. Chem. Biol.* **2015**, *25*, 98 – 102.
[221] Y. Zhou, X.-M. Xia, C. J. Lingle, *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 5237 – 5242.
[222] M. Iwamoto, H. Shimizu, F. Inoue, T. Konno, Y. C. Sasaki, S. Oiki, *J. Biol. Chem.* **2006**, *281*, 28379 – 28386.
[223] P. Kele, G. Mezö, D. Achatz, O. S. Wolfbeis, *Angew. Chem. Int. Ed.* **2009**, *48*, 344 – 347; *Angew. Chem.* **2009**, *121*, 350 – 353.
[224] J. J. Smith, D. W. Conrad, M. J. Cuneo, H. W. Hellinga, *Protein Sci.* **2005**, *14*, 64 – 73.
[225] J. Rodríguez, J. Mosquera, O. Vázquez, M. E. Vázquez, J. L. Mascareñas, *Chem. Commun.* **2014**, *50*, 2258 – 2260.
[226] J. M. Kuiper, R. Pluta, W. H. Huibers, F. Fusetti, E. R. Geertsma, B. Poolman, *Protein Sci.* **2009**, *18*, 1033 – 1041.
[227] M. C. Puljung, W. N. Zagotta, *Biophys. J.* **2011**, *100*, 2513 – 2521.
[228] M. C. Puljung, W. N. Zagotta, *Curr. Protoc. Protein Sci.* **2012**, *1*, 1 – 10.
[229] A. O.-Y. Chan, J. L.-L. Tsai, V. K.-Y. Lo, G.-L. Li, M.-K. Wong, C.-M. Che, *Chem. Commun.* **2013**, *49*, 1428 – 1430.
[230] J.-J. Zhang, R. W.-Y. Sun, C.-M. Che, *Chem. Commun.* **2012**, *48*, 3388 – 3390.
[231] K. K.-Y. Kung, H.-M. Ko, J.-F. Cui, H.-C. Chong, Y.-C. Leung, M.-K. Wong, *Chem. Commun.* **2014**, *50*, 11899 – 11902.
[232] R. Kundu, Z. T. Ball, *Chem. Commun.* **2013**, *49*, 4166 – 4168.
[233] E. V. Vinogradova, C. Zhang, A. M. Spokoyny, B. L. Pentelute, S. L. Buchwald, *Nature* **2015**, *526*, 687 – 691.
[234] G. Casi, N. Huguenin-Dezot, K. Zuberbühler, J. Scheuermann, D. Neri, *J. Am. Chem. Soc.* **2012**, *134*, 5887 – 5892.
[235] G. J. L. Bernardes, M. Steiner, I. Hartmann, D. Neri, G. Casi, *Nat. Protoc.* **2013**, *8*, 2079 – 2089.
[236] T. H. Fife, R. Natarajan, C. C. Shen, R. Bembi, *J. Am. Chem. Soc.* **1991**, *113*, 3071 – 3079.
[237] S. Shaunak, A. Godwin, J.-W. Choi, S. Balan, E. Pedone, D. Vijayarangam, S. Heidelberger, I. Teo, M. Zloh, S. Brocchini, *Nat. Chem. Biol.* **2006**, *2*, 312 – 313.
[238] H. Khalili, A. Godwin, J. W. Choi, R. Lever, S. Brocchini, *Bioconjugate Chem.* **2012**, *23*, 2262 – 2277.
[239] A. Pfisterer, K. Eisele, X. Chen, M. Wagner, K. Müllen, T. Weil, *Chem. Eur. J.* **2011**, *17*, 9697 – 9707.
[240] T. Wang, D. Y. W. Ng, Y. Wu, J. Thomas, T. TamTran, T. Weil, *Chem. Commun.* **2014**, *50*, 1116 – 1118.
[241] T. Wang, A. Pfisterer, S. L. Kuan, Y. Wu, O. Dumele, M. Lamla, K. Mullen, T. Weil, *Chem. Sci.* **2013**, *4*, 1889 – 1894.
[242] T. Wang, Y. Wu, S. L. Kuan, O. Dumele, M. Lamla, D. Y. W. Ng, M. Arzt, J. Thomas, J. O. Mueller, C. Barner-Kowollik, T. Weil, *Chem. Eur. J.* **2015**, *21*, 228 – 238.
[243] S. Brocchini, G. Badescu, P. Bryant, J. Swierkosz, F. Khayrzad, E. Pawlisz, M. Farys, Y. Cong, N. Rumpf, A. Godwin, *Bioconjugate Chem.* **2013**, *25*, 460 – 469.
[244] D. Zhang, N. O. Devarie-Baez, Q. Li, J. R. Lancaster, M. Xian, *Org. Lett.* **2012**, *14*, 3396 – 3399.
[245] N. Toda, S. Asano, C. F. Barbas, *Angew. Chem. Int. Ed.* **2013**, *52*, 12592 – 12596; *Angew. Chem.* **2013**, *125*, 12824 – 12828.
[246] J. T. Patterson, S. Asano, X. Li, C. Rader, C. F. Barbas, *Bioconjugate Chem.* **2014**, *25*, 1402 – 1407.
[247] X. Li, J. T. Patterson, M. Sarkar, L. Pedzisa, T. Kodadek, W. R. Roush, C. Rader, *Bioconjugate Chem.* **2015**, *26*, 2243 – 2248.
[248] M. S. Masri, M. Friedman, *J. Protein Chem.* **1988**, *7*, 49 – 54.
[249] Z. Wu, L. Li, S. Liu, F. Yakushijin, K. Yakushijin, D. Horne, P. S. Conti, Z. Li, F. Kandeel, J. E. Shively, *J. Nucl. Med.* **2014**, *55*, 1178 – 1184.
[250] M. Morpurgo, F. M. Veronese, D. Kachensky, J. M. Harris, *Bioconjugate Chem.* **1996**, *7*, 363 – 368.

These are not the final page numbers! ↗↗

[251] J. Morales-Sanfrutos, J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Perez-Balderas, F. Hernandez-Mateo, F. Santoyo-Gonzalez, *Org. Biomol. Chem.* **2010**, *8*, 667 – 675.
[252] A. Megia-Fernandez, F. Hernandez-Mateo, F. Santoyo-Gonzalez, *Org. Biomol. Chem.* **2013**, *11*, 2586 – 2596.
[253] J. Morales-Sanfrutos, F. J. Lopez-Jaramillo, F. Hernandez-Mateo, F. Santoyo-Gonzalez, *J. Org. Chem.* **2010**, *75*, 4039 – 4047.
[254] M. Ortega-Muñoz, J. Morales-Sanfrutos, A. Megia-Fernandez, F. J. Lopez-Jaramillo, F. Hernandez-Mateo, F. Santoyo-Gonzalez, *J. Mater. Chem.* **2010**, *20*, 7189 – 7196.
[255] J. A. Traverso, F. J. López-Jaramillo, A. J. Serrato, M. Ortega-Muñoz, D. Aguado-Llera, M. Sahrawy, F. Santoyo-Gonzalez, J. L. Neira, A. Chueca, *J. Plant Physiol.* **2010**, *167*, 423 – 429.
[256] A. L. Medina-Castillo, J. Morales-Sanfrutos, A. Megia-Fernandez, J. F. Fernandez-Sanchez, F. Santoyo-Gonzalez, A. Fernandez-Gutierrez, *J. Polym. Sci. Part A* **2012**, *50*, 3944 – 3953.
[257] M. Stanley, C. Han, A. Knebel, P. Murphy, N. Shpiro, S. Virdee, *ACS Chem. Biol.* **2015**, *10*, 1542 – 1554.
[258] G. Liang, H. Ren, J. Rao, *Nat. Chem.* **2010**, *2*, 54 – 60.
[259] H. Ren, F. Xiao, K. Zhan, Y. P. Kim, H. Xie, Z. Xia, J. Rao, *Angew. Chem. Int. Ed.* **2009**, *48*, 9658 – 9662; *Angew. Chem.* **2009**, *121*, 9838 – 9842.
[260] P. Wang, C.-J. Zhang, G. Chen, Z. Na, S. Q. Yao, H. Sun, *Chem. Commun.* **2013**, *49*, 8644 – 8646.
[261] H.-C. Wang, C. C. Yu, C. F. Liang, L. De Huang, J. R. Hwu, C. C. Lin, *ChemBioChem* **2014**, *15*, 829 – 835.
[262] J. Jeon, B. Shen, L. Xiong, Z. Miao, K. H. Lee, J. Rao, F. T. Chin, *Bioconjugate Chem.* **2012**, *23*, 1902 – 1908.
[263] Y. Yuan, G. Liang, *Org. Biomol. Chem.* **2014**, *12*, 865 – 871.
[264] Y. Yuan, D. Li, J. Zhang, X. Chen, C. Zhang, Z. Ding, L. Wang, X. Zhang, J. Yuan, Y. Li, Y. Kang, G. Liang, *Chem. Sci.* **2015**, *6*, 6425 – 6431.
[265] V. Hong, A. A. Kislukhin, M. G. Finn, *J. Am. Chem. Soc.* **2009**, *131*, 9986 – 9994.
[266] A. A. Kislukhin, C. J. Higginson, V. P. Hong, M. G. Finn, *J. Am. Chem. Soc.* **2012**, *134*, 6491 – 6497.
[267] S. Arumugam, J. Guo, N. E. Mbua, F. Friscourt, N. Lin, E. Nekongo, G.-J. Boons, V. V. Popik, *Chem. Sci.* **2014**, *5*, 1591 – 1598.
[268] A. Abbas, B. Xing, T.-P. Loh, *Angew. Chem. Int. Ed.* **2014**, *53*, 7491 – 7494; *Angew. Chem.* **2014**, *126*, 7621 – 7624.
[269] O. Koniev, G. Leriche, M. Nothisen, J.-S. Remy, J.-M. Strub, C. Schaeffer-Reiss, A. Van Dorsselaer, R. Baati, A. Wagner, *Bioconjugate Chem.* **2014**, *25*, 202 – 206.
[270] J. Dadová, P. Orság, R. Pohl, M. Brázdová, M. Fojta, M. Hocek, *Angew. Chem. Int. Ed.* **2013**, *52*, 10515 – 10518; *Angew. Chem.* **2013**, *125*, 10709 – 10712.
[271] J. Dadová, M. Vrábel, M. Adámik, M. Brázdová, R. Pohl, M. Fojta, M. Hocek, *Chem. Eur. J.* **2015**; DOI: 10.1002/chem.201502209.

Manuscript received: December 14, 2015
Accepted article published: January 20, 2016
Final article published: ■■ ▮▮, 0000

Cysteine residues and bioconjugation: Chemical protein modification is used to construct proteins with enhanced and/or altered properties useful for a vast range of applications. Modification at only a single site ensures homogeneity, and the relatively uncommon and uniquely reactive natural residue cysteine allows for selective reactions with a diverse range of coupling partners.



*S. B. Gunnoo, A. Madder**

■■–■■

Chemical Protein Modification through Cysteine

Please check that the ORCID identifiers listed below are correct. We encourage all authors to provide an ORCID identifier for each coauthor. ORCID is a registry that provides researchers with a unique digital identifier. Some funding agencies recommend or even require the inclusion of ORCID IDs in all published articles, and authors should consult their funding agency guidelines for details. Registration is easy and free; for further information, see http://orcid.org/.

Smita B. Gunnoo
Annemieke Madder