CHEMICAL REVIEWS

Review

Advances in Chemical Protein Modification

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1. INTRODUCTION

Chemical protein modification has emerged as an invaluable tool for the development of modified proteins. The complementary use of both genetic and chemical methods has provided a large toolbox that allows the preparation of almost unlimited protein constructs with either natural or synthetically modified residues.[1] Such a protein chemodiversity, usually achieved after translation and commonly referred to as post-translational protein modifications (PTMs), is often responsible for the vast biodiversity found in nature. These modifications include acylation, methylation, phosphorylation, sulfation, farnesylation, ubiquitination, and glycosylation, among others, and play a pivotal role in important cellular processes including trafficking, differentiation, migration, and signaling.[2] Consequently, reproducing in a highly efficient and controlled way such *natural* modifications of proteins (by introducing natural PTMs) would provide an invaluable tool to study their precise function. Additionally, the possibility offered by the introduction and (bio)orthogonal modification of *unnatural* moieties/amino acids (usually improving the properties of natural PTMs during isolation, analysis, and processing)[3] makes site-selective modification of proteins a key tool for interrogating and intervening biological systems both in vitro and in vivo.[4]

Given the range of chemical modification methods available, it is now possible to decide which residue to target and which modification to attach in order to confer the desired property/ function (affinity probes, fluorophores, reactive tags, etc.). For example, increasing the circulation half-life of a therapeutic protein may be achieved by the addition of polyethylene glycol (PEG). On the other hand, the use of a spectroscopic label to monitor biomolecule distribution in vivo enables the construction of highly selective imaging agents. Despite the vast progress in the field of bioconjugation chemistry, scientists still face many challenges, not only synthetically but also from a processing, manufacturing, safety, and stability perspective.[5] A number of methods have been developed and applied for the modification of particular proteins and therefore may not be applicable to any protein of interest. Thus, there remains a need for the development of complementary reactions for the site-selective chemical modification of proteins that are mild, efficient, and robust. Several reviews covering different aspects of the chemical synthesis of proteins, from general native chemical ligation strategies[6] and the modification of endoge-

Scheme 1. Classical Methods for the Modification of Cys and Lys: (a) Amide Formation, (b) Urea and Thiourea Formation, (c) Reductive Amination, (d) Cys-Specific Disulfide Exchange, (e) Alkylation, and (f) Conjugate Addition to a Representative Maleimide Michael Acceptor
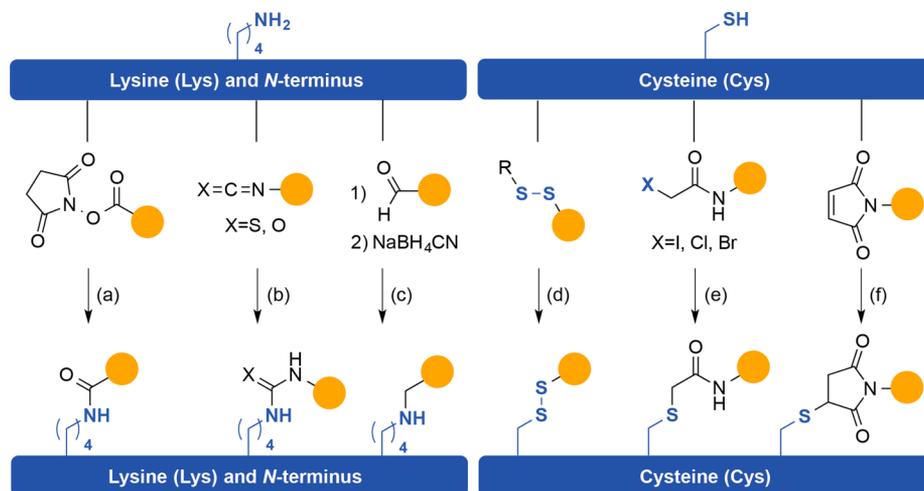
nous amino acids[7] to more specialized topics such as click modification protocols,[8] the introduction of particular PTMs including glycosylation,[9] PEGylation,[5b,10] and polymerization of protein-based initiators,[11] and the challenging labeling of a specific protein of interest in a complex cellular mixture using the so-called "bioorthogonal" reactions,[12] have been published during the past decade.

While the later publications describe different protein syntheses/modifications in detail, the aim of the present review is not to be an exhaustive survey of all available bioconjugation methodologies but to discuss recent chemical strategies for the site-selective modification of proteins such as fast sulfur exchange or stable thioether formation, photo and metal-free cycloadditions, and other particularly challenging metal-mediated protocols. This review will be divided into two sections: transition metal-free and transition metal-mediated approaches. For clarity, we will use the following terminology throughout this manuscript: *residue/amino acid/site-selective* (or simply *site-selective*) reactions are those transformations that preferentially modify one amino acid residue over the others (e.g., cysteine versus lysine) and, thus, can be considered examples of chemoselective reactions; on the other hand transformations described as *regioselective* preferentially modify only one of a set of the same amino acid, in particular when more than one is present in the same molecule (e.g., solvent-exposed lysine versus internal lysine).

2. TRANSITION METAL-FREE APPROACHES

2.1. Classical Methods

The chemical modification of proteins aims to obtain new bioconjugates by performing chemical reactions on their original structures, maintaining both protein integrity and function. The ideal requirements for such reactions are those amenable with proteins such as functional group tolerance/compatibility, selectivity, water as a reaction media, at or near neutral pH and room temperature (or up to 40 °C), high reaction rates, low reactant concentrations, and nontoxic reagents, all to ensure high modification efficiencies that may avoid tedious and often inefficient purification/characterization protocols. Such a method would be a priori suitable for in vivo studies because it would not interfere with normal cell life.

Classical protein bioconjugation methods have traditionally relied upon reactions at nucleophilic amino acids, particularly cysteine (Cys) or lysine (Lys) residues (Scheme 1).[13]

Cysteine easily undergoes disulfide exchange to form mixed disulfides[14] and also alkylation with suitable electrophiles that include α-halocarbonyls (e.g., iodoacetamide)[14] and Michael acceptors (e.g., maleimides or vinyl sulfones)[15] (Scheme 1d−f). The stability of some of the resulting conjugates, particularly those with a 1-*S*-3-carbonyl motif, may be compromised under certain conditions due to retro-Michael side-reactions and other thiol-promoted exchange processes,[16] yet this issue can be minimized by reducing the electron-withdrawing ketone to secondary alcohol under mild conditions in a second step.[17] Although the nucleophilicity of Lys may interfere with classical methods used for Cys modification, the low abundance of Cys in combination with fine-tuning the pH of the media or by modulating the residue's pK_a with different steric and chemical environment enables a certain degree of site-selectivity. Lys modification is a popular protocol because of the availability of methods to preferentially modify primary amines (although often these methods also modify the *N*-terminus).[18] These protocols involve direct reaction with electrophilic reagents such as activated acids, vinyl sulfones, sulfonyl chlorides, iso(thio)cyanates, and squaric acids as well as reductive amination reactions with aldehydes (Scheme 1a−c). More sophisticated alkylation agents, such as cyclohexene sulfonamide derivatives, have recently been developed for the site-selective labeling of Lys in human serum albumin (HSA), although cross-reactivity with Cys may occur in other proteins.[19] Indeed, the modification of Lys is often the first of a subsequent modification step by a second bioorthogonal reaction (e.g., click chemistry). However, the recent needs of well-defined therapeutic proteins such as homogeneous glycoprotein vaccines[20] have promoted extensive work on protocols that enable access to pure constructs even when using such classical methods, which typically show a limited degree of site-selectivity and regioselectivity. Thus, a careful control of reaction conditions allows traditional, robust, and operationally simple albeit a priori nonselective Lys modification protocols to be used with a level of regioselectivity as recently demonstrated by Adamo and co-workers.[21] In this study, the authors have

Scheme 2. Recent Site-Selective Methods for the Modification of Lys and the *N*-Terminal Position: (a) Lys Labeling by Rapid 6π-Aza-electrocyclization Reaction or (b) 2-Imino-2-methoxyethyl Reagents (IME), (c) Reversible Lys and *N*-Terminal Modification via Formation of Stable Iminobornates, (D) Reaction With Diazonium Salts, and (e) Selective *N*-Terminal Modification Using Ketenes
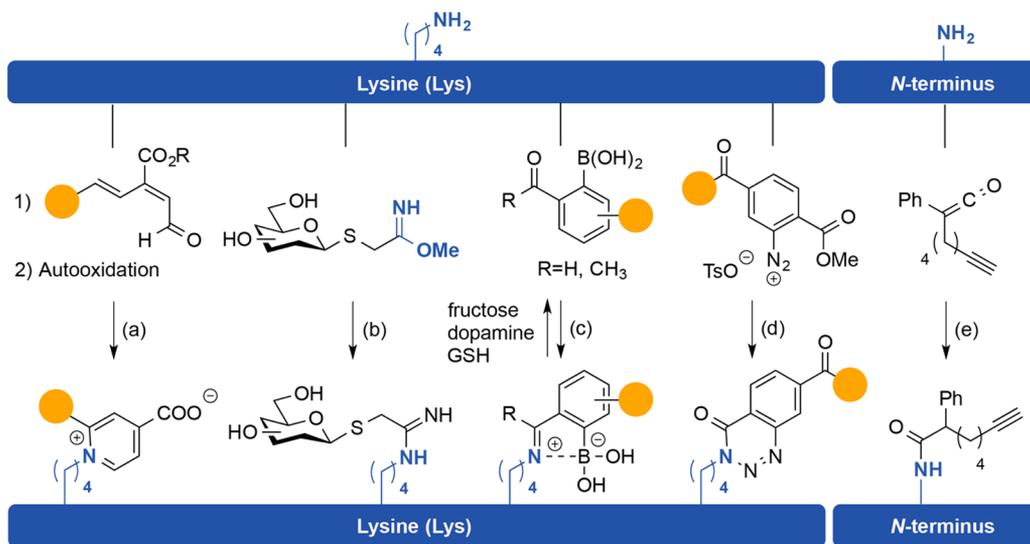
developed reproducible batch-to-batch Lys alkylations of CRM$_{197}$ after mapping their reactivity profile using semi-quantitative LC-MS/MS as well as computational analyses. Similarly, Weil and co-workers reported a site- and regio-selective Lys modification protocol via kinetically controlled acylation by stepwise addition of substoichiometric amounts of an activated *N*-hydroxysuccinimide (NHS) ester under mild conditions.[22] Although this protocol enables the modification of a single Lys, the enhanced reactivity of this residue is determined by a number of factors including solvent accessibility and chemical environment of the surrounding residues; therefore, the regioselective acylation of a different Lys may be difficult. The rational introduction of a Lys in a highly solvent-exposed site may overcome this limitation. Other scarcely utilized nucleophilic residues in classical alkylation/acylation methods include histidine (His), which reacts preferentially over Lys in a cAb-An33 nanobody using vinyl sulfones as alkylating agents,[23] arginine (Arg) and the acidic moieties, aspartic (Asp), and glutamic (Glu) or the *C*-terminus, which have been used, for example, to modulate lipase activity.[24] More recently, diazo compounds also proved suitable for the reversible alkylation of protein carboxylic moieties.[25] Although most of these methods are nonselective, their reliability and robustness (usually generating amide bonds) make them the methods of choice for worldwide biotechnological applications such as immobilization protocols for enzyme-linked immunosorbent assays (ELISAs), microarrays, or polymer beads. Methionine (Met) alkylation has also been exploited for the modification of synthetic polypeptides.[26] The alkylation of thioether groups in Met-containing peptides enable the reversible (by adding several *S*-Nu) introduction of a broad range of functional and reactive groups to yield stable sulfonium derivatives including carbohydrate probes, alkenes, alkynes, azides, boronic acids, and PEG chains. Remarkably, this transformation is compatible with the deprotection of other functional groups and proceeds at low pH (in dimethylforma-mide (DMF), H$_2$O, or 0.2 M aqueous formic acid) unlike other thiol alkylations that require higher pH values. Other nonselective methods not covered in this review include the

protein cross-linking due to generation of highly reactive species, such as carbenes, nitrenes, and radicals.[7]

2.2. Modern Methods for Targeting Natural Amino Acids

2.2.1. Lysine and *N*-Terminus. Despite the general use of nonselective protein modification methods, new chemical tools to access homogeneous proteins for in vitro and in vivo applications have been developed for the modification of natural Cys, Lys, and tyrosine (Tyr) residues. Selected examples of the next generation of Lys modifications are depicted in Scheme 2. A highly selective Lys modification protocol, based on the rapid 6π-aza-electrocyclization reaction, has been successfully developed by Tanaka and Fukase (Scheme 2a).[27] The efficiency of this procedure depends on the accessibility of the primary amino group; hence, excellent selectivities due to very rapid reaction kinetics toward solvent-accessible Lys (10−30 min at 24 °C) were observed when compared with internal and *N*-terminal amines (>5 h at 24 °C). Importantly, this protocol has been not only used for the asymmetric synthesis of pyridine/indole alkaloid-type natural products but also developed into a *nondestructive Lys-labeling Kit named 'Stella$^+$'*, which proved effective as a labeling strategy for biomolecules (e.g., by positron emission tomography (PET) or fluorescence) and living cells.

Another method for selective Lys modification involves the use of 2-imino-2-methoxyethyl reagents (IME) through the formation of an amidine linkage (Scheme 2b). In particular, 2-imino-2-methoxy-1-thioglycosides have been successfully used for the selective modification of an α-L-rhamnopyranosidase[28] and more recently for the glycosylation of RNase A leading to semisynthetic neoglycoconjugates with mannose mono- and disaccharides.[29] Gois and co-workers have also developed a strategy to reversibly modify both the ε-amino group of Lys and the *N*-terminal position, based on the formation of stable iminoboronates in aqueous media (20 mM NH$_4$OAc, pH 7.0) at room temperature (Scheme 2c).[30] This transformation proceeds via formation of a dative B−N linkage, and examples of peptides and proteins successfully modified using this protocol include somatostatin and model proteins such as

Scheme 3. Recent Site-Selective Methods for the Modification of Tyr: (a) Reaction with Diazonium Salts, (b) Three-Component Mannich Reaction, (c) Reaction with Preformed Imines, and (d) Ene-type Reaction with Diazodicarboxylate Reagents
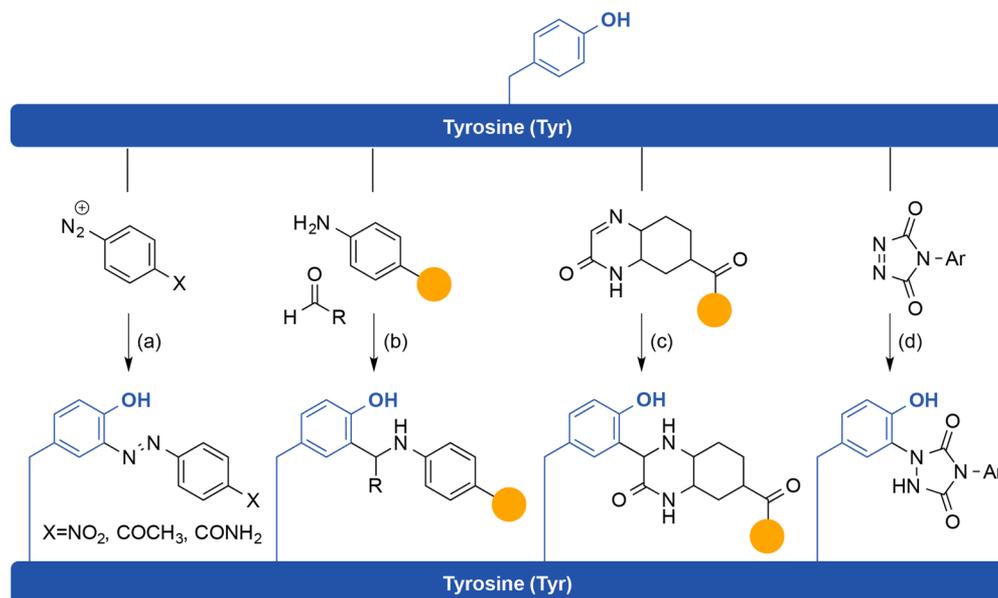
lysozyme, cytochrome c, ribonuclease A, and myoglobin. The modification is stable over a period of 5 h but proved reversible upon the addition of fructose, dopamine, or glutathione, most likely by the disruption of the B−N bond. Although residue-selective, this method is nonregioselective since multiple Lys and N-terminal positions are modified. An application of this technology includes the introduction of a fluorescent motif into the 2-acetylbenzene boronic acid reagent that enabled the modification of not only lysozyme but also N-(2-aminoethyl) folic acid, leading to conjugates that are selectively uptaked by cancer cells that overexpress folic acid receptors.[31] Similarly to the arene diazonium bioconjugation with Tyr described in the following section, Carreira and co-workers have developed an amine-selective method using diazonium terephthalates that ensures the irreversibility of this transformation via formation of a stable triazin-4(3H)-one ring (Scheme 2d).[32] The amino acid selectivity and high rate of this reaction is demonstrated by labeling several small peptide models containing Tyr, primary OH, and CO_2H as "potentially reactive" nucleophilic moieties and myoglobin, which is indeed nonregioselectively labeled at Lys and N-terminal positions with up to six modifications.

Selective modification of N-terminal positions in the presence of a primary ε-amino group from Lys residues is anticipated to be a difficult task. However, an N-terminal modification protocol using ketenes has been recently developed by Wong, Che, and co-workers (Scheme 2e).[33] A series of different peptides and proteins including insulin, lysozyme, RNase A, and the therapeutic protein for cancer treatment BCArg were selectively N-terminally modified using an alkyne-functionalized ketene at moderate temperatures (room temperature or 37 °C) and pH 6.3−9.2, resulting in excellent selectivity (N-terminal amino/ε-amino up to >99:1). The authors rationalize this selectivity on the basis of the lower pK_a of the N-terminal position (~8) versus that for the ε-amino group of Lys (~10), which seems to favor the acylation of the N-terminal amino using ketenes. The local environment of the N-terminal position and its solvent accessible area may also play a role in the observed selectivity. Although not the focus of the

present review, selective N-terminal protein modification can also be efficiently achieved through native chemical ligation (NCL) strategies[4] as well as with a flexible chemoenzymatic approach using simple aminoacyl and fatty acid transferase substrates.[34]

2.2.2. Tyrosine. Several nontransition metal-catalyzed strategies have emerged for the modification of the electron-rich side-chain of Tyr residues in proteins, in a chemoselective fashion (Scheme 3). Initially Francis and co-workers[35] and more recently Barbas and co-workers[36] have independently demonstrated that several diazonium salts bearing electron-withdrawing para substituents (NO_2, $COCH_3$, and $CONH_2$) react chemoselectively with Tyr ortho to the phenol group (Scheme 3a). Interestingly, the introduction of ketones, aldehydes, and more recently alkynes[37] as an additional bioorthogonal handle at the para position allows for a second modification round, for example, with fluorophore reagents. Alternatively, the azo bond can be further reduced and the corresponding aniline can be acylated with, for example, acrylamides, introducing additional reaction points. An interesting complementary variant that works with diazonium derivatives that are substituted with an electron-donating moiety (ArNHCOR with R = CH_3, PEG) employs a genetically encoded 2-naphthol analogue of Tyr (NpOH).[38] The coupling is rapid (~2 h), is selective (NpOH reacts preferentially over Tyr), and requires mild conditions (0 °C and pH 7.0).

Francis and co-workers have also developed a three-component Mannich-type reaction with aldehydes and anilines for the modification of Tyr residues (Scheme 3b).[39] The high selectivity of this transformation is demonstrated by the successful modification of solvent-exposed Tyr such as those found in lysozyme, RNase A, and chymotrypsinogen A. Drawbacks of the method include the unreactivity of more buried Tyr residues (e.g., in horse heart myoglobin) and cross-reactivity with both exposed tryptophan (Trp) residues and reduced disulfides. Interestingly, while Cys residues do not interfere with this reaction, reduced disulfides form dithioacetals. This conjugation method has enabled the introduction of

Scheme 4. Site-Selective Chemical Protein Modification at Cys and Dha: (a) Nucleophilic Disulfide Formation, (b) Diselenide Exchange, (c) Electrophilic Disulfide Formation, (d) Thioether Formation
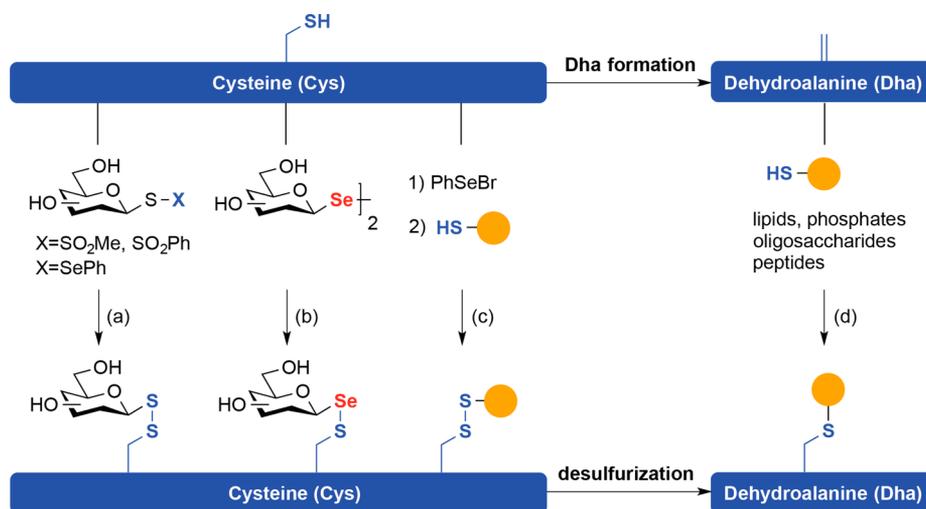
isoindoline-based nitroxide spin labels for electron paramagnetic resonance (EPR) in a small chloroplastic protein CP12, which possess a single tyrosine together with disulfide bonds and Trp residues that remain unmodified.[40] Other methods for Tyr modification include the reaction with preformed imines (Scheme 3c)[41] and also with azomaleimides[42] via an aqueous ene-type reaction that involves a concerted [1,5]-hydride shift from the phenol residue (Tyr) to the RN=NR moiety (Scheme 3d). Recent examples of the later Tyr selective modification approach include the preparation of well-defined glycoconjugate vaccines[43] and DNA−protein conjugates in model streptavidin and myoglobin proteins.[44] A potential drawback of this method is the instability of cyclic diazodicarboxamides via formation of isocyanates in water, which readily react with Lys residues leading to urea byproducts. Yet, the authors overcome this issue by simply using tris(hydroxymethyl)aminomethane (Tris) buffer that functions as an isocyanate scavenger, thus yielding only Tyr-labeled conjugates.[43a]

2.2.3. Cysteine. Unlike the nonselective methods for Cys modification described in the previous section, the development of more selective protocols has grown exponentially.[13] In particular, the recent work by Davis and co-workers based on a "tag-and-modify" approach[45] describes a series of complementary methods for the site-selective Cys modification (Scheme 4). The time line of this rational conjugation approach starts from the easy disulfide exchange reaction to form mixed disulfides as well as a conceptually similar reaction using diselenides (Scheme 4a−c)[46] that produces more stable SeS-linked conjugates; it continues by the transformation of potentially cleavable disulfide linkages to more stable thioethers by desulfurization of disulfides and finishes by developing complementary Cys-elimination methods that give access to dehydroalanine (Dha),[47] a useful Michael acceptor from which thioethers can be obtained directly upon conjugate addition of thiol nucleophiles (Scheme 4d). A recent example by Brik and co-workers exploits the formation of Dha for the preparation of diubiquitin activity probes.[48] Remarkably, these methods allowed not only the incorporation of most of the common PTM mimics such as S-glycosides, thiophosphates, and thioprenyl units in a rapid and selective way but also the creation of more sophisticated proteins by introducing [19]F- and

[18]F-fluorosugars[49] as potential imaging labels, the preparation of PEGylated proteins[50] (e.g., recombinant human granulocyte colony-stimulating factor, rhG-CSF), and homogeneous glycoconjugate vaccine candidates[51] as well as synthetic histone mimetics.[52] Indeed, some of the corresponding glycoproteins are amenable to being enzymatically modified by endoglycosidases (Endo A), giving access to higher glycoprotein complexity without the need of any other round of chemical or genetic protein manipulations.[53] One limitation of the conjugate addition to Dha approach is the potential racemization at the modification site and consequent formation of D/L diastereoisomers. However, thioether PTM mimics of histones obtained by this method were found to be functional both in immunoblot and enzymatic analysis.[52] Chiral dehydroalanines have been proposed to achieve stereoselective sulfa-Michael additions.[54] However, this approach seems limited to simple amino acid models.

Site-selective modification strategies have received particularly attention for the attachment of potent cytotoxic drugs to recombinant antibodies engineered with Cys residues at predetermined sites. Most antibody−drug conjugates (ADCs)[5c] are built through conjugation of N-ethylmaleimide linkers that contain a cleavable site for drug release (e.g., valine−citruline dipeptide).[55] More recently, Neri and co-workers proposed and described two traceless strategies for the modification of antibody fragments where the linkage formed after conjugation between antibody fold and drug is the cleavable site for drug release. The first method (Scheme 5a) explores the reactivity of C-terminal cysteines in the site-selective formation of mixed disulfides and uses Ellman's reagent for the preactivation of the Cys residue.[56] The second (Scheme 5b) targets N-terminal Cys residues and explores the reactivity of the sulfhydryl side-chain with aldehyde-containing drugs[57] or 2-cyanobenzothiazoles[58] (CBTs) to form thiazolidine-linked conjugates.

Other modern Cys modification methods are summarized in Schemes 6−8. These include both alkylation (polar reactions) and more sophisticated photochemical activation methods based on thiol−ene/yne chemistry (radical reactions). The work by Baker, Caddick, and co-workers (Scheme 6a) relies on tunable bromo- and dibromomaleimides for multifunctional bioconjugation by either reversible or permanent Cys alkylation

Scheme 5. Site-Selective Modification of Antibodies at Cys (a) at the *C*-Terminus via Disulfide and (b) at the *N*-Terminus via Thiazolidine
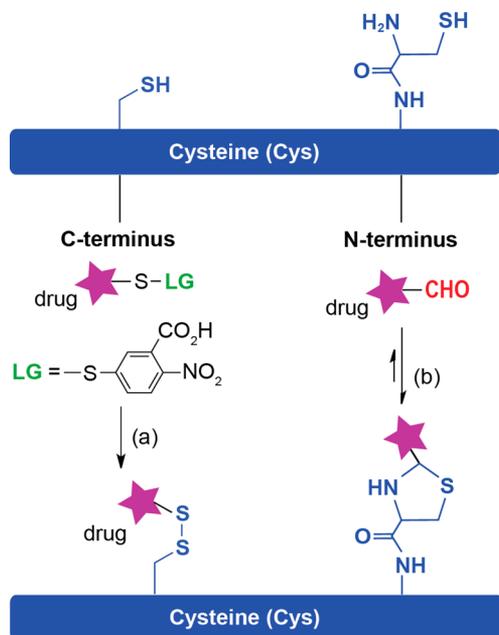
both in vitro[59] and in mammalian cells.[60] Aryloxymaleimides are also used as the next generation of tunable maleimides with attenuated reactivity enabling Cys modification strategies, disulfide bridging, and the dual functionalization of disulfide bonds.[61] While the thioether succinimide motifs that result from conjugate addition of the sulfhydryl group side-chain of Cys to maleimide readily undergo retro-Michael addition with biological thiols (e.g., Cys34 of HSA, the major constituent of plasma),[16] stable conjugates may be obtained using electron-

withdrawing maleimides that are rapidly hydrolyzed to their ring-opened counterparts to ensure in vivo stability.[62] Bromo- and dibromopyridazinediones have also been used for the reversible modification of proteins at Cys and disulfides with four potential points of chemical attachment (Scheme 6b).[63] While this conjugation is reversible by a thiol exchange mechanism, the use of a tris(2-carboxyethyl)phosphine (TCEP)-based *para*-azidobenzyl temporary protecting group (R^2) readily transforms these reversible reagents into irreversible conjugates.[64] A conceptually similar alkylation strategy (Scheme 6c)[65] revealed that bis-alkylation of the Cys residue (S147C) of a "superfolder" green fluorescent protein (GFP) with the 2,5-dibromohexanediamide developed by Davis and co-workers enables a two-step modification protocol by first creating an unexpectedly stable cyclic sulfonium intermediate that is subsequently opened with thiol, selenium, and nitrogen nucleophiles with good conversions (up to >95%) and mild conditions (21−37 °C). Analogously, the preparation of a double Cys mutant of the superfolder GFP by rational positioning of two cysteines with different local chemical environments and reactivities allows precise control of the regioselective formation of both Dha and cyclic sulfonium intermediate on the surface of the same protein. Subsequent ring opening of the cyclic ion with sodium azide generates two orthogonal handles (Dha and N_3) that enable dual labeling upon reaction with thiol and alkyne probes, respectively.[66]

Cys can also add to electron-deficient alkynoic amides/esters and alkynones, leading to the corresponding vinyl sulfides in aqueous media (Scheme 6d).[67] This reaction proved to be fast (terminal alkynone ≫ internal alkynone = alkynoic ester > alkynoic amide), compatible with other nucleophilic amino acid residues (Ser, His, Met, Lys, and Tyr with only limited cross-reactivity with *N*-terminal Ala and certain alkynones) and reversible in nature; therefore, Cys can be recovered by addition of an external thiol via a base-induced thiolate

Scheme 6. Site-Selective Methods for Cys Modification Using Polar Reactions—Part A: (a) Bromo- (X = H, Y = Br), Dibromo- (X = Y = Br), and Aryloxy- (X = H, Y = OAr) Maleimides, (b) Bromo- (X = H) and Dibromo- (X = Br) Pyridazinediones, (c) Dibromo Bisamide (2,5-Dibromohexanediamide) and Addition to the Electron-Deficient Triple Bonds (d) Alkynones and (e) 3-Arylpropiolonitriles
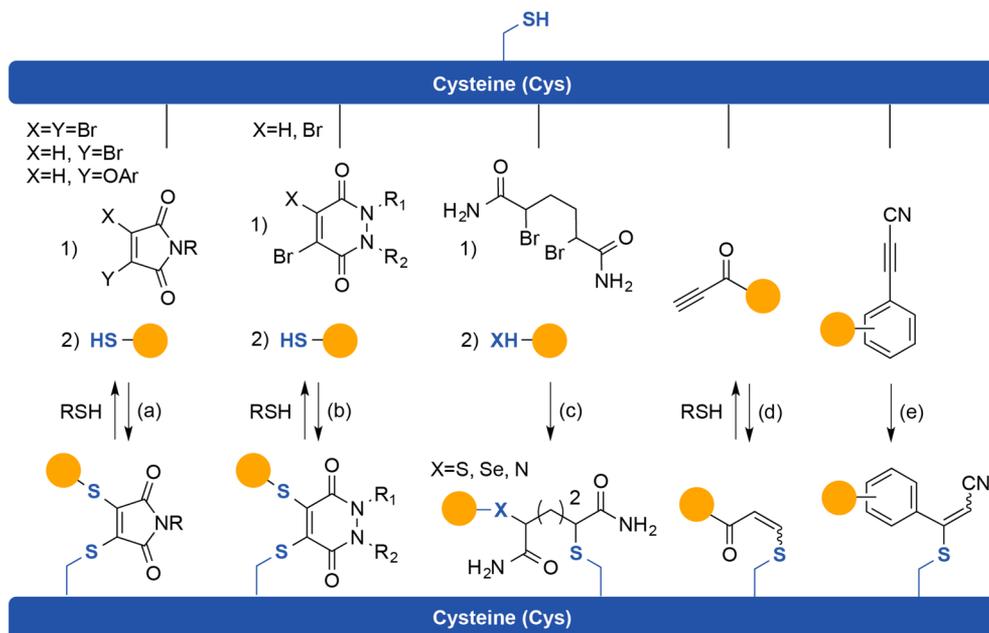
exchange. A recent application of this technology involves the preparation of phosphorescent proteins by introducing luminescent cyclometalated iridium(III) complexes as potential bioimaging agents.[68] Other electron-deficient triple bonds that have been employed for chemoselective Cys alkylations include 3-arylpropiolonitriles (Scheme 6e).[69] The reaction is fast and provides hydrolytically stable conjugates that may be useful in Cys-selective bioconjugation strategies. The authors attribute this stability to the presence of an aryl moiety that reduces the electronic density of the alkene unit by delocalization on the aromatic ring.

Boons, Popik, and co-workers have demonstrated the selective and reversible photochemical derivatization of cysteine residues on proteins using 3-(hydroxymethyl)-2-naphthol derivatives (NQMPs) (Scheme 7a).[70] The formation of

Scheme 7. Site-Selective Methods for Cys Modification Using Polar Reactions—Part B: (e) 3-(Hydroxymethyl)-2-naphthol, (f) Julia–Kocienski-like Reagents, and (g) Addition to Allenamides
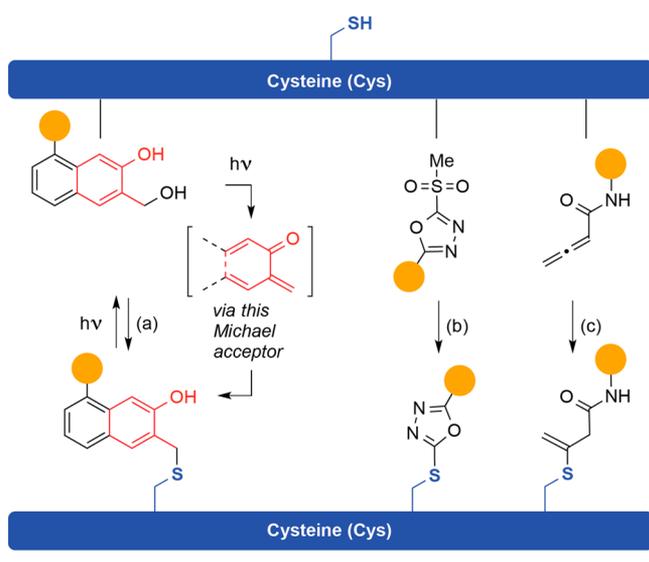
transient Michael acceptors upon irradiation of NQMPs at 350 nm enables the rapid attack of Cys and the formation of hydrolytically stable thioether linkages as demonstrated by the conjugation of several probes (PEG, dyes, carbohydrates, and other alkynes, azides, and biotin-labeled units) into model bovine serum albumin (BSA). Interestingly, this transformation is reversible upon irradiation of dilute solutions or in the presence of vinyl ethers (see also Scheme 15i).

Xian and co-workers first reported the Julia–Kocienski-like reagent methylsulfonyl benzothiazole (MSBT) as a selective protein thiol blocking agent in glyceraldehyde 3-phosphate dehydrogenase (GAPDH),[71] and later Barbas and co-workers expanded this methodology as a rapid thiol-selective protein modification strategy in a recombinant HSA and a fusion maltose-binding-HA peptide protein (MBP-C-HA), generating fluorophore and PEGylated conjugates superior in stability to maleimide-conjugated proteins in human plasma (Scheme 7b).[72] Indeed, this novel protocol also enabled the site-selective labeling of engineered Cys but also selenocysteine (Sec) residues of antibody conjugates, successfully improving their stability in human plasma.[73]

Recently, Abbas, Xing, and Loh have employed allenamides as privileged orthogonal handles for Cys-selective protein

modification in aqueous media (Scheme 7c).[74] The Michael-type addition enables the fast formation of a stable and irreversible vinyl sulfide conjugate, and its utility was demonstrated for the selective labeling at Cys (or disulfides after treatment with dithiothreitol (DTT)) of small peptides (e.g., glutathione (GSH)) as well as on BSA and the antibiotic-resistant bacterial enzyme TEM 1 β-lactamase (Bla).

Most of the site-selective methods described above rely on the unique reactivity of Cys in comparison to other nucleophilic residues. However, it is rather challenging when the introduction of two different modifications in two Cys units is required. To achieve this goal, the identification of Cys environments that render sufficient differences in the reactivity of the sulfhydryl side-chain of Cys may allow dual-selective Cys modifications. It is well-known that solvent accessibility and local charge strongly modulates Cys reactivity. In one example, the introduction of charged amino acids proximal to a Cys decreases the pK_a of the sulfhydryl moiety (by electrostatic interactions) and therefore accelerates its reaction with electrophiles, whereas the opposite trend is observed with negatively charged residues.[75] In another example, the protonation state of the N in vinyl-substituted pyridine derivatives (Michael-like acceptors) in combination with the local environment of the Cys also led to differences in reactivity toward these vinyl tags.[76] Additionally, cysteine mutations at specific positions in designed ankyrin repeat proteins (DARPins) allow the sequential alkylation of the strongest nucleophilic Cys with a weak thiol-reactive reagent (bromoacetamide) followed by treatment with a more reactive maleimide that reacts with the less nucleophilic Cys.[77] A conceptually similar approach exploits the preferential succinimide hydrolysis occurring in positively charged environments, which leads to a more stable conjugate and prevents detrimental maleimide exchange reactions with plasma thiols. This has been utilized for the modulation of the in vivo stability and therapeutic activity of antibody–drug conjugates (ADCs).[78]

Alternatively, the formation of stable thioethers can also be efficiently achieved using the emerging thiol–ene[45,79] and thiol–yne[80] couplings with native Cys proteins where radical species are typically generated using either radical initiators or light (Scheme 8). While thiol–ene coupling enables the introduction of a single molecule, the complementary use of alkynes allows for double functionalization and, thus, the incorporation of different modifications such as glycoconjugates, peptides, and fluorescent probes. The transformation proceeds by a first radical addition to form a stable vinyl sulfide moiety that undergoes a second radical addition with a different thiol, to finally obtain a 1,2-modified thioether under mild conditions. Despite advances of both thiol–ene/yne reactions for site-selective chemical protein modification of native proteins, the implementation of these methods is limited because the formation of thiyl radicals may damage the protein structure.

Although nonselective Cys alkylation is often expected when using electrophilic reagents, some degree of site-selectivity is achieved by fine-tuning the reaction conditions (without any additional mutation) and particularly by using a Cys-rich Cys-$(X)_m$-Cys-$(X)_n$-Cys (m and n = 3–6) motif with electrophilic trihalides (Scheme 9a). Heinis and co-workers have successfully exploited this strategy for generating cyclic peptide regions by alkylation of Cys side-chains with the trivalent tris-(bromomethyl)benzene (TBMB) under mild conditions in

Scheme 8. Site-Selective Methods for Cys Modification Using Radical (a) Thiol−Ene and (b) Thiol−Yne Reactions

Scheme 9. Chemical Synthesis of Uniformly Sized Cyclic Peptides by Selective Cys Alkylation with (a) Electrophilic Trihalides and (b) Perfluorinated Benzene Derivatives. GST = Glutathione S-Transferase, TCEP = Tris(2-carboxyethyl)phosphine

functionalized fluorescent dyes, one of the first examples of a bioorthogonal approach in Chemical Biology.[85] For more information on this as well as other peptide motifs that can be modified through chelation with transition metals (e.g., Ni(II)-His-tag pair)[86] via ionic interactions or by enzymatic reactions,[87] the reader is directed to these general reviews.[12a−h,88] Alternative site-selective protein modification methods, typically exploiting those nucleophilic natural amino acid residues (Cys, Lys, Glu, Asp, etc.) employed in nonselective protocols, have been also reported in conjugation protocols based on proximity-induced reactions and recognition or ligand-directed strategies using a wide range of conjugation chemistries including alkylation with electrophilic handles[89] (e.g., α-chloroacetamides, tosylates, DMAP/imidazole-activated acyl units, genetically encoded vinyl sulfonamides, etc.), disulfide formation,[90] and radical trapping via local single-electron transfer catalysis.[91] These robust transformations provide a certain degree of selectivity in contrast to the nonrecognition, old-fashioned methods.

2.2.4. Disulfides. The modification of native disulfide bonds through bis-alkylation protocols has attracted growing interest in recent years since the rise of novel therapeutic proteins in the global biotechnology market has stimulated the development of new methods for improving the properties and safety of these protein-based pharmaceuticals (Scheme 10).

Scheme 10. Site-Selective Methods for the Modification of Exposed Disulfides: Bis-alkylation with (a) α,β-Unsaturated-β′-monosulfones and (b) Dibromo/Dithiophenol (X = Y = Br, SPh) or Aryloxy (X = H, Y = OAr) Maleimides


aqueous media, leading to a general protocol for attaching and evaluating the proteolytic stability, half-life, and other important biological properties in phages, antibodies, and peptide fragments.[81] Crown ether-like supramolecular libraries[82] and phage selection of photoswitchable peptides[83] are also accessible using this technology. Similarly, Pentelute and co-workers have reported the preparation of hybrid macrocyclic peptide libraries via a chemoselective S$_N$Ar with perfluorinated benzene derivatives, which allow the fine-tuning of the dimensions and chemical composition of the peptide macrocycles (Scheme 9b).[84]

The genetic incorporation of Cys-rich peptide sequences has also been exploited for the chemical labeling of proteins, for example, using a tetracycline fluorophore with biarsenical-

Among these technologies, the work by Shaunak and co-workers was found to be particularly useful (Scheme 10a). Disulfide modification allows the site-selective conjugation of poly(ethylene glycol) (PEG) or other polymers to proteins from which optimal pharmacokinetics are achieved, hence ensuring protein purity, maintenance of tertiary structure, and stability.[92] Mechanistically, the conjugation is conducted by a sequential, iterative bis-alkylation using α,β-unsaturated-β′-monosulfone functionalized PEG reagents, leading to a stable thioether bridge. This protocol efficiently modifies a broad

Scheme 11. Bioorthogonal Reactions at Ketone and Aldehyde Functionalities: (a) Oxime and (b) Hydrazone Formation, (c) Pictet–Spengler Reaction and Its Improved Versions (d) Pictet–Spengler Ligation (X = O) and Hydrazino-Pictet–Spengler Ligation (X = NHMe), (e) Wittig Reaction
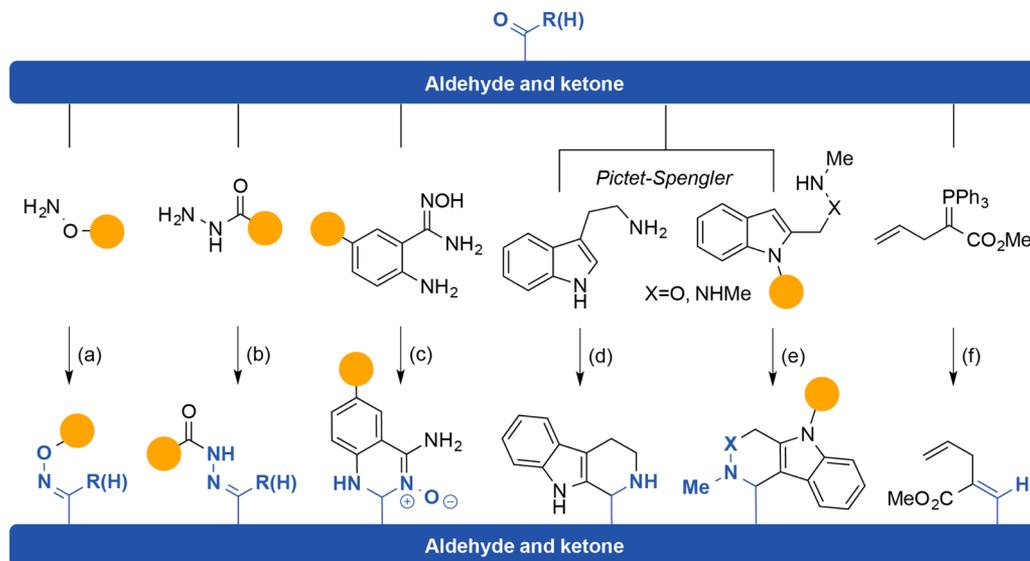
range of peptides, proteins, enzymes, and antibody fragments that can be site-selectively PEGylated or conjugated to potent cytotoxic payloads using a native and solvent-accessible disulfide moiety.[93] This methodology may also be applied to proteins bearing a polyhistidine tag (His-tag) through the same bis-alkylation principle of reactivity.[94]

Similarly, Haddleton, Caddick, and co-workers employed dibromo-[95] and dithiophenolmaleimides[96] and Baker and co-workers used aryloxymaleimides[61] as bis-alkylating reagents for the in situ bridging of disulfides allowing the incorporation of PEG chains in a very efficient manner at pH 6.2 (Scheme 10b). This reaction benefits from the rapid reaction kinetics, high efficiency, and reduced purification due to the use of equimolar equivalents of both peptide and polymer. Importantly, while this thiomaleamic acid linker is stable under physiological conditions, it is readily cleaved at lysosomal pH (around 4.5) and temperature (37 °C) enabling the preparation of homogeneous ADCs (trastuzumab Fab fragment) at disulfide sites and their subsequent controlled payload release.[97] Homogenous bispecific antibodies (able to bind two different antigens) have been also built using a bis-dibromomaleimide cross-linker.[98]

## 2.3. Modern Methods for Targeting Unnatural Amino Acids

2.3.1. Carbonyl Handles. The incorporation of unnatural amino acids (UAAs) combined with chemical protein synthesis enables access to chemically modified biomolecules that are difficult to obtain using standard molecular biology methods. Often the unnatural amino acid (e.g., alkene/alkyne groups, azido groups, or carbonyl groups) serves as a privileged reaction handle that can undergo further bioorthogonal modifications.[12] Several strategies, both chemical and genetic, have been developed for the incorporation of carbonyl functionalities into proteins that enable subsequent chemical modification (Scheme 11).

Early strategies used genetically encoded aldehydes and ketones that can be selectively labeled with hydrazides and aminooxy groups to form hydrazones and oximes, respectively (Scheme 11a and b) as well as Trp derivatives (Scheme 11d). Alternative (chemo)enzymatic strategies use two-step proce-

dures involving, first, a chemical oxidation with (i) sodium periodate (including those methods based on oxidation of carbohydrate moieties),[99] (ii) a site-selective transamination reaction[100] with pyridoxal 5′-phosphate or related reagents (Rapoport's salt), or (iii) an enzymatic tagging with formylglycine-generating enzymes (FGEs)[101] to efficiently install an aldehyde (or ketone) handle that can be modified with a second, carbonyl-selective reaction.[102] Among these, relevant examples include the well-known oxime (Scheme 11a)[103] and hydrazide (Scheme 11b)[104] ligations together with a related reaction with 2-amino benzamidoxime (ABAO) derivatives (Scheme 11c),[105] the Pictet–Spengler ligation (Scheme 11e),[106] which is more rapid than its parent version (Scheme 11d), and, more recently, the so-called hydrazino-Pictet–Spengler (HIPS) ligation (Scheme 11e).[107] The HIPS ligation is fast ($k_2 = 4.17 \pm 0.19$ M^{-1} s^{-1}); proceeds at near neutral pH 6, allowing the direct labeling of proteins under mild conditions; and, importantly, yields stable conjugates. Moreover, the HIPS ligation has been recently employed for the preparation of ADCs by site-selective conjugation at different sites, hence with variable in vivo efficacy and pharmacokinetics.[108] Aside from the aforementioned reactions, the aldehyde moiety can also be used for selective protein modification, generating stable C—C bonds by the Wittig reaction.[109] Moreover, the biocompatibility of such a reaction enables the application of stabilized phosphorus ylides in live cell affinity isolation and fluorescence labeling of proteins (Scheme 11f).[110]

2.3.2. Alkynes, Alkenes, and Dipole Handles. The development of complementary chemical, enzymatic, and genetic methods for introducing and using azide moieties[111] (a privileged 1,3-dipole handle) as bioorthogonal reporter groups has made this reactive functional group a routine choice for protein modification strategies. Schemes 12 and 13 summarize the bioorthogonal reactions developed for the modification of proteins equipped with azides and other dipoles. Early methods employed azide-functionalized proteins together with the classic Staudinger reaction to install amide-linked modifications (Scheme 12a).[12a−h] While in the original

Scheme 12. Metal-Free Bioorthogonal Reactions at Azides: (a) Staudinger, (b) Traceless Staudinger and (c) Staudinger-Phosphite/Phosphonite Ligations, and (d) Strain-Promoted Cycloadditions
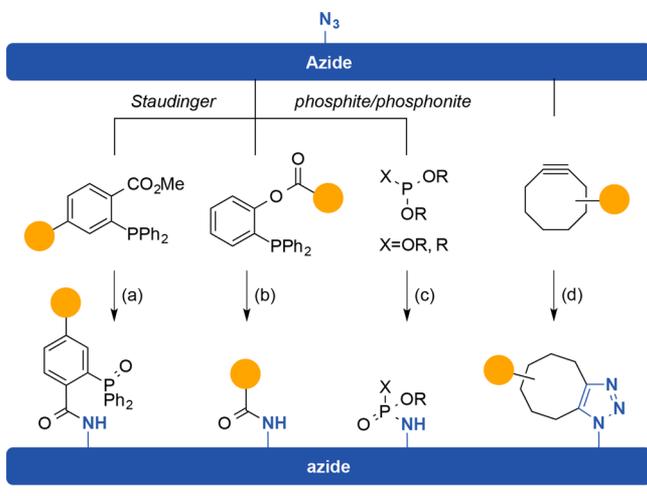
Scheme 13. Metal-Free Bioorthogonal Reactions at Other Dipole Handles: (a) Nitrones, (b) Nitrile Oxides and (c) 4-Halosydnones with Cyclooctyne (Metal-Free Click Reactions), and (d) Isonitriles with Tetrazine
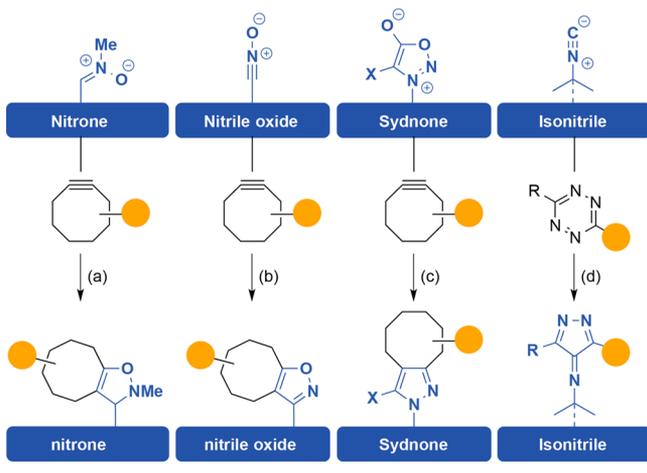
strategy the phosphine oxide generated remains incorporated into the protein, the "traceless" version (Scheme 12b)[112] overcomes this limitation, hence improving the overall transformation, especially in applications where a minimalist amide bond is required. Recent variants developed by Hackenberger and co-workers (Scheme 12c) expanded this type of chemistry to other phosphorus moieties, developing the corresponding Staudinger-phosphite[113] and Staudinger-phosphonite ligation protocols,[114] leading to phosphoramidate or phosphonamidate linkages, respectively. These linkages mimic natural occurring phosphoric esters and have the potential of introducing either one or two molecular probes into the same protein as exemplified by the successful PEGylation of Calmodulin.

Alternatively, azide handles also participate in [3 + 2] cycloadditions with strained alkynes (Scheme 12d), in a reaction usually referred to as a copper-free azide–alkyne "click" reaction,[115] which represents a significant improvement of the former uncatalyzed Huisgen reaction and other metal-catalyzed versions with Cu(I) and Ru(II) (Schemes 19a–c and

20a and b) . The strain-promoted alkyne–azide cycloaddition (SPAAC) represents a powerful tool not only for protein and antibody labeling (for example, for the construction of homogeneous ADCs)[116] but also for other applications such as antibody-free Western Blot analysis.[117] This is mainly due to the fact that no additional reagents or toxic metals that may damage proteins are required; the reaction proceeds with a rapid rate (k_2 up to 0.96 M^{-1} s^{-1} when the appropriate cyclooctyne is employed). Indeed, the success of this reaction is related to the fine-tuning of reactivity/selectivity ratio, taking into account important parameters such as lipophilicity (nonselective binding) and cross-reactivity with the biological thiols (leading to alkenyl sulfides).[80,118] Moreover, other derivatives present in the media in small quantities such as sulfenic acids (RSOH), which are formed by the reaction of reactive oxygen species (ROS) with protein thiols, rapidly cross-react with strained cyclooctynes to afford alkenyl sulfoxides.[119] This reaction may become a valuable tool for dissecting the roles of protein sulfenic acids in vitro and in vivo.

Other 1,3-dipoles such as nitrones and nitrile oxides have gained popularity during the past few years as handles for bioorthogonal strain-promoted cycloadditions (Scheme 13). van Delft, Boons, and co-workers developed the so-called bioorthogonal strain-promoted alkyne–nitrone cycloaddition (SPANC) following a one-pot, three-step protocol consisting of the chemical introduction of a terminal aldehyde handle by serine oxidation with periodate, its subsequent transformation into a nitrone using N-methylhydroxylamine, and finally reaction with the corresponding cyclooctyne (Scheme 13a).[120] This efficient site-selective modification protocol is fast (typically 10 times faster than SPAAC), and its robustness has been demonstrated not only for the modification of the chemokine interleukin-8 (IL-8) but also in the recent site-selective conjugation of ScFvs antibodies to nanoparticles.[121] A valuable variant of the SPAAC reaction with nitrones employs protein equipped with nitrile oxides (Scheme 13b) as reactive handles to explore an operationally similar (oxime formation–oxidation–cycloaddition) strain-promoted alkyne–nitrile oxide cycloaddition (SPANOC).[122] Recently, a fast copper-free strain-promoted sydnone–alkyne cycloaddition (SPSAC) between 4-halosydnones and bicyclo-[6.1.0]-nonyne has been reported (Scheme 13c).[123] Structural motifs increasing the partial positive charge at the N-3 atom of the sydnone ring (N-aryl moieties bearing strong electron-withdrawing groups) proved beneficial to the reaction. Among those, 4-Cl N-aryl sydnone was the best performer, with model bicyclo-[6.1.0]-nonyne showing reaction rates of up to $k_2 = 1.593 \pm 0.034$ M^{-1} s^{-1}, and was subsequently utilized for the modification of a sydnone-functionalized BSA with a fluorescent cyclooctyne derivative.

Isonitriles also react via [4 + 1] cycloaddition with tetrazines in aqueous media as demonstrated by the introduction of a fluorophore into the tertiary isonitrile-labeled C2A domain of synaptotagmin-I (Scheme 13d).[124] Hydrolysis of the isonitrile moiety to the corresponding amine can be minimized using tertiary analogues.

Alkynes and alkenes can also be incorporated into proteins and used in a wide range of bioorthogonal reactions (Schemes 14 and 15). Simple alkynes such as alkynyl–pyrrolysine analogues are incorporated and modified without any metal catalyst using a site-selective thiol–yne coupling (Scheme 14a).[125] Alternatively, protein encoding with cyclooctynes (Scheme 14b and c) using either chemical[126] or genetic[127]

Scheme 14. Metal-Free Bioorthogonal Reactions at Alkynes and Cyclooctynes: (a) Thiol–Yne and Strain-Promoted Cycloadditions with (b) Azides, (c) Tetrazines, and (d) Sydnones
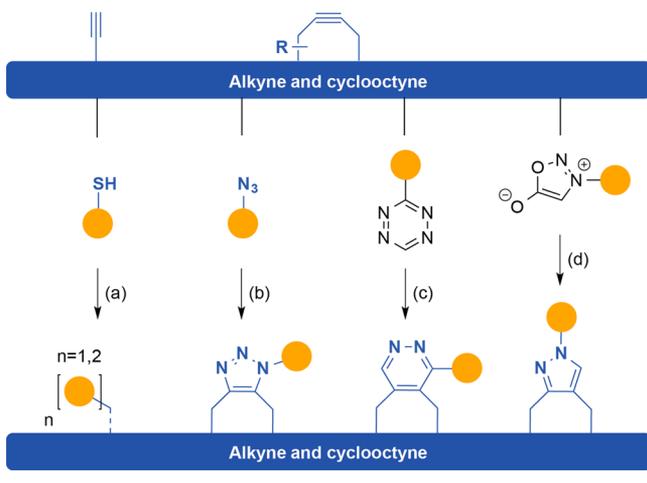
methods has been exploited in metal-free protocols with azides or tetrazine reagents as shown in previous sections. An interesting application includes the postfunctionalization of virus particles via azide–alkyne click chemistry (including the comparison with the Cu(I) variant) demonstrating the preservation of capsid integrity and its self-assembly properties.[128]

More recently, Wallace and Chin have reported a strain-promoted sydnone 1,3-dipole bicyclo-[6.1.0]-nonyne cycloaddition,[129] demonstrating that this metal-free transformation is biocompatible and proceeds in aqueous buffer at physiological

temperature with complete conversion/selectivity albeit with moderate rates (0.054 M^{-1} s^{-1}) (Scheme 14d).

While oxanorbornadienes react with azides (Scheme 15a) and norbornenes equipped with electron-deficient sulfonyl azides (Scheme 15b),[130] other alkenes such as strained *trans*-cyclooctenes, cyclopropenes, norbornenes, acrylamides, or simple vinyl/allyl alcohols have received considerable attention because of their fast reaction rates in inverse-demand Diels–Alder reactions with tetrazines[131] and 1,3-dipolar cycloadditions with in situ chemical or photogenerated nitrile–imine dipoles (Scheme 15c–g).[131c,132] Of particular relevance is the selective inverse-electron-demand Diels–Alder reaction of tetrazines with isomeric 1,3-disubstituted cyclopropenes, as well as the 1,3-dipolar cycloaddition of nitrile imines (generated by "photoclick" reaction from tetrazole) with 3,3-disubstituted cyclopropenes[133] or spiro[2.3]hex-1-enes (with an impressive k_2 up to 34 000 M^{-1} s^{-1}).[134] This unique, orthogonal reactivity resulting from rational positioning of a single methyl group into a cyclopropene moiety will be beneficial in dual labeling strategies both in vitro and in vivo. The reverse protocol, using tetrazine-modified proteins instead, is also an alternative for such labeling methods.[109,135] For a more in depth description of the reaction kinetics as well as other particular aspects of these transformations, the reader is directed to recent and comprehensive reviews.[12a–h] Indeed, these highly efficient transformations enable double labeling strategies such as the modification of several pairs of sites in Calmodulin with fluorophores for determining protein structure and dynamics.[136] Acyclic alkenes such as homoallylglycine (Hag),[51,137] alkenyl–pyrrolysine analogues, and acrylamides also react in a site-selective manner with thiol probes under radical conditions by a thiol–ene ligation (Scheme 15h).[125] Other chemistries have also been explored with a dienophile(vinyl sulfide)

Scheme 15. Metal-Free Bioorthogonal Reactions at Alkenes: (a, b) Reactions of Oxanorbornadienes and Norbornenes with Azides, (c) Inverse-Demand Diels–Alder of *trans*-Cyclooctenes and (d) Norbornenes and 1,3-Cyclopropenes with Tetrazines, (e) In Situ Nitrile Imine Generation from Hydrazonoyl Chloride and (f, g) Tetrazole and Subsequent 1,3-Dipolar Cycloaddition with Alkenes Including 3,3-Cyclopropenes and Spiro[2.3]hex-1-ene; (h) Thiol–Ene, and (i) Hetero-Diels–Alder with Vinyl Sulfides
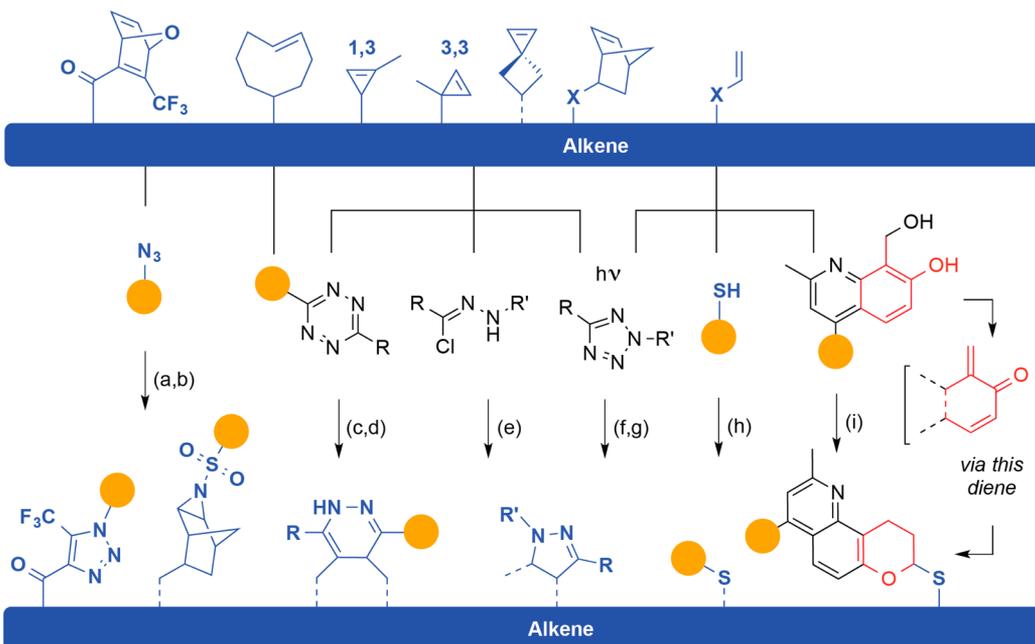
functionalized protein and *o*-quinolinone quinone methide as diene[138] or with maleimide-tagged proteins with 5-alkoxyoxazoles (via Kondrat'eva irreversible cycloaddition)[139] in hetero-Diels−Alder reactions (Schemes 15i and 16b) as well as with

Scheme 16. Metal-Free Bioorthogonal Reactions at Dienes and Maleimides: (a) Classical Diels−Alder Reaction and (b) the Kondrat'eva Hetero-Diels−Alder Irreversible Ligation
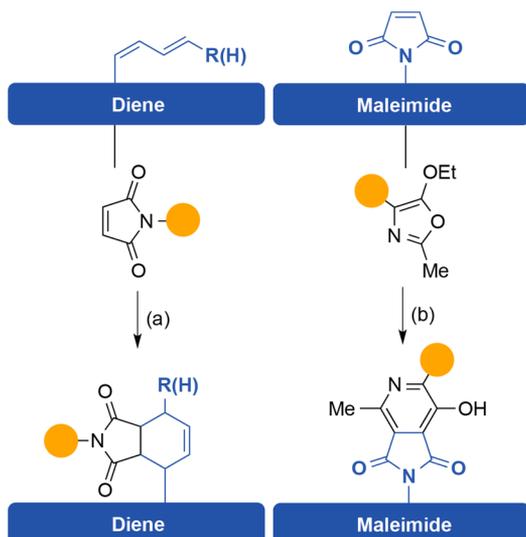
diene-functionalized proteins in classical Diels−Alder reactions with maleimides (Scheme 16a).[8] Importantly, and unlike most of the reversible Diels−Alder-based reactions, the Kondrat'eva hetero-Diels−Alder is an irreversible ligation that has recently been added to the click chemistry toolkit. This reaction occurs under slightly acidic conditions (0.1 M NaOAc buffer, pH 5.0 at 37 °C) between 5-alkoxyoxazoles and maleimides, affording stable pyridine moieties as demonstrated by the labeling of BSA−maleimide with a fluorescent dye.[139]

N-Acylazetine (the *N*-analogue of cyclobutene) is another dienophile that has not been used yet for protein modification

but holds a great potential.[140] This achiral and minimalistic non-native functional group has been successfully employed in the two-step activity-based protein profiling of catalytically active proteasome subunits using tetrazine-based bioorthogonal chemistry ($k_2 = 0.39 \pm 0.1$ M^{-1} s^{-1}).

3. TRANSITION METAL-MEDIATED APPROACHES

Significant advances in both genetic and synthetic methods over the past few years have allowed the use of nonbiological metal-mediated reactions for protein modification protocols.[141] Some of the limitations that traditionally faced organometallic transformations such as the use of anhydrous solvents and catalyst poisoning (typically associated with the reaction of the catalyst with soft, nucleophilic residues) have been progressively solved, and a range of novel reactions have emerged, expanding the toolkit of selective bioorthogonal transformations.[1,4,142] These new protocols should fulfill not only the "on protein" reaction conditions—aqueous media, high efficiency, and chemoselectivity at or near room temperature and over a narrow pH range—but also those of an efficient, environmentally benign catalytic system: moisture and air-stable ligands or ligandless systems that enable mild, fast, and economic processes. The most significant contributions and challenges associated with metal-mediated protein modification protocols targeting both natural or unnatural amino acid residues will be highlighted in the following sections.

3.1. Methods for Targeting Natural Amino Acids

3.1.1. Lysine and Tyrosine with Iridium and Palladium Complexes. Early metal-mediated protein modification methods focused on the modification of nucleophilic natural residues such as Lys and Tyr (Scheme 17). Reductive alkylation of surface-exposed Lys residues using a Cp*−Ir bipyridyl complex enabled the condensation of aliphatic and aromatic aldehydes.[143] The formed imine is subsequently reduced by an active Ir−hydride species, which is generated in situ from sodium formate at pH 7.4 and room temperature (Scheme 17a). However, low selectivity is observed because the protein *N*-terminus is also modified. Alternatively, Francis and coworkers explored the palladium-catalyzed Tsuji−Trost mod-

Scheme 17. Lysine and Tyrosine Modifications with Iridium and Palladium Complexes: (a) Reductive Alkylation of Lysine and *N*-Terminus with Ir Complex and (b) Pd-Catalyzed Allylic *O*-Alkylation of Tyrosine
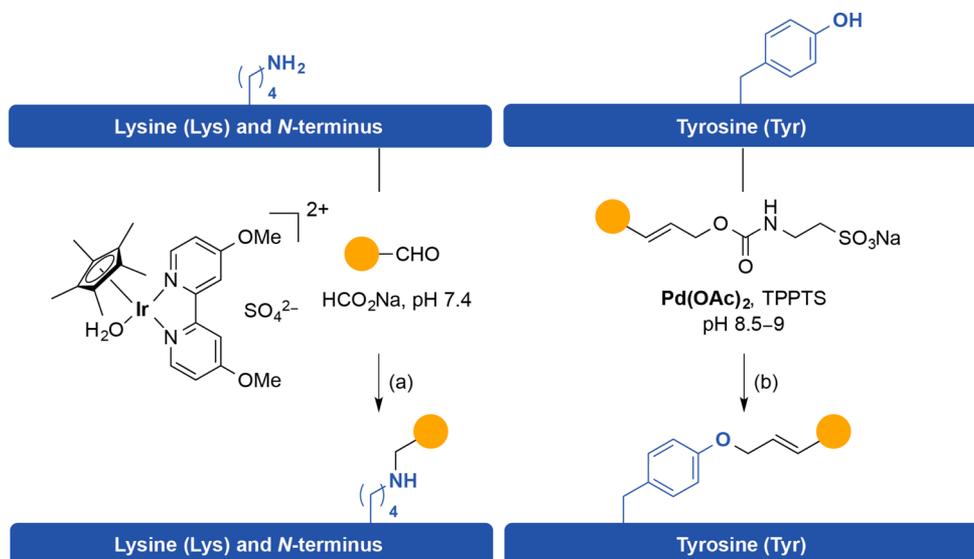
Scheme 18. Metal-Mediated Tryptophan, Cysteine, and "Proximity-Driven or Recognition" Modifications: (a−c) Modifications with Rhodium Carbenoids and (d) Au-Catalyzed Oxidative Allene−Thiol Coupling of Cysteine
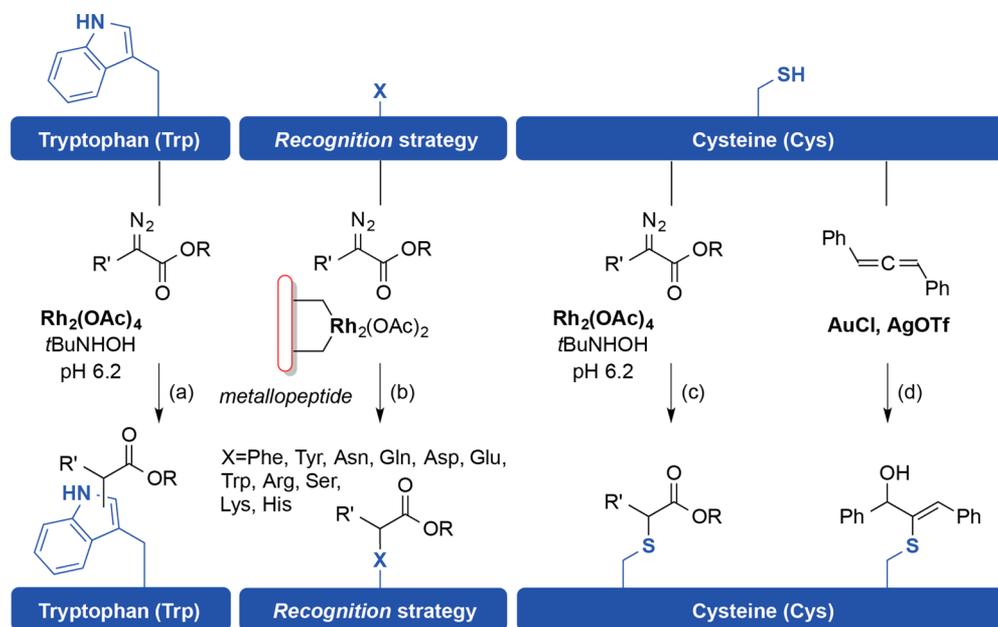
ification of Tyr (Scheme 17b). The authors reported the use of π-allylpalladium complexes, which are generated from allyl acetates/carbamates and Pd(OAc)$_2$/triphenylphosphine tris-(sulfonate) (TPPTS) as a catalytic system, for selective Tyr O-alkylation with a rhodamine dye and lipophilic moieties.[144] Moderate conversion (50−65%) to a singly solvent-exposed alkylated product was obtained although a small amount of doubly alkylated product was also observed. Examples of this Pd-catalyzed Tyr-selective strategy include the selective introduction of different fluorophores into bovine erythrocyte Cu/Zn superoxide dismutase (SOD)[145] and HSA.[146]

Other metal-mediated modifications of Tyr, Trp, N-terminal amino acids (e.g., proline), and other unnatural residues involving oxidative couplings with Ni(II), Ce(IV), or Fe(III) are not covered in the present work because they simply act as oxidants in these transformations, yet they have also been explored for chemical protein modification.[1,142,147]

3.1.2. Natural Residues with Rhodium and Gold Complexes. Pioneering work by Francis and co-workers shed light into the use of rhodium carbenoids for protein modification targeting Trp residues, yielding mixtures of N-1 and C-1 regioisomers (Scheme 18a).[148] However, the general applicability of this transformation is limited due to the low pH <3 required. Improved reaction conditions requiring only a slightly acidic media (pH 6) were reported, but the conversions were only moderate.[149] Solvent-accessible Cys residues also react with rhodium carbenoids via S−H insertion of a metallocarbene intermediate as exemplified by the modification of the PDZ domain of the cystic fibrosis transmembrane conductance regulator-associated ligand (CALP) (Scheme 18c).[150] Alternatively, peptide-based molecular recognition strategies (Scheme 18b) with metallopeptides have also been used to site-selectively modify proteins by targeting proteinogenic residues including Phe, Tyr, Asn, Gln, Lys, and His, among others that were unreactive under standard conditions with simple Rh$_2$(OAc)$_4$ catalysts.[45,151]

Wong, Che, and co-workers have described the gold-mediated selective cysteine modification of peptides and

proteins using allenes, which represents the first utilization of gold complexes for protein modification (Scheme 18d).[152] The reaction is formally an oxidative allene−thiol coupling in aqueous media at room temperature using AuCl/AgOTf as a catalytic system. Although this protocol has been successfully employed in peptides (up to 97% conv.), the modification of RNase A resulted in reduced conversion and regioselectivity (24% single modification together with 9% of the doubly modified protein).

## 3.2. Methods for Targeting Unnatural Amino Acids

3.2.1. Azides, Sydnones, Alkynes, and Alkenes. Recent advances in the incorporation of unnatural amino acids into proteins[153] have stimulated the development of novel methodologies for their modification as well as the recycling of well-known and established protocols. As such, *unnatural reactive handles* provide a privileged chemical point from which metal-mediated strategies, usually developed earlier, may efficiently work by reassigning the reaction conditions for such transformations using aqueous, mild, and atmospheric-tolerable catalytic systems. In this section, we describe recent developments that have allowed these protocols to become attractive tools for selective chemical protein modification. Schemes 19−21 illustrate a range of methods used for the transition metal-mediated modification of azides, sydnones, alkynes, and alkenes.

Early examples include the modified Huisgen cycloaddition also referred to as Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) or "click reaction", which represents one of the most important bioconjugation methods for protein modification reported to date (Schemes 19a and 20a).[154] Catalytic amounts of Cu(I) are sufficient to promote a mild chemoselective [3 + 2] cycloaddition in aqueous media that produces hydrolytically stable and amide isosteric 1,4-disubstituted triazole rings. This method has been used to access and study a wide range of important PTMs,[4,8] including cases of dual, orthogonal PTMs,[155] or in site-specific immobilization protocols.[156] In addition, the introduction of [19]F- and radiolabeled ^{18}F-glycosyl

Scheme 19. Transition Metal-Mediated Modifications of Sydnones and Azides: (a−c) Cu(I)- and Ru(II)-Catalyzed Azide−Alkyne and Sydnone Cycloadditions, Respectively
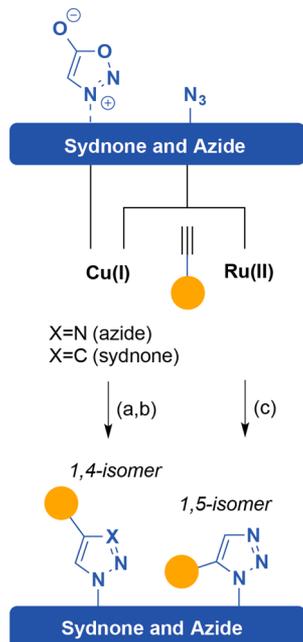

Scheme 20. Transition Metal-Mediated Modifications of Alkynes: (a, b) Cu(I)- and Ru(II)-Catalyzed Azide−Alkyne Cycloadditions, Respectively, (c) Copper-Free Sonogashira Cross-Coupling at Homopropargylglycine (Hpg) and Its (d) Ligandless Version at Pyrrolysine (Pyl)-Based Systems, (e) Modification of Hpg with Palladacycles


Scheme 21. Transition Metal-Mediated Modification of Alkenes: Olefin Cross-Metathesis with (a) S-Allylcysteine and (b) Se-Allylselenocysteine, and (c) Pd-Catalyzed Oxidative Heck Reaction
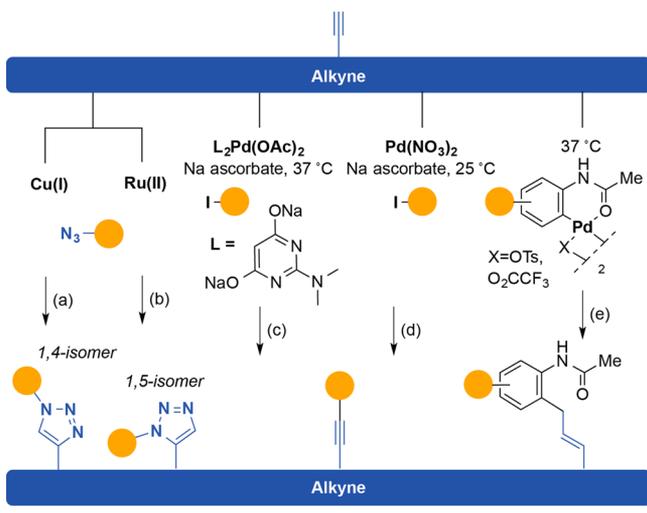
probes on an Hpg-functionalized globular protein SsβG (β-galactosidase) and also on a multivalent and self-assembled virus-like bacteriophage particle Qβ (with 180 F-glycosyl units incorporated) was achieved under CuAAC conditions. This approach allows the design of homogeneous glycoprotein probes for structural dynamics (e.g., ^{19}F−NMR) and imaging (e.g., ^{18}F-positron emission tomography (PET)) purposes.[157] However, and despite the enormous success of this protocol and the progress made on the development of ligands and complexes (e.g., N-heterocyclic carbene−Cu catalysts)[158] that stabilize and maintain the active catalytic Cu(I) species, there

are still some limitations including protein damage and associated toxicity. This is likely due to the generation of ROS, which have progressively favored the utilization of more friendly Cu-free protocols (Schemes 12 and 13).[159] Using a high-throughput immunoassay screening, Taran and co-workers discovered sydnone 1,3-dipoles as an alternative handle that also reacts with Cu(I) in chemoselective and biocompatible fashion in the so-called Cu-catalyzed sydnone−alkyne cycloaddition (CuSAC) (Scheme 19b),[160] similar to the metal-free variant depicted in Scheme 14d. This transformation was demonstrated with a sydnone-functionalized BSA that was reacted (ca. 75% conv.) with a dansyl-labeled alkyne using CuSO$_4$/Na ascorbate/ligand system in PBS buffer (pH 8.0) at 37 °C. This transformation, however, does not proceed with N-alkyl sydnones. Ru(II) has recently emerged as an alternative metal able to catalyze [3 + 2] cycloadditions of both terminal and internal alkynes with azides (RuAAC), leading to otherwise elusive 1,5-disubstituted triazoles (Schemes 19c and 20b). Thus, RuAAC has been used as a way of introducing cis-peptide bond surrogates in pancreatic ribonuclease (RNase A).[161]

Another transformation that takes advantage of an alkyne handle is the palladium-catalyzed Sonogashira cross-coupling in aqueous media (Scheme 20c and d). Palladium catalyzed cross-coupling reactions in aqueous media[162] have been widely studied and developed over the last 30 years on small-molecule models. As such, reactions with peptide substrates are not covered in the present section, which highlights recent achievements using functionalized proteins bearing suitable unnatural residues. Thus, copper-free Sonogashira cross-coupling at homopropargylglycine (Hpg) is a useful method for C−C bond formation between alkynes (Csp) and aryl halides (Csp^2) enabling the efficient attachment of aromatic groups, including fluorophores and PEG chain motifs to ubiquitin,[163] ubiquitin-fused peptides selected from phage display,[164] and a model dihydrofolate reductase (eDHFR) from E. coli, which was designed with an N-terminal 13 amino acid lipoic acid acceptor peptide (LAP) sequence.[165] This

Scheme 22. Pd-Mediated Protein Cross-Couplings at Aryl Halides: (a) Mizoroki−Heck, (b) Sonogashira and (c) Copper-Free Ligandless Version, (d) Phosphine-Free Suzuki−Miyaura with ADHP or NHC Ligands, and (e) Ligandless Suzuki−Miyaura Cross-Couplings
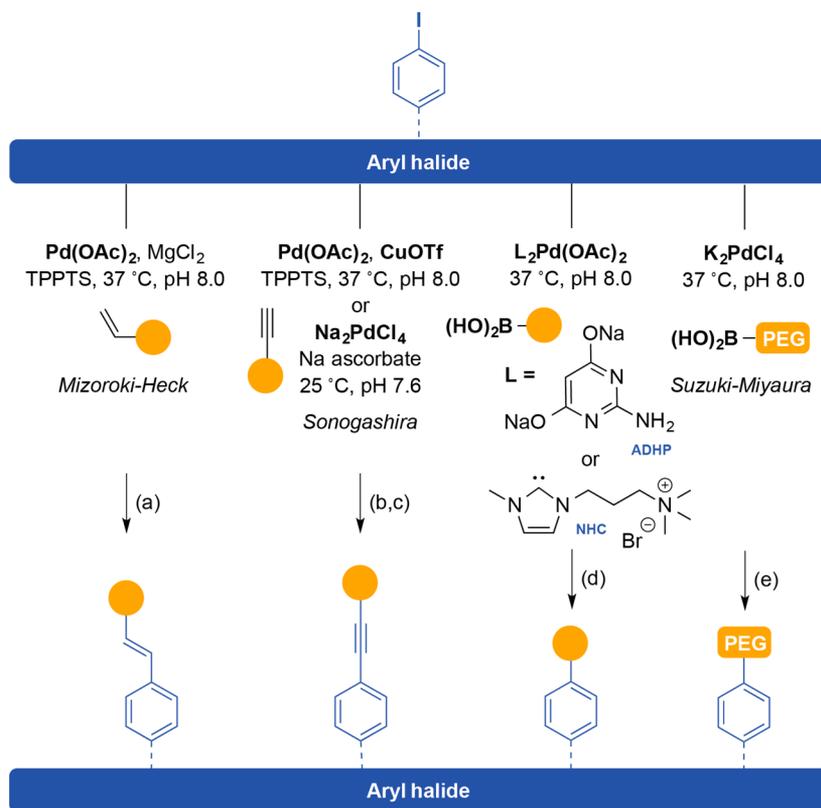
approach has also been successfully used for the labeling of proteins in bacterial cells (*E. coli*)[163] and in the surface of mammalian cells.[166] The success of this transformation in live cells is dependent on the use of water-soluble 2-amino-4,6-dihydroxypyrimidine (ADHP) ligands (Scheme 20c). Recently, an improved copper-free Sonogashira cross-coupling at pyrrolysine (Pyl)-based systems has been reported by Chen and co-workers (Scheme 20d).[167] The authors identified Pd(NO$_3$)$_2$ as an effective nontoxic (with diminished membrane damage potential) catalyst that works efficiently at room temperature (25 °C) in live *E. coli* and *Shigella* cells. This method was applied for the intracellular labeling and visualization of type-III Secretion (T3S) toxin-OspF.[167] The authors suggest the generation of Pd(0) nanoparticles from the Pd(II) source and an electron donor—sodium ascorbate in the reaction mechanism of this process. The use of similar catalysts enabled Pd-triggered deprotection and subsequent activation of N^ε-propargyloxycarbonyl-L-lysine and N^ε-allyloxycarbonyl-L-lysine containing proteins in cells.[168] Finally, another site-selective metal-catalyzed cross-coupling at Hpg-functionalized ubiquitin exploits water-stable palladacycles to form styrene adducts with fluorescent and PEG modifications in moderate to high yields in PBS buffer at 37 °C (Scheme 20e).[169]

The Nobel-winning olefin cross-metathesis (CM) reaction represents another well-known C−C bond-forming reaction that has recently been added to the pool of bioorthogonal transformations for chemical protein modification (Scheme 21).[170] Davis and co-workers successfully demonstrated the first cross-metathesis couplings with an *S*-allylcysteine (Sac)-functionalized protein.[171] In this case, the first step involves the chemoselective installation of a Sac handle that may be

achieved using complementary methods.[172] The second step is an aqueous cross-metathesis reaction catalyzed by air-stable Hoveyda−Grubbs second-generation catalyst, which is enhanced by the presence of *S*-allyl moieties and suggested to proceed via a chalcogen-relayed mechanism (Scheme 21a). This behavior is also known as allylic chalcogen effect[173] and rationalizes the reactivity enhancement of allylic chalogenides by a chelation mechanism that can be exploited to efficiently attach a wide variety of alkenes. Important issues of this transformation include (i) the need to use 30% *t*BuOH as a solvent system to reduce the poor water solubility of conventional Ru catalysts, (ii) the use of MgCl$_2$ as a mild Lewis acid to prevent nonproductive chelation of other nucleophilic moieties, and, finally, (iii) the fact that reaction completion was only observed with allyl alcohol. This observation points out steric hindrance as a possible limitation of this approach. This limitation was addressed by the use of a linker-extended version together with a more reactive *Se*-allylselenocysteine (Seac) handle, which allowed successful reaction with previously unreactive metathesis partners (Scheme 21b).[171] Next, the direct installation of a rare C−Se bond on a protein surface via biomimetic addition of allylselenoate to Dha-functionalized proteins suppressed the need for the linker and enabled cross-metathesis to proceed with alkene counterparts (allyl GlcNAc and *N*-allyl acetamide) that were previously not possible with the corresponding Sac analogue.[174] Reaction kinetics revealed rate constants (k_2 = 0.3 M^{-1} s^{-1}) comparable or superior to many current bioorthogonal reactions. Interestingly, this transformation is reversible upon treatment with H$_2$O$_2$ via Cope-type selenoxide elimination, which was exploited in a synthetic write (CM)−

read (antibody)−erase (elimination)−rewrite (CM)−read (antibody) cycle at the epigenetic site 9 (N-acetyl Lys derivative) of histone H3. Moreover, using the same catalytic system, Schultz and co-workers performed a related ring-closing reaction at genetically encoded alkenes in yeast.[175]

Another bioorthogonal Pd-mediated transformation has been recently added to the alkene modifications toolbox. Dekker and co-workers employed a single Cys mutant of enzyme 4-oxalocrotonate tautomerase (4-OT), which was alkylated with several maleimide-containing alkenes, to develop an oxidative Heck reaction for mild bioconjugation in vitro (Scheme 21c).[176] This strategy enabled the chemoselective introduction of several boronic acids with excellent conversions into a single alkene-functionalized protein using a base-free catalytic system with Pd(OAc)$_2$ and the bis-imine of acenaphthenequinone and mesitylamine (BIAN) as a ligand in the presence of complex protein mixtures and importantly under aerobic conditions. Alkene tolerance was remarkable because not only terminal but also cis ≫ trans internal alkenes, which represent an interesting cis-selective alkene modification protocol, were modified, leading to mixtures of linear/branched final products as demonstrated in small-molecule models.

Alternative couplings exploiting allylic disulfides have also been utilized but only in peptide models. These include the desulfurization of allylic disulfides followed by a silver-mediated allylic rearrangement[177] and the Kirmse−Doyle reaction, which consists of the generation of a sulfur ylide upon attack of a rhodium carbenoid and subsequent rearrangement.[178]

3.2.2. Aryl Halides and Boronates. Aside from the aforementioned copper-free palladium-catalyzed Sonogashira cross-couplings at proteins equipped with alkyne handles (Scheme 20c and d), other transformations using the same metal have emerged as powerful tools for protein modification at different groups. Similarly to cross-metathesis, palladium-catalyzed cross-coupling of aryl halides or boronates with organometallic partners represents one the most useful methods for C−C bond formation, being awarded with the Nobel Prize in 2010.[179] Advantages of these methods include (i) high functional group tolerance, (ii) reduced toxicity, (iii) chemical bioorthogonality, (iv) the use of air- and moisture-stable ligands avoiding inert atmosphere or degassed solvents, and finally (v) stability of the product and irreversibility of the transformation, which make these transformations amenable and attractive protocols for generating stable protein conjugates (Schemes 22 and 23). Preliminary examples of Suzuki−Miyaura cross-couplings on a p-iodophenylalanine-encoded (pIPhe) peptide revealed the feasibility of this transformation using Na$_2$PdCl$_4$ with a series of boronic acid partners in a 1:1 mixture of glycerol and water, resulting in high conversions at 40 °C.[180] Early examples using pIPhe-encoded proteins included a Mizoroki−Heck reaction (alkene cross-coupling partner)[181] with a Pd(OAc)$_2$/TPPTS catalytic system (Scheme 22a) together with MgCl$_2$, which inhibits protein precipitation due to palladium binding, and a Sonogashira reaction (alkyne cross-coupling partner)[182] with Pd(OAc)$_2$/TPPTS and CuOTf (Scheme 22b). Conversions were, however, rather low (2−25%). A copper-free ligandless Sonogashira cross-coupling analogous, albeit less efficient, to that described in Scheme 20d also has been applied to the labeling of a GFP−pyrrolysine carrying a terminal iodophenyl group (up to 15% conversion with Na$_2$PdCl$_4$) (Scheme 22c).[167] The genetic incorporation of a boronate handle (Scheme 23) opened up an opportunity to perform Suzuki−Miyaura cross-couplings at this residue,

Scheme 23. Pd-Mediated Protein Cross-Couplings at Aryl Boronates: (a) Suzuki−Miyaura
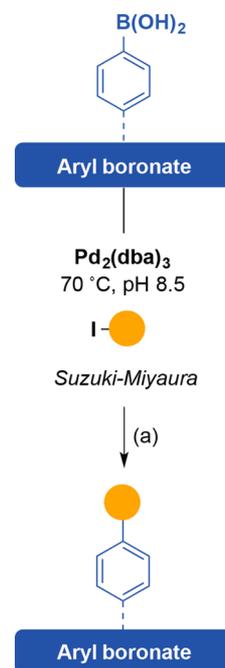
however only with limited success (30% conversion with a fluorescent bodipy aryl iodide partner) using Pd$_2$(dba)$_3$. This is likely a result of the harsh reaction conditions employed (12 h at 70 °C).[183] These results highlight the need for the development of a general, mild Pd catalyst for such transformations.

Davis and co-workers contributed significantly to the use of palladium chemistry for protein modification strategies by employing water-soluble and air-stable 2-amino-4,6-dihydroxypyrimidine (ADHP) ligands for a phosphine-free Suzuki−Miyaura reaction (Scheme 22d), which afforded unprecedented complete conversions (>95%) with a model cysteine-alkylated protein bearing a p-I-benzyl handle.[184] This transformation occurred under mild conditions (up to 37 °C in aqueous media) without the need of a cosolvent in only 30 min. Indeed, a huge range of aryl and vinyl boronic acid cross-coupling partners were successfully employed, allowing the installation of biaryl moieties and glycosides.[184]

Recent progress include the expansion of such a reaction at genetically encoded pIPhe,[185] which revealed the importance of Pd scavenging with 3-mercaptopropionic acid to prevent protein precipitation or Cys interference, similarly to the role of MgCl$_2$ in early Mizoroki−Heck examples.[181] Other inherent issues associated with this transformation such as dehydrohalogenation remain a challenge. Finally, genetic encoding and a site-selective labeling approach for protein modification by Suzuki−Miyaura cross-coupling has been successfully applied for cell surface labeling in E. coli by attaching either fluorescent molecules[186] or glycosides, thus modulating the interaction of bacterial glycocalyx with several binding partners.[187] The covalent labeling of molecules on the bacterial cell surface was efficiently achieved via Suzuki−Miyaura chemistry after incorporation of pIPhe into the outer membrane channel protein C (OmpC) using an amber stop codon suppression technique. Cross-couplings with either a fluorophore or a sugar boronic acid/fluorescent lectin reporter were visualized by

fluorescence microscopy, demonstrating a reduced toxicity at the catalyst loadings required for efficient labeling. Although Pd(OAc)$_2$·(ADHP)$_2$ catalysts have proved efficient in both Suzuki−Miyaura and copper-free Sonogashira cross-couplings, the use of alternative catalytic systems (e.g., *N*-heterocyclic carbene (NHC)−Pd catalysts for the cell surface labeling of mammalian cells) (Scheme 22d)[188] or the development of ligandless protocols such as the site-selective protein PEGylation by Suzuki−Miyaura cross-coupling recently (Scheme 22e) will be of enlarged value in particularly important biotechnology applications such as the development of therapeutic proteins with improved properties.[189]

4. CONCLUSION

This review has highlighted the state of the art of recent aqueous reaction methodology developed for site-selective chemical protein modification. The awareness of the need to develop site-selective protein modification methods is still recent. Despite several advances reported in the last 10 years, there remains a need for additional and complementary site-selective reactions with improved kinetics and selectivities. These methods will expand the current available chemical toolbox and help chemists and biologists further improve their understanding of complex biochemical processes. We anticipate a key role for metal-mediated transformations in protein chemistry and highlight challenges associated with adapting such chemistries to biological systems. These include (i) enlarging the plethora of metal-mediated transformations for protein modification by developing catalytic systems able to efficiently work in benign aqueous systems, at physiological temperature and pH, and in the presence of oxygen; (ii) progressing toward "real" catalytic systems because almost all metal-mediated strategies reported to date require an excess of metal to achieve moderate-to-good conversions (improve the catalyst turnover numbers); (iii) moving to *inter*species couplings because most of the current metal-mediated protocols are limited to prokaryotic systems; (iv) designing and developing cell-permeable reagents and catalysts to perform intracellular reactions because most of the current methods are limited to cell lysate or surface labeling; and finally (v) addressing toxicity problems that may allow in vivo couplings with whole-body organisms. In addition, site-selective protein modification will be central for the preparation of the next generation of biopharmaceuticals, including ADCs for cancer therapy and glycoproteins for vaccination. Such approaches will lead to defined constructs that enable a clear molecular dissection of modified protein structure on therapeutic function[5a,56a] and hopefully result in therapeutic candidates with improved safety and efficacy.

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Notes

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Biographies

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REFERENCES

(1) Stephanopoulos, N.; Francis, M. B. *Nat. Chem. Biol.* **2011**, *7*, 876.

(2) Walsh, C. T.; Garneau-Tsodikova, S.; Gregory J. Gatto, J. *Angew. Chem., Int. Ed.* **2005**, *44*, 7342.

(3) Kee, J.-M.; Muir, T. W. *ACS Chem. Biol.* **2011**, *7*, 44.

(4) Bernardes, G. J. L.; Chalker, J. M.; Davis, B. G. *Chemical Protein Modification*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2010.

(5) (a) Sun, S. B.; Schultz, P. G.; Kim, C. H. *ChemBioChem.* **2014**, *15*, 1721. (b) Pelegri-O'Day, E. M.; Lin, E.-W.; Maynard, H. D. *J. Am. Chem. Soc.* **2014**, *136*, 14323. (c) Chari, R. V. J.; Miller, M. L.; Widdison, W. C. *Angew. Chem., Int. Ed.* **2014**, *53*, 3796. (d) Schumacher, D.; Hackenberger, C. P. R. *Curr. Opin. Chem. Biol.* **2014**, *22*, 62.

(6) (a) Siman, P.; Brik, A. *Org. Biomol. Chem.* **2012**, *10*, 5684. (b) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338. (c) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030.

(7) Baslé, E.; Joubert, N.; Pucheault, M. *Chem. Biol.* **2010**, *17*, 213.

(8) (a) Tasdelen, M. A.; Yagci, Y. *Angew. Chem., Int. Ed.* **2013**, *52*, 5930. (b) Palomo, J. M. *Org. Biomol. Chem.* **2012**, *10*, 9309. (c) van Berkel, S. S.; van Eldijk, M. B.; van Hest, J. C. M. *Angew. Chem., Int. Ed.* **2011**, *50*, 8806. (d) Lallana, E.; Riguera, R.; Fernandez-Megia, E. *Angew. Chem., Int. Ed.* **2011**, *50*, 8794.

(9) (a) Villalonga, M. L.; Díez, P.; Sánchez, A.; Gamella, M.; Pingarrón, J. M.; Villalonga, R. *Chem. Rev.* **2014**, *114*, 4868. (b) Wang, L.-X.; Amin, M. N. *Chem. Biol.* **2014**, *21*, 51. (c) Schmaltz, R. M.; Hanson, S. R.; Wong, C.-H. *Chem. Rev.* **2011**, *111*, 4259. (d) Gamblin, D. P.; Scanlan, E. M.; Davis, B. G. *Chem. Rev.* **2009**, *109*, 131.

(10) Nischan, N.; Hackenberger, C. P. R. *J. Org. Chem.* **2014**, *79*, 10727.

(11) (a) Matyjaszewski, K.; Tsarevsky, N. V. *J. Am. Chem. Soc.* **2014**, *136*, 6513. (b) Wallat, J. D.; Rose, K. A.; Pokorski, J. K. *Polym. Chem.* **2014**, *5*, 1545.

(12) (a) King, M.; Wagner, A. *Bioconjugate Chem.* **2014**, *25*, 825. (b) Lang, K.; Chin, J. W. *Chem. Rev.* **2014**, *114*, 4764. (c) Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. *ACS Chem. Biol.* **2014**, *9*, 592. (d) Lang, K.; Chin, J. W. *ACS Chem. Biol.* **2014**, *9*, 16. (e) Takaoka, Y.; Ojida, A.; Hamachi, I. *Angew. Chem., Int. Ed.* **2013**, *52*, 4088. (f) Debets, M. F.; van Hest, J. C. M.; Rutjes, F. P. J. T. *Org. Biomol. Chem.* **2013**, *11*, 6439. (g) Ramil, C. P.; Lin, Q. *Chem. Commun.* **2013**, *49*, 11007. (h) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974. (i) Shih, H.-W.; Kamber, D. N.; Prescher, J. A. *Curr. Opin. Chem. Biol.* **2014**, *21*, 103. (j) McKay, C. S.; Finn, M. G. *Chem. Biol.* **2014**, *21*, 1075. (k) Spicer, C. D.; Davis, B. G. *Nat. Commun.* **2014**, *5*, 4740.

(13) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem.—Asian J.* **2009**, *4*, 630.

(14) Hemantha, H. P.; Bavikar, S. N.; Herman-Bachinsky, Y.; Haj-Yahya, N.; Bondalapati, S.; Ciechanover, A.; Brik, A. *J. Am. Chem. Soc.* **2014**, *136*, 2665.

(15) (a) Massa, S.; Xavier, C.; De Vos, J.; Caveliers, V.; Lahoutte, T.; Muyldermans, S.; Devoogdt, N. *Bioconjugate Chem.* **2014**, *25*, 979. (b) Morales-Sanfrutos, J.; Lopez-Jaramillo, J.; Ortega-Munoz, M.; Megia-Fernandez, A.; Perez-Balderas, F.; Hernandez-Mateo, F.; Santoyo-Gonzalez, F. *Org. Biomol. Chem.* **2010**, *8*, 667. (c) Morales-Sanfrutos, J.; Lopez-Jaramillo, F. J.; Hernandez-Mateo, F.; Santoyo-Gonzalez, F. *J. Org. Chem.* **2010**, *75*, 4039.

(16) Cal, P. M. S. D.; Bernardes, G. J. L.; Gois, P. M. P. *Angew. Chem., Int. Ed.* **2014**, *53*, 10585.

(17) Badescu, G.; Bryant, P.; Swierkosz, J.; Khayrzad, F.; Pawlisz, E.; Farys, M.; Cong, Y.; Muroni, M.; Rumpf, N.; Brocchini, S.; Godwin, A. *Bioconjugate Chem.* **2013**, *25*, 460.

(18) (a) Bailey, J. J.; Bundle, D. R. *Org. Biomol. Chem.* **2014**, *12*, 2193. (b) Wurm, F. R.; Klok, H.-A. *Chem. Soc. Rev.* **2013**, *42*, 8220. (c) Patel, M. K.; Vijayakrishnan, B.; Koeppe, J. R.; Chalker, J. M.; Doores, K. J.; Davis, B. G. *Chem. Commun.* **2010**, *46*, 9119. (d) Liu, Z.; Liu, T.; Lin, Q.; Bao, C.; Zhu, L. *Chem. Commun.* **2014**, *50*, 1256.

(19) Asano, S.; Patterson, J. T.; Gaj, T.; Barbas, C. F. *Angew. Chem., Int. Ed.* **2014**, *53*, 11783.

(20) Adamo, R.; Nilo, A.; Castagner, B.; Boutureira, O.; Berti, F.; Bernardes, G. J. L. *Chem. Sci.* **2013**, *4*, 2995.

(21) Crotti, S.; Zhai, H.; Zhou, J.; Allan, M.; Proietti, D.; Pansegrau, W.; Hu, Q.-Y.; Berti, F.; Adamo, R. *ChemBioChem* **2014**, *15*, 836.

(22) Chen, X.; Muthoosamy, K.; Pfisterer, A.; Neumann, B.; Weil, T. *Bioconjugate Chem.* **2012**, *23*, 500.

(23) del Castillo, T.; Morales-Sanfrutos, J.; Santoyo-González, F.; Magez, S.; Lopez-Jaramillo, F. J.; Garcia-Salcedo, J. A. *ChemMedChem* **2014**, *9*, 383.

(24) Díaz-Rodríguez, A.; Davis, B. G. *Curr. Opin. Chem. Biol.* **2011**, *15*, 211.

(25) McGrath, N. A.; Andersen, K. A.; Davis, A. K. F.; Lomax, J. E.; Raines, R. T. *Chem. Sci.* **2015**, *6*, 752.

(26) (a) Kramer, J. R.; Deming, T. J. *Biomacromolecules* **2012**, *13*, 1719. (b) Kramer, J. R.; Deming, T. J. *Chem. Commun.* **2013**, *49*, 5144.

(27) Tanaka, K.; Fukase, K.; Katsumura, S. *Synlett* **2011**, *2011*, 2115.

(28) Robinson, M. A.; Charlton, S. T.; Garnier, P.; Wang, X.-T.; Davis, S. S.; Perkins, A. C.; Frier, M.; Duncan, R.; Savage, T. J.; Wyatt, D. A.; Watson, S. A.; Davis, B. G. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14527.

(29) Bavaro, T.; Filice, M.; Temporini, C.; Tengattini, S.; Serra, I.; Morelli, C. F.; Massolini, G.; Terreni, M. *RSC Adv.* **2014**, *4*, 56455.

(30) Cal, P. M. S. D.; Vicente, J. B.; Pires, E.; Coelho, A. V.; Veiros, L. F.; Cordeiro, C.; Gois, P. M. P. *J. Am. Chem. Soc.* **2012**, *134*, 10299.

(31) Cal, P. M. S. D.; Frade, R. F. M.; Chudasama, V.; Cordeiro, C.; Caddick, S.; Gois, P. M. P. *Chem. Commun.* **2014**, *50*, 5261.

(32) Diethelm, S.; Schafroth, M. A.; Carreira, E. M. *Org. Lett.* **2014**, *16*, 3908.

(33) Chan, A. O.-Y.; Ho, C.-M.; Chong, H.-C.; Leung, Y.-C.; Huang, J.-S.; Wong, M.-K.; Che, C.-M. *J. Am. Chem. Soc.* **2012**, *134*, 2589.

(34) (a) Kulkarni, C.; Kinzer-Ursem, T. L.; Tirrell, D. A. *ChemBioChem* **2013**, *14*, 1958. (b) Wagner, A. M.; Fegley, M. W.; Warner, J. B.; Grindley, C. L. J.; Marotta, N. P.; Petersson, E. J. *J. Am. Chem. Soc.* **2011**, *133*, 15139.

(35) (a) Schlick, T. L.; Ding, Z.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127*, 3718. (b) Hooker, J. M.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 3718.

(36) Gavrilyuk, J.; Ban, H.; Nagano, M.; Hakamata, W.; Barbas, C. F. *Bioconjugate Chem.* **2012**, *23*, 2321.

(37) Zhang, J.; Ma, D.; Du, D.; Xi, Z.; Yi, L. *Org. Biomol. Chem.* **2014**, *12*, 9528.

(38) Chen, S.; Tsao, M.-L. *Bioconjugate Chem.* **2013**, *24*, 1645.

(39) (a) Lorenzi, M.; Puppo, C.; Lebrun, R.; Lignon, S.; Roubaud, V.; Martinho, M.; Mileo, E.; Tordo, P.; Marque, S. R. A.; Gontero, B.; Guigliarelli, B.; Belle, V. *Angew. Chem., Int. Ed.* **2011**, *50*, 9108. (b) McFarland, J. M.; Joshi, N. S.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 7639. (c) Romanini, D. W.; Francis, M. B. *Bioconjugate Chem.* **2008**, *19*, 153. (d) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942.

(40) Mileo, E.; Etienne, E.; Martinho, M.; Lebrun, R.; Roubaud, V.; Tordo, P.; Gontero, B.; Guigliarelli, B.; Marque, S. R. A.; Belle, V. *Bioconjugate Chem.* **2013**, *24*, 1110.

(41) Guo, H.-M.; Minakawa, M.; Ueno, L.; Tanaka, F. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1210.

(42) Ban, H.; Gavrilyuk, J.; Barbas, C. F. *J. Am. Chem. Soc.* **2010**, *132*, 1523.

(43) (a) Hu, Q.-Y.; Allan, M.; Adamo, R.; Quinn, D.; Zhai, H.; Wu, G.; Clark, K.; Zhou, J.; Ortiz, S.; Wang, B.; Danieli, E.; Crotti, S.; Tontini, M.; Brogioni, G.; Berti, F. *Chem. Sci.* **2013**, *4*, 3827. (b) Adamo, R.; Hu, Q.-Y.; Torosantucci, A.; Crotti, S.; Brogioni, G.; Allan, M.; Chiani, P.; Bromuro, C.; Quinn, D.; Tontini, M.; Berti, F. *Chem. Sci.* **2014**, *5*, 4302. (c) Nilo, A.; Allan, M.; Brogioni, B.; Proietti, D.; Cattaneo, V.; Crotti, S.; Sokup, S.; Zhai, H.; Margarit, I.; Berti, F.; Hu, Q.-Y.; Adamo, R. *Bioconjugate Chem.* **2014**, *25*, 2105.

(44) Bauer, D. M.; Ahmed, I.; Vigovskaya, A.; Fruk, L. *Bioconjugate Chem.* **2013**, *24*, 1094.

(45) Chalker, J. M.; Bernardes, G. J. L.; Davis, B. G. *Acc. Chem. Res.* **2011**, *44*, 730.

(46) Boutureira, O.; Bernardes, G. J. L.; Fernández-González, M.; Anthony, D. C.; Davis, B. G. *Angew. Chem., Int. Ed.* **2012**, *51*, 1432.

(47) (a) Chalker, J. M.; Gunnoo, S. B.; Boutureira, O.; Gerstberger, S. C.; Fernández-González, M.; Bernardes, G. J. L.; Griffin, L.; Hailu, H.; Schofield, C. J.; Davis, B. G. *Chem. Sci.* **2011**, *2*, 1666. (b) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 5052. (c) Bernardes, G. J. L.; Grayson, E. J.; Thompson, S.; Chalker, J. M.; Errey, J. C.; Oualid, F. E.; Claridge, T. D. W.; Davis, B. G. *Angew. Chem., Int. Ed.* **2008**, *47*, 2244.

(48) Haj-Yahya, N.; Hemantha, H. P.; Meledin, R.; Bondalapati, S.; Seenaiah, M.; Brik, A. *Org. Lett.* **2014**, *16*, 540.

(49) Boutureira, O.; Bernardes, G. J. L.; D'Hooge, F.; Davis, B. G. *Chem. Commun.* **2011**, *47*, 10010.

(50) Kunstelj, M.; Fidler, K.; Škrajnar, Š.; Kenig, M.; Smilović, V.; Kusterle, M.; Caserman, S.; Zore, I.; Porekar, V. G.; Jevševar, S. *Bioconjugate Chem.* **2013**, *24*, 889.

(51) Grayson, E. J.; Bernardes, G. J. L.; Chalker, J. M.; Boutureira, O.; Koeppe, J. R.; Davis, B. G. *Angew. Chem., Int. Ed.* **2011**, *50*, 4127.

(52) Chalker, J. M.; Lercher, L.; Rose, N. R.; Schofield, C. J.; Davis, B. G. *Angew. Chem., Int. Ed.* **2012**, *51*, 1835.

(53) Fernández-González, M.; Boutureira, O.; Bernardes, G. J. L.; Chalker, J. M.; Young, M. A.; Errey, J. C.; Davis, B. G. *Chem. Sci.* **2010**, 709.

(54) Aydillo, C.; Compañón, I.; Avenoza, A.; Busto, J. H.; Corzana, F.; Peregrina, J. M.; Zurbano, M. M. *J. Am. Chem. Soc.* **2014**, *136*, 789.

(55) Junutula, J. R.; Raab, H.; Clark, S.; Bhakta, S.; Leipold, D. D.; Weir, S.; Chen, Y.; Simpson, M.; Tsai, S. P.; Dennis, M. S.; Lu, Y.; Meng, Y. G.; Ng, C.; Yang, J.; Lee, C. C.; Duenas, E.; Gorrell, J.; Katta, V.; Kim, A.; McDorman, K.; Flagella, K.; Venook, R.; Ross, S.; Spencer, S. D.; Lee Wong, W.; Lowman, H. B.; Vandlen, R.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Mallet, W. *Nat. Biotechnol.* **2008**, *26*, 925.

(56) (a) Steiner, M.; Hartmann, I.; Perrino, E.; Casi, G.; Brighton, S.; Jelesarov, I.; Bernardes, G. J. L.; Neri, D. *Chem. Sci.* **2013**, *4*, 297. (b) Bernardes, G. J. L.; Casi, G.; Trüssel, S.; Hartmann, I.; Schwager, K.; Scheuermann, J.; Neri, D. *Angew. Chem., Int. Ed.* **2012**, *51*, 941. (c) Perrino, E.; Steiner, M.; Krall, N.; Bernardes, G. J. L.; Pretto, F.; Casi, G.; Neri, D. *Cancer Res.* **2014**, *74*, 2569. (d) Bernardes, G. J. L.; Steiner, M.; Hartmann, I.; Neri, D.; Casi, G. *Nat. Protoc.* **2013**, *8*, 2079.

(57) Casi, G.; Huguenin-Dezot, N.; Zuberbühler, K.; Scheuermann, J. r.; Neri, D. *J. Am. Chem. Soc.* **2012**, *134*, 5887.

(58) Yuan, Y.; Liang, G. *Org. Biomol. Chem.* **2014**, *12*, 865.

(59) (a) Nathani, R. I.; Chudasama, V.; Ryan, C. P.; Moody, P. R.; Morgan, R. E.; Fitzmaurice, R. J.; Smith, M. E. B.; Baker, J. R.; Caddick, S. *Org. Biomol. Chem.* **2013**, *11*, 2408. (b) Smith, M. E. B.; Schumacher, F. F.; Ryan, C. P.; Tedaldi, L. M.; Papaioannou, D.; Waksman, G.; Caddick, S.; Baker, J. R. *J. Am. Chem. Soc.* **2010**, *132*, 1960.

(60) Moody, P.; Smith, M. E. B.; Ryan, C. P.; Chudasama, V.; Baker, J. R.; Molloy, J.; Caddick, S. *ChemBioChem* **2012**, *13*, 39.

(61) Marculescu, C.; Kossen, H.; Morgan, R. E.; Mayer, P.; Fletcher, S. A.; Tolner, B.; Chester, K. A.; Jones, L. H.; Baker, J. R. *Chem. Commun.* **2014**, *50*, 7139.

(62) (a) Lyon, R. P.; Setter, J. R.; Bovee, T. D.; Doronina, S. O.; Hunter, J. H.; Anderson, M. E.; Balasubramanian, C. L.; Duniho, S. M.; Leiske, C. I.; Li, F.; Senter, P. D. *Nat. Biotechnol.* **2014**, *32*, 1059. (b) Tumey, L. N.; Charati, M.; He, T.; Sousa, E.; Ma, D.; Han, X.; Clark, T.; Casavant, J.; Loganzo, F.; Barletta, F.; Lucas, J.; Graziani, E. I. *Bioconjugate Chem.* **2014**, *25*, 1871. (c) Fontaine, S. D.; Reid, R.; Robinson, L.; Ashley, G. W.; Santi, D. V. *Bioconjugate Chem.* **2015**, *26*, 145.

(63) Chudasama, V.; Smith, M. E. B.; Schumacher, F. F.; Papaioannou, D.; Waksman, G.; Baker, J. R.; Caddick, S. *Chem. Commun.* **2011**, *47*, 8781.

(64) Maruani, A.; Alom, S.; Canavelli, P.; Lee, M. T. W.; Morgan, R. E.; Chudasama, V.; Caddick, S. *Chem. Commun.* **2015**, DOI: 10.1039/C4CC08515A.

(65) Nathani, R.; Moody, P.; Smith, M. E. B.; Fitzmaurice, R. J.; Caddick, S. *ChemBioChem* **2012**, *13*, 1283.

(66) Nathani, R. I.; Moody, P.; Chudasama, V.; Smith, M. E. B.; Fitzmaurice, R. J.; Caddick, S. *Chem. Sci.* **2013**, *4*, 3455.

(67) Shiu, H.-Y.; Chan, T.-C.; Ho, C.-M.; Liu, Y.; Wong, M.-K.; Che, C.-M. *Chem.—Eur. J.* **2009**, *15*, 3839.

(68) Shiu, H.-Y.; Chong, H.-C.; Leung, Y.-C.; Zou, T.; Che, C.-M. *Chem. Commun.* **2014**, *50*, 4375.

(69) Koniev, O.; Leriche, G.; Nothisen, M.; Remy, J.-S.; Strub, J.-M.; Schaeffer-Reiss, C.; Van Dorsselaer, A.; Baati, R.; Wagner, A. *Bioconjugate Chem.* **2014**, *25*, 202.

(70) Arumugam, S.; Guo, J.; Mbua, N. E.; Friscourt, F.; Lin, N.; Nekongo, E.; Boons, G.-J.; Popik, V. V. *Chem. Sci.* **2014**, *5*, 1591.

(71) Zhang, D.; Devarie-Baez, N. O.; Li, Q.; Lancaster, J. R.; Xian, M. *Org. Lett.* **2012**, *14*, 3396.

(72) Toda, N.; Asano, S.; Barbas, C. F. *Angew. Chem., Int. Ed.* **2013**, *52*, 12592.

(73) Patterson, J. T.; Asano, S.; Li, X.; Rader, C.; Barbas, C. F. *Bioconjugate Chem.* **2014**, *25*, 1402.

(74) Abbas, A.; Xing, B.; Loh, T.-P. *Angew. Chem., Int. Ed.* **2014**, *53*, 7491.

(75) Lutolf, M. P.; Tirelli, N.; Cerritelli, S.; Cavalli, L.; Hubbell, J. A. *Bioconjugate Chem.* **2001**, *12*, 1051.

(76) Ma, F.-H.; Chen, J.-L.; Li, Q.-F.; Zuo, H.-H.; Huang, F.; Su, X.-C. *Chem.—Asian J.* **2014**, *9*, 1808.

(77) Moody, P.; Chudasama, V.; Nathani, R. I.; Maruani, A.; Martin, S.; Smith, M. E. B.; Caddick, S. *Chem. Commun.* **2014**, *50*, 4898.

(78) Shen, B.-Q.; Xu, K.; Liu, L.; Raab, H.; Bhakta, S.; Kenrick, M.; Parsons-Reponte, K. L.; Tien, J.; Yu, S.-F.; Mai, E.; Li, D.; Tibbitts, J.; Baudys, J.; Saad, O. M.; Scales, S. J.; McDonald, P. J.; Hass, P. E.; Eigenbrot, C.; Nguyen, T.; Solis, W. A.; Fuji, R. N.; Flagella, K. M.; Patel, D.; Spencer, S. D.; Khawli, L. A.; Ebens, A.; Wong, W. L.; Vandlen, R.; Kaur, S.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Junutula, J. R. *Nat. Biotechnol.* **2012**, *30*, 184.

(79) (a) Valkevich, E. M.; Guenette, R. G.; Sanchez, N. A.; Chen, Y.-C.; Ge, Y.; Strieter, E. R. *J. Am. Chem. Soc.* **2012**, *134*, 6916. (b) Li, F.; Allahverdi, A.; Yang, R.; Lua, G. B. J.; Zhang, X.; Cao, Y.; Korolev, N.; Nordenskiöld, L.; Liu, C.-F. *Angew. Chem., Int. Ed.* **2011**, *50*, 9611.

(80) Conte, M. L.; Staderini, S.; Marra, A.; Sánchez-Navarro, M.; Davis, B. G.; Dondoni, A. *Chem. Commun.* **2011**, *47*, 11086.

(81) (a) Chen, S.; Touati, J.; Heinis, C. *Chem. Commun.* **2014**, *50*, 5267. (b) Chen, S.; Bertoldo, D.; Angelini, A.; Pojer, F.; Heinis, C. *Angew. Chem., Int. Ed.* **2014**, *53*, 1602. (c) Angelini, A.; Diderich, P.; Morales-Sanfrutos, J.; Thurnheer, S.; Hacker, D.; Menin, L.; Heinis, C. *Bioconjugate Chem.* **2012**, *23*, 1856. (d) Angelini, A.; Heinis, C. *Curr. Opin. Chem. Biol.* **2011**, *15*, 355. (e) Heinis, C.; Rutherford, T.; Freund, S.; Winter, G. *Nat. Chem. Biol.* **2009**, *5*, 502.

(82) Fukunaga, K.; Hatanaka, T.; Ito, Y.; Minami, M.; Taki, M. *Chem. Commun.* **2014**, *50*, 3921.

(83) Bellotto, S.; Chen, S.; Rentero Rebollo, I.; Wegner, H. A.; Heinis, C. *J. Am. Chem. Soc.* **2014**, *136*, 5880.

(84) (a) Zou, Y.; Spokoyny, A. M.; Zhang, C.; Simon, M. D.; Yu, H.; Lin, Y.-S.; Pentelute, B. L. *Org. Biomol. Chem.* **2014**, *12*, 566. (b) Zhang, C.; Dai, P.; Spokoyny, A. M.; Pentelute, B. L. *Org. Lett.* **2014**, *16*, 3652. (c) Zhang, C.; Spokoyny, A. M.; Zou, Y.; Simon, M. D.; Pentelute, B. L. *Angew. Chem., Int. Ed.* **2013**, *52*, 14001.

(85) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269.

(86) Kim, T. H.; Swierczewska, M.; Oh, Y.; Kim, A.; Jo, D. G.; Park, J. H.; Byun, Y.; Sadegh-Nasseri, S.; Pomper, M. G.; Lee, K. C.; Lee, S. *Angew. Chem., Int. Ed.* **2013**, *52*, 6880.

(87) (a) Rashidian, M.; Dozier, J. K.; Distefano, M. D. *Bioconjugate Chem.* **2013**, *24*, 1277. (b) Rashidian, M.; Kumarapperuma, S. C.; Gabrielse, K.; Fegan, A.; Wagner, C. R.; Distefano, M. D. *J. Am. Chem. Soc.* **2013**, *135*, 16388.

(88) Uchinomiya, S.; Ojida, A.; Hamachi, I. *Inorg. Chem.* **2014**, *53*, 1816.

(89) (a) Chen, X.-H.; Xiang, Z.; Hu, Y. S.; Lacey, V. K.; Cang, H.; Wang, L. *ACS Chem. Biol.* **2014**, *9*, 1956. (b) Furman, J. L.; Kang, M.; Choi, S.; Cao, Y.; Wold, E. D.; Sun, S. B.; Smider, V. V.; Schultz, P. G.; Kim, C. H. *J. Am. Chem. Soc.* **2014**, *136*, 8411. (c) Lu, Y.; Huang, F.; Wang, J.; Xia, J. *Bioconjugate Chem.* **2014**, *25*, 989. (d) Xiang, Z.;

Lacey, V. K.; Ren, H.; Xu, J.; Burban, D. J.; Jennings, P. A.; Wang, L. *Angew. Chem., Int. Ed.* **2014**, *53*, 2190. (e) Masuya, T.; Murai, M.; Ifuku, K.; Morisaka, H.; Miyoshi, H. *Biochemistry* **2014**, *53*, 2307. (f) Hayashi, T.; Sun, Y.; Tamura, T.; Kuwata, K.; Song, Z.; Takaoka, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2013**, *135*, 12252. (g) Uchinomiya, S.; Nonaka, H.; Wakayama, S.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2013**, *49*, 5022. (h) Tamura, T.; Tsukiji, S.; Hamachi, I. *J. Am. Chem. Soc.* **2012**, *134*, 2216. (i) Fujishima, S.-h.; Yasui, R.; Miki, T.; Ojida, A.; Hamachi, I. *J. Am. Chem. Soc.* **2012**, *134*, 3961. (j) Wang, H.; Koshi, Y.; Minato, D.; Nonaka, H.; Kiyonaka, S.; Mori, Y.; Tsukiji, S.; Hamachi, I. *J. Am. Chem. Soc.* **2011**, *133*, 12220. (k) Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T.; Hamachi, I. *Nat. Chem. Biol.* **2009**, *5*, 341. (l) Koshi, Y.; Nakata, E.; Miyagawa, M.; Tsukiji, S.; Ogawa, T.; Hamachi, I. *J. Am. Chem. Soc.* **2008**, *130*, 245.

(90) Lodge, J. M.; Justin Rettenmaier, T.; Wells, J. A.; Pomerantz, W. C.; Mapp, A. K. *MedChemComm* **2014**, *5*, 370.

(91) Sato, S.; Nakamura, H. *Angew. Chem., Int. Ed.* **2013**, *52*, 8681.

(92) Brocchini, S.; Godwin, A.; Balan, S.; Choi, J.-W.; Zloh, M.; Shaunak, S. *Adv. Drug Delivery Rev.* **2008**, *60*, 3.

(93) (a) Badescu, G.; Bryant, P.; Bird, M.; Henseleit, K.; Swierkosz, J.; Parekh, V.; Tommasi, R.; Pawlisz, E.; Jurlewicz, K.; Farys, M.; Camper, N.; Sheng, X.; Fisher, M.; Grygorash, R.; Kyle, A.; Abhilash, A.; Frigerio, M.; Edwards, J.; Godwin, A. *Bioconjugate Chem.* **2014**, *25*, 1124. (b) Wang, T.; Ng, D. Y. W.; Wu, Y.; Thomas, J.; TamTran, T.; Weil, T. *Chem. Commun.* **2014**, *50*, 1116. (c) Wang, T.; Wu, Y.; Kuan, S. L.; Dumele, O.; Lamla, M.; Ng, D. Y. W.; Arzt, M.; Thomas, J.; Mueller, J. O.; Barner-Kowollik, C.; Weil, T. *Chem.—Eur. J.* **2015**, *21*, 228.

(94) Cong, Y.; Pawlisz, E.; Bryant, P.; Balan, S.; Laurine, E.; Tommasi, R.; Singh, R.; Dubey, S.; Peciak, K.; Bird, M.; Sivasankar, A.; Swierkosz, J.; Muroni, M.; Heidelberger, S.; Farys, M.; Khayrzad, F.; Edwards, J.; Badescu, G.; Hodgson, I.; Heise, C.; Somavarapu, S.; Liddell, J.; Powell, K.; Zloh, M.; Choi, J.-W.; Godwin, A.; Brocchini, S. *Bioconjugate Chem.* **2012**, *23*, 248.

(95) (a) Bryden, F.; Maruani, A.; Savoie, H.; Chudasama, V.; Smith, M. E. B.; Caddick, S.; Boyle, R. W. *Bioconjugate Chem.* **2014**, *25*, 611. (b) Jones, M. W.; Strickland, R. A.; Schumacher, F. F.; Caddick, S.; Baker, J. R.; Gibson, M. I.; Haddleton, D. M. *J. Am. Chem. Soc.* **2012**, *134*, 1847.

(96) (a) Jones, M. W.; Strickland, R. A.; Schumacher, F. F.; Caddick, S.; Baker, J. R.; Gibson, M. I.; Haddleton, D. M. *Chem. Commun.* **2012**, *48*, 4064. (b) Schumacher, F. F.; Nunes, J. P. M.; Maruani, A.; Chudasama, V.; Smith, M. E. B.; Chester, K. A.; Baker, J. R.; Caddick, S. *Org. Biomol. Chem.* **2014**, *12*, 7261.

(97) Castañeda, L.; Maruani, A.; Schumacher, F. F.; Miranda, E.; Chudasama, V.; Chester, K. A.; Baker, J. R.; Smith, M. E. B.; Caddick, S. *Chem. Commun.* **2013**, *49*, 8187.

(98) Hull, E. A.; Livanos, M.; Miranda, E.; Smith, M. E. B.; Chester, K. A.; Baker, J. R. *Bioconjugate Chem.* **2014**, *25*, 1395.

(99) (a) Dhal, P. K.; Polomoscanik, S. C.; Gianolio, D. A.; Starremans, P. G.; Busch, M.; Alving, K.; Chen, B.; Miller, R. J. *Bioconjugate Chem.* **2013**, *24*, 865. (b) Zuberbühler, K.; Casi, G.; Bernardes, G. J. L.; Neri, D. *Chem. Commun.* **2012**, *48*, 7100. (c) Zeng, Y.; Ramya, T. N. C.; Dirksen, A.; Dawson, P. E.; Paulson, J. C. *Nat. Methods* **2009**, *6*, 207. (d) Zhang, H.; Li, X.-j.; Martin, D. B.; Aebersold, R. *Nat. Biotechnol.* **2003**, *21*, 660.

(100) Witus, L. S.; Netirojjanakul, C.; Palla, K. S.; Muehl, E. M.; Weng, C.-H.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2013**, *135*, 17223.

(101) Carrico, I. S.; Carlson, B. L.; Bertozzi, C. R. *Nat. Chem. Biol.* **2007**, *3*, 321.

(102) El-Mahdi, O.; Melnyk, O. *Bioconjugate Chem.* **2013**, *24*, 735.

(103) (a) Smith, E. L.; Giddens, J. P.; Iavarone, A. T.; Godula, K.; Wang, L.-X.; Bertozzi, C. R. *Bioconjugate Chem.* **2014**, *25*, 788. (b) Hudak, J. E.; Barfield, R. M.; de Hart, G. W.; Grob, P.; Nogales, E.; Bertozzi, C. R.; Rabuka, D. *Angew. Chem., Int. Ed.* **2012**, *51*, 4161. (c) Ng, S.; Jafari, M. R.; Matochko, W. L.; Derda, R. *ACS Chem. Biol.* **2012**, *7*, 1482. (d) Kularatne, S. A.; Deshmukh, V.; Ma, J.; Tardif, V.; Lim, R. K. V.; Pugh, H. M.; Sun, Y.; Manibusan, A.; Sellers, A. J.;

Barnett, R. S.; Srinagesh, S.; Forsyth, J. S.; Hassenpflug, W.; Tian, F.; Javahishvili, T.; Felding-Habermann, B.; Lawson, B. R.; Kazane, S. A.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2014**, *53*, 11863.

(104) (a) Lu, Y.; Ngo Ndjock Mbong, G.; Liu, P.; Chan, C.; Cai, Z.; Weinrich, D.; Boyle, A. J.; Reilly, R. M.; Winnik, M. A. *Biomacromolecules* **2014**, *15*, 2027. (b) Zhou, Z.; Zhang, J.; Sun, L.; Ma, G.; Su, Z. *Bioconjugate Chem.* **2013**, *25*, 138.

(105) Kitov, P. I.; Vinals, D. F.; Ng, S.; Tjhung, K. F.; Derda, R. *J. Am. Chem. Soc.* **2014**, *136*, 8149.

(106) Agarwal, P.; van der Weijden, J.; Sletten, E. M.; Rabuka, D.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 46.

(107) Agarwal, P.; Kudirka, R.; Albers, A. E.; Barfield, R. M.; de Hart, G. W.; Drake, P. M.; Jones, L. C.; Rabuka, D. *Bioconjugate Chem.* **2013**, *24*, 846.

(108) Drake, P. M.; Albers, A. E.; Baker, J.; Banas, S.; Barfield, R. M.; Bhat, A. S.; de Hart, G. W.; Garofalo, A. W.; Holder, P.; Jones, L. C.; Kudirka, R.; McFarland, J.; Zmolek, W.; Rabuka, D. *Bioconjugate Chem.* **2014**, *25*, 1331.

(109) Han, M.-J.; Xiong, D.-C.; Ye, X.-S. *Chem. Commun.* **2012**, *48*, 11079.

(110) Lum, K. M.; Xavier, V. J.; Ong, M. J. H.; Johannes, C. W.; Chan, K.-P. *Chem. Commun.* **2013**, *49*, 11188.

(111) van Hest, J. C. M.; van Delft, F. L. *ChemBioChem* **2011**, *12*, 1309.

(112) (a) Ahad, A. M.; Jensen, S. M.; Jewett, J. C. *Org. Lett.* **2013**, *15*, 5060. (b) Bernardes, G. J. L.; Linderoth, L.; Doores, K. J.; Boutureira, O.; Davis, B. G. *ChemBioChem* **2011**, *12*, 1383.

(113) Serwa, R.; Majkut, P.; Horstmann, B.; Swiecicki, J.-M.; Gerrits, M.; Krause, E.; Hackenberger, C. P. R. *Chem. Sci.* **2010**, 596.

(114) Vallée, M. R. J.; Majkut, P.; Wilkening, I.; Weise, C.; Müller, G.; Hackenberger, C. P. R. *Org. Lett.* **2011**, *13*, 5440.

(115) Debets, M. F.; van Berkel, S. S.; Dommerholt, J.; Dirks, A. J.; Rutjes, F. P. J. T.; van Delft, F. L. *Acc. Chem. Res.* **2011**, *44*, 805.

(116) (a) Li, X.; Fang, T.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2014**, *53*, 7179. (b) Zeglis, B. M.; Davis, C. B.; Aggeler, R.; Kang, H. C.; Chen, A.; Agnew, B. J.; Lewis, J. S. *Bioconjugate Chem.* **2013**, *24*, 1057. (c) Zhou, Q.; Gui, J.; Pan, C.-M.; Albone, E.; Cheng, X.; Suh, E. M.; Grasso, L.; Ishihara, Y.; Baran, P. S. *J. Am. Chem. Soc.* **2013**, *135*, 12994.

(117) Jang, S.; Sachin, K.; Lee, H.-j.; Kim, D. W.; Lee, H. S. *Bioconjugate Chem.* **2012**, *23*, 2256.

(118) van Geel, R.; Pruijn, G. J. M.; van Delft, F. L.; Boelens, W. C. *Bioconjugate Chem.* **2012**, *23*, 392.

(119) Poole, T. H.; Reisz, J. A.; Zhao, W.; Poole, L. B.; Furdui, C. M.; King, S. B. *J. Am. Chem. Soc.* **2014**, *136*, 6167.

(120) Ning, X.; Temming, R. P.; Dommerholt, J.; Guo, J.; Ania, D. B.; Debets, M. F.; Wolfert, M. A.; Boons, G.-J.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 3065.

(121) Colombo, M.; Sommaruga, S.; Mazzucchelli, S.; Polito, L.; Verderio, P.; Galeffi, P.; Corsi, F.; Tortora, P.; Prosperi, D. *Angew. Chem., Int. Ed.* **2012**, *51*, 496.

(122) Sanders, B. C.; Friscourt, F.; Ledin, P. A.; Mbua, N. E.; Arumugam, S.; Guo, J.; Boltje, T. J.; Popik, V. V.; Boons, G.-J. *J. Am. Chem. Soc.* **2011**, *133*, 949.

(123) Plougastel, L.; Koniev, O.; Specklin, S.; Decuypere, E.; Créminon, C.; Buisson, D.-A.; Wagner, A.; Kolodych, S.; Taran, F. *Chem. Commun.* **2014**, *50*, 9376.

(124) Stöckmann, H.; Neves, A. A.; Stairs, S.; Brindle, K. M.; Leeper, F. J. *Org. Biomol. Chem.* **2011**, *9*, 7303.

(125) (a) Li, Y.; Pan, M.; Li, Y.; Huang, Y.; Guo, Q. *Org. Biomol. Chem.* **2013**, *11*, 2624. (b) Li, Y.; Yang, M.; Huang, Y.; Song, X.; Liu, L.; Chen, P. R. *Chem. Sci.* **2012**, *3*, 2766.

(126) (a) Thomas, J. D.; Cui, H.; North, P. J.; Hofer, T.; Rader, C.; Burke, T. R. *Bioconjugate Chem.* **2012**, *23*, 2007. (b) Kele, P.; Mező, G.; Achatz, D.; Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 344.

(127) (a) Plass, T.; Milles, S.; Koehler, C.; Szymański, J.; Mueller, R.; Wießler, M.; Schultz, C.; Lemke, E. A. *Angew. Chem., Int. Ed.* **2012**, *51*, 4166. (b) Plass, T.; Milles, S.; Koehler, C.; Schultz, C.; Lemke, E. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 3878.

(128) Hommersom, C. A.; Matt, B.; van der Ham, A.; Cornelissen, J. J. L. M.; Katsonis, N. *Org. Biomol. Chem.* **2014**, *12*, 4065.

(129) Wallace, S.; Chin, J. W. *Chem. Sci.* **2014**, *5*, 1742.

(130) Gattner, M. J.; Ehrlich, M.; Vrabel, M. *Chem. Commun.* **2014**, *50*, 12568.

(131) (a) Schneider, S.; Gattner, M. J.; Vrabel, M.; Flügel, V.; López-Carrillo, V.; Prill, S.; Carell, T. *ChemBioChem* **2013**, *14*, 2114. (b) Han, H.-S.; Devaraj, N. K.; Lee, J.; Hilderbrand, S. A.; Weissleder, R.; Bawendi, M. G. *J. Am. Chem. Soc.* **2010**, *132*, 7838. (c) Kurra, Y.; Odoi, K. A.; Lee, Y.-J.; Yang, Y.; Lu, T.; Wheeler, S. E.; Torres-Kolbus, J.; Deiters, A.; Liu, W. R. *Bioconjugate Chem.* **2014**, *25*, 1730.

(132) (a) Wang, X. S.; Lee, Y.-J.; Liu, W. R. *Chem. Commun.* **2014**, *50*, 3176. (b) Lee, Y.-J.; Wu, B.; Raymond, J. E.; Zeng, Y.; Fang, X.; Wooley, K. L.; Liu, W. R. *ACS Chem. Biol.* **2013**, *8*, 1664. (c) Li, F.; Zhang, H.; Sun, Y.; Pan, Y.; Zhou, J.; Wang, J. *Angew. Chem., Int. Ed.* **2013**, *52*, 9700. (d) Yu, Z.; Ohulchanskyy, T. Y.; An, P.; Prasad, P. N.; Lin, Q. *J. Am. Chem. Soc.* **2013**, *135*, 16766. (e) Kaya, E.; Vrabel, M.; Deiml, C.; Prill, S.; Fluxa, V. S.; Carell, T. *Angew. Chem., Int. Ed.* **2012**, *51*, 4466. (f) Lang, K.; Davis, L.; Torres-Kolbus, J.; Chou, C.; Deiters, A.; Chin, J. W. *Nat. Chem.* **2012**, *4*, 298. (g) Yu, Z.; Pan, Y.; Wang, Z.; Wang, J.; Lin, Q. *Angew. Chem., Int. Ed.* **2012**, *51*, 10600.

(133) Kamber, D. N.; Nazarova, L. A.; Liang, Y.; Lopez, S. A.; Patterson, D. M.; Shih, H.-W.; Houk, K. N.; Prescher, J. A. *J. Am. Chem. Soc.* **2013**, *135*, 13680.

(134) Yu, Z.; Lin, Q. *J. Am. Chem. Soc.* **2014**, *136*, 4153.

(135) Darko, A.; Wallace, S.; Dmitrenko, O.; Machovina, M.; Mehl, R.; Chin, J. W.; Fox, J. *Chem. Sci.* **2014**, *5*, 3770.

(136) (a) Wang, K.; Sachdeva, A.; Cox, D. J.; Wilf, N. W.; Lang, K.; Wallace, S.; Mehl, R. A.; Chin, J. W. *Nat. Chem.* **2014**, *6*, 393. (b) Sachdeva, A.; Wang, K.; Elliott, T.; Chin, J. W. *J. Am. Chem. Soc.* **2014**, *136*, 7785.

(137) Floyd, N.; Vijayakrishnan, B.; Koeppe, J. R.; Davis, B. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 7798.

(138) Li, Q.; Dong, T.; Liu, X.; Lei, X. *J. Am. Chem. Soc.* **2013**, *135*, 4996.

(139) Jouanno, L.-A.; Chevalier, A.; Sekkat, N.; Perzo, N.; Castel, H.; Romieu, A.; Lange, N.; Sabot, C.; Renard, P.-Y. *J. Org. Chem.* **2014**, *79*, 10353.

(140) Engelsma, S. B.; Willems, L. I.; van Paaschen, C. E.; van Kasteren, S. I.; van der Marel, G. A.; Overkleeft, H. S.; Filippov, D. V. *Org. Lett.* **2014**, *16*, 2744.

(141) (a) Yang, M.; Li, J.; Chen, P. R. *Chem. Soc. Rev.* **2014**, *43*, 6511. (b) Chankeshwara, S. V.; Indrigo, E.; Bradley, M. *Curr. Opin. Chem. Biol.* **2014**, *21*, 128.

(142) (a) Sasmal, P. K.; Streu, C. N.; Meggers, E. *Chem. Commun.* **2013**, *49*, 1581. (b) Antos, J. M.; Francis, M. B. *Curr. Opin. Chem. Biol.* **2006**, *10*, 253.

(143) McFarland, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127*, 13490.

(144) Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 1080.

(145) Chen, S.; Li, X.; Ma, H. *ChemBioChem* **2009**, *10*, 1200.

(146) Cserép, G. B.; Herner, A.; Wolfbeis, O. S.; Kele, P. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 5776.

(147) (a) Obermeyer, A. C.; Jarman, J. B.; Netirojjanakul, C.; El Muslemany, K.; Francis, M. B. *Angew. Chem., Int. Ed.* **2014**, *53*, 1057. (b) Ji, A.; Ren, W.; Ai, H.-W. *Chem. Commun.* **2014**, *50*, 7469. (c) Obermeyer, A. C.; Jarman, J. B.; Francis, M. B. *J. Am. Chem. Soc.* **2014**, *136*, 9572. (d) Seim, K. L.; Obermeyer, A. C.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133*, 16970.

(148) Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256.

(149) Antos, J. M.; McFarland, J. M.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2009**, *131*, 6301.

(150) Kundu, R.; Ball, Z. T. *Chem. Commun.* **2013**, *49*, 4166.

(151) Gillingham, D.; Fei, N. *Chem. Soc. Rev.* **2013**, *42*, 4918.

(152) On-Yee Chan, A.; Lui-Lui Tsai, J.; Kar-Yan Lo, V.; Li, G.-L.; Wong, M.-K.; Che, C.-M. *Chem. Commun.* **2013**, *49*, 1428.

(153) Liu, C. C.; Schultz, P. G. *Annu. Rev. Biochem.* **2010**, *79*, 413.

(154) (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596. (b) Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057.

(155) van Kasteren, S. I.; Kramer, H. B.; Jensen, H. H.; Campbell, S. J.; Kirkpatrick, J.; Oldham, N. J.; Anthony, D. C.; Davis, B. G. *Nature* **2007**, *446*, 1105.

(156) Raliski, B. K.; Howard, C. A.; Young, D. D. *Bioconjugate Chem.* **2014**, *25*, 1916.

(157) Boutureira, O.; D'Hooge, F.; Fernández-González, M.; Bernardes, G. J. L.; Sánchez-Navarro, M.; Koeppe, J. R.; Davis, B. G. *Chem. Commun.* **2010**, *46*, 8142.

(158) Diaz Velazquez, H.; Ruiz Garcia, Y.; Vandichel, M.; Madder, A.; Verpoort, F. *Org. Biomol. Chem.* **2014**, *12*, 9350.

(159) (a) Besanceney-Webler, C.; Jiang, H.; Zheng, T.; Feng, L.; Soriano del Amo, D.; Wang, W.; Klivansky, L. M.; Marlow, F. L.; Liu, Y.; Wu, P. *Angew. Chem., Int. Ed.* **2011**, *50*, 8051. (b) Kennedy, D. C.; McKay, C. S.; Legault, M. C. B.; Danielson, D. C.; Blake, J. A.; Pegoraro, A. F.; Stolow, A.; Mester, Z.; Pezacki, J. P. *J. Am. Chem. Soc.* **2011**, *133*, 17993. (c) Hong, V.; Presolski, S. I.; Ma, C.; Finn, M. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 9879.

(160) Kolodych, S.; Rasolofonjatovo, E.; Chaumontet, M.; Nevers, M.-C.; Créminon, C.; Taran, F. *Angew. Chem., Int. Ed.* **2013**, *52*, 12056.

(161) Tam, A.; Arnold, U.; Soellner, M. B.; Raines, R. T. *J. Am. Chem. Soc.* **2007**, *129*, 12670.

(162) Li, J.; Chen, P. R. *ChemBioChem* **2012**, *13*, 1728.

(163) Li, N.; Lim, R. K. V.; Edwardraja, S.; Lin, Q. *J. Am. Chem. Soc.* **2011**, *133*, 15316.

(164) Lim, R. K. V.; Li, N.; Ramil, C. P.; Lin, Q. *ACS Chem. Biol.* **2014**, *9*, 2139.

(165) Hauke, S.; Best, M.; Schmidt, T. T.; Baalmann, M.; Krause, A.; Wombacher, R. *Bioconjugate Chem.* **2014**, *25*, 1632.

(166) Li, N.; Ramil, C. P.; Lim, R. K. V.; Lin, Q. *ACS Chem. Biol.* **2015**, DOI: 10.1021/cb500649q.

(167) Li, J.; Lin, S.; Wang, J.; Jia, S.; Yang, M.; Hao, Z.; Zhang, X.; Chen, P. R. *J. Am. Chem. Soc.* **2013**, *135*, 7330.

(168) Li, J.; Yu, J.; Zhao, J.; Wang, J.; Zheng, S.; Lin, S.; Chen, L.; Yang, M.; Jia, S.; Zhang, X.; Chen, P. R. *Nat. Chem.* **2014**, *6*, 352.

(169) (a) Cheng, G.; Lim, R. K. V.; Li, N.; Lin, Q. *Chem. Commun.* **2013**, *49*, 6809. (b) Cheng, G.; Lim, R. K. V.; Ramil, C. P.; Lin, Q. *Chem. Commun.* **2014**, *50*, 11679.

(170) Lin, Y. A.; Chalker, J. M.; Davis, B. G. *ChemBioChem* **2009**, *10*, 959.

(171) (a) Lin, Y. A.; Chalker, J. M.; Davis, B. G. *J. Am. Chem. Soc.* **2010**, *132*, 16805. (b) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 9642.

(172) Chalker, J. M.; Lin, Y. A.; Boutureira, O.; Davis, B. G. *Chem. Commun.* **2009**, 3714.

(173) Lin, Y. A.; Davis, B. G. *Beilstein J. Org. Chem.* **2010**, *6*, 1219.

(174) Lin, Y. A.; Boutureira, O.; Lercher, L.; Bhushan, B.; Paton, R. S.; Davis, B. G. *J. Am. Chem. Soc.* **2013**, *135*, 12156.

(175) Ai, H.-W.; Shen, W.; Brustad, E.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2010**, *49*, 935.

(176) Ourailidou, M. E.; van der Meer, J.-Y.; Baas, B.-J.; Jeronimus-Stratingh, M.; Gottumukkala, A. L.; Poelarends, G. J.; Minnaard, A. J.; Dekker, F. J. *ChemBioChem* **2014**, *15*, 209.

(177) Crich, D.; Subramanian, V.; Karatholuvhu, M. *J. Org. Chem.* **2009**, *74*, 9422.

(178) Crich, D.; Zou, Y.; Brebion, F. *J. Org. Chem.* **2006**, *71*, 9172.

(179) Croft, L. *Nat. Chem.* **2010**, *2*, 1009.

(180) Ojida, A.; Tsutsumi, H.; Kasagi, N.; Hamachi, I. *Tetrahedron Lett.* **2005**, *46*, 3301.

(181) Kodama, K.; Fukuzawa, S.; Nakayama, H.; Kigawa, T.; Sakamoto, K.; Yabuki, T.; Matsuda, N.; Shirouzu, M.; Takio, K.; Tachibana, K.; Yokoyama, S. *ChemBioChem* **2006**, *7*, 134.

(182) Kodama, K.; Fukuzawa, S.; Nakayama, H.; Sakamoto, K.; Kigawa, T.; Yabuki, T.; Matsuda, N.; Shirouzu, M.; Takio, K.; Yokoyama, S.; Tachibana, K. *ChemBioChem* **2007**, *8*, 232.

(183) Brustad, E.; Bushey, M. L.; Lee, J. W.; Groff, D.; Liu, W.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2008**, *47*, 8220.

(184) Chalker, J. M.; Wood, C. S. C.; Davis, B. G. *J. Am. Chem. Soc.* **2009**, *131*, 16346.

(185) Spicer, C. D.; Davis, B. G. *Chem. Commun.* **2011**, *47*, 1698.

(186) Spicer, C. D.; Triemer, T.; Davis, B. G. *J. Am. Chem. Soc.* **2012**, *134*, 800.

(187) Spicer, C. D.; Davis, B. G. *Chem. Commun.* **2013**, *49*, 2747.

(188) Ma, X.; Wang, H.; Chen, W. *J. Org. Chem.* **2014**, *79*, 8652.

(189) Dumas, A.; Spicer, C. D.; Gao, Z.; Takehana, T.; Lin, Y. A.; Yasukohchi, T.; Davis, B. G. *Angew. Chem., Int. Ed.* **2013**, *52*, 3916.