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Residue-Specific Peptide Modification: A Chemist's Guide

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Abstract

Advances in bioconjugation and native protein modification are appearing at a blistering pace, making it increasingly time consuming for practitioners to identify the best chemical method for modifying a specific amino acid residue in a complex setting. The purpose of this perspective is to provide an informative, graphically rich manual highlighting significant advances in the field over the past decade. This guide will help triage candidate methods for peptide alteration and will serve as a starting point for those seeking to solve long-standing challenges.

Graphical abstract

Just as some of the most pivotal advances in total synthesis can be tied to overcoming obstacles in chemoselectivity,¹ advances in bioconjugation chemistry are intimately linked to the development of targeted amino acid modifications for the precise engineering of proteins. Given the sheer number of functional groups present and the general requirement for aqueous reaction media, protein modification is perhaps the ultimate expression of a chemoselective reaction. A growing appreciation for the therapeutic potential of peptides^{2,3} has led to numerous initiatives that necessitate an equivalent degree of chemoselectivity in diverse, late-stage peptide functionalizations. This challenge has mobilized peptide and organic chemists alike, a point illustrated by the upsurge of interdisciplinary collaborations

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Author Contributions

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Supporting Information

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Delineation of Amino Acid Side-Chain Modification Report Card assessments, including references (PDF)

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Notes

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over the past decade. Such endeavors have uniquely altered the landscape of traditional amino acid modification strategies for both peptide functionalization and protein bioconjugation, and will be the focus of this perspective.

This survey is not intended to provide a comprehensive history of bioconjugation and related chemistry;^{4–7} rather, it should serve as a graphically rich catalog for the practitioner interested in recently developed transformations for the discrete modification of polypeptide and protein substrates. Ligation,⁸ stapling,⁹ macrocyclization,¹⁰ and cross-linking¹¹ strategies will not be covered, nor will methods that rely on enzymatic transformations¹² or the incorporation of designer residues.¹³ Instead, chemical modifications specific to proteinogenic residues, select noncanonical but naturally occurring residues [e.g., dehydroalanine (Dha)], and peptide C- and N-termini will be highlighted. The format is pedagogically similar to *The Portable Chemist's Consultant*,¹⁴ an e-book recently published for those engaged in chemical synthesis.

A model protein bioconjugation should be site-selective and robust and should proceed under mild conditions (physiological pH, ambient temperature and pressure, and aqueous solvent preferred). Moreover, preservation of the protein structure and conservation of activity are among the most important considerations. While shorter peptide sequences allow for some deviation, the operational simplicity epitomized by these standards is an ideal goal for the development of versatile peptide modification strategies. With these (and other) factors in mind, the aim of this user guide is to provide an etic assessment of both the advantages offered and limitations imposed by the strategies featured herein. Organized by amino acid, this schematic manual will detail key elements of each reaction, including demonstrated or implied compatibility (indicated by brackets) with common peptide functional groups, attributes unique to the method described, and challenges that may arise in reagent preparation or reaction protocol. Specific examples and general trends are highlighted in the text to guide the reader and provide essential information in a succinct manner.

Finally, this guide is supplemented with a high-level view of the field [see Table 3 (Amino Acid Side-Chain Modification Report Card) and the Supporting Information] that offers a snapshot of the available literature in the area. These resources have been compiled with an eye toward gaps in current methodology and opportunities for innovation.

ALIPHATIC SIDE CHAINS

Without an apparent functional handle, there are relatively few methods available (*vide infra*) for derivatization of the aliphatic residues. However, fundamental advances in the direct functionalization of C–H bonds have ushered in new opportunities for targeted modifications.¹⁵ In perhaps the most notable recent example, Yu and co-workers disclosed a palladium-catalyzed C–H arylation of N-terminal alanine (Ala) residues facilitated by coordination of the metal catalyst to the peptide backbone.¹⁶ Although currently limited to di-, tri-, and tetrapeptide substrates, this seminal report illustrates the vast potential of postassembly C(sp³)–H functionalization as an enabling tool for hydrocarbon modifications.

A promising metal-mediated approach to the γ -arylation of valine (Val) and isoleucine (Ile) within dipeptide substrates has also been reported¹⁷ (Table 1).

POLAR, NONIONIZABLE SIDE CHAINS

Similar to aliphatic residues, the primary amides of asparagine (Asn) and glutamine (Gln) are all but immovable and have proven to be difficult targets for selective modification. One notable exception is the molecular recognition strategy employed by Popp and Ball, which enabled a targeted, proximity-driven modification of Asn and Gln using dirhodium metallopeptides.¹⁹ In contrast, creative reactions seeking to modify methionine (Met) are far more common. With a relatively high oxidation potential, the reversible oxidation of the thioether to the corresponding sulfoxide or sulfone is a well-defined Met reaction pathway, both synthetically and biochemically.⁹⁶ Strikingly, it is the only native residue that can be alkylated under acidic conditions.⁹⁷ In fact, early approaches to protein degradation for sequencing exploited these unique properties; cyanogen bromide in formic or hydrochloric acid buffer, for example, is a reliable method for Met-specific peptide bond hydrolysis that is still in use today.⁹⁸ The methylthioether side chain can also be used as a homocysteine proxy, as in entry 2.²¹ Alkylation and subsequent demethylation provides the homologated cysteine (Cys) analogue.

In a recent joint effort, Toste, Chang, and co-workers reported the use of redox-activated chemical tagging (ReACT) for site-selective Met modification (entry 4).²³ This bioconjugation strategy harnesses the previously underexplored nitrogen-transfer properties of oxaziridines to form the functionalized sulfimide. The resulting conjugates demonstrate remarkable stability when exposed to a wide pH range, disulfide reducing agents, and bioorthogonal reaction conditions. Furthermore, the ReACT approach exhibits exclusive selectivity for Met in both protein and antibody substrates.

AROMATIC SIDE CHAINS

Among the most abundant targets for new synthetic transformations, residues with aromatic side chains can be further divided into those that are readily ionized (histidine, His, and tyrosine, Tyr) and those that are not (tryptophan, Trp, and phenylalanine, Phe). It should be noted, however, that side-chain pK_a is not an exclusive predictor of reactivity in this case, as indicated by the wealth of transformations specific to the indole of Trp. Importantly, the nucleophilicity of both His and Tyr can be modulated by the protonation state of the side chain. As such, the standard modes of Tyr reactivity can be effectively tuned by pH control: in acidic or neutral environments, the aromatic carbons *ortho* to the hydroxyl group may undergo ene-type reactions (entry 5),^{26,27} diazonium couplings (entry 6),²⁸ and Mannich-type condensation reactions (entry 9),^{31,32} while at a pH near the pK_a of phenol (~ 10), alkylation or acylation of the oxygen is observed (entries 8 and 10).^{30,33,34} In 2009, Barbas and co-workers reported a robust aqueous ene-type reaction that enables click-like Tyr bioconjugation (entry 5).^{26,27} Further investigation has expanded the scope and utility of this method, allowing for the installation of diverse handles, including PEG chains and bifunctional linkers.²⁷ In the case of Trp, modifications involving the indole C-2 position dominate the reaction landscape. A recent, metal-free organoradical conjugation strategy

(entry 12)³⁶ supplements a variety of powerful metal-based methods (entries 13–15).^{19,37–43} Metal-catalyzed C–H functionalization is a particularly useful strategy that enables alkynylation (entry 13)^{37,38} and arylation (entry 14)^{39–41} of the indole side chain. While not yet fully realized, emerging trends in C–H functionalization are expected to facilitate the development of robust modification protocols for Phe and His.

POLAR, IONIZABLE SIDE CHAINS

As the most readily modified of amino acids, residues with polar, ionizable side chains have been widely examined. Cys, undoubtedly the most well-studied residue, occupies a distinguished place in the bioconjugation literature.^{4,99} The inherently low pK_a (~8.3) and substantial nucleophilic character provide a convenient handle for site-selective derivatization. Historically, cysteine–maleimide conjugation has been the most common manner of thiol modification, but the low stability of the adducts has rendered this particular reaction outmoded.¹⁰⁰ However, as a mild and selective modification, conjugate addition remains a popular approach for functionalization. With recent improvements in conjugate stability (entries 22–25),^{52–56} including the design of self-hydrolyzing maleimides,¹⁰¹ as well as the development of bifunctional Michael acceptors (entries 22 and 24),^{52,54,55} it is expected that this venerable reaction will retain prominence for years to come.

In an effort to improve the delivery of compact, high-value bioisosteres—namely, cyclobutanes, cyclopentanes, and related analogues—our lab reported the development of a variety of designer, spring-loaded reagents for strain-release functionalization.^{45,46} Remarkably, a number of bicyclobutane sulfones and chiral “housanes” were shown to rapidly and selectively functionalize Cys (entry 17)^{45,46} in the presence of several unprotected, nucleophilic peptide functional groups.

The use of transition metals in bioconjugation chemistry has gained recent traction, with the combined efforts of Pentelute, Buchwald, and co-workers at the forefront of these endeavors.^{47,60} In 2015, the researchers reported a palladium(II)-promoted arylation of Cys under mild conditions (entry 18).⁴⁷ Of note, the palladium(II) complexes used are readily synthesized and easy to handle. In addition, the arylation reaction proved to be an effective tool in peptide stapling and antibody–drug conjugation, leading to stable bioconjugates. The site-guided palladium-mediated arylation of metal-binding proteins¹⁰² and expansion of transition metal functionalizations to the alkynylation (entry 21) and alkenylation of Cys by Messaoudi and co-workers⁵⁰ demonstrate the adaptability of this approach.

While a myriad of acid-specific reactions exists, differentiation between peptide α - and side-chain carboxylic acids (e.g., aspartic acid, Asp, and glutamic acid, Glu) has proven to be a formidable challenge in bioconjugation chemistry. Initially reported by our group in 2016,¹⁰³ the use of “redox-active esters” (RAEs) as a means to activate and engage carboxylic acids in a nickel-catalyzed cross-coupling event has since been parlayed into a broad approach for the construction of carbon–carbon (entries 27, 28, and 44)^{58,59,79} and carbon–heteroatom (entry 46) bonds.⁸² The robust procedure, which repurposes activating reagents typically employed in amide bond formation (e.g., HOAt, *N*-hydroxyphthalimides), has been successfully applied to both resin-bound^{58,79} and solution-phase peptide substrates,

^{59,79,82} allowing for the introduction of discrete cross-coupled products at either side-chain positions or α -positions.

Approaches to lysine (Lys) modification encounter similar obstacles in α - versus ϵ -amine differentiation, but selective acylation or alkylation can be achieved through careful pH control. Recent advances in arylation (entry 29)⁶⁰ and condensation (entry 30)⁶¹ reactions have also expanded the diversity of functionalizations. The potential for reversible labeling (entries 31 and 32)^{62,63} of Lys is particularly intriguing, with envisioned applications toward the development of antibody–drug conjugates (ADCs). Finally, modifications to arginine (Arg) are currently limited in scope; acylation and condensation are common approaches (entries 35 and 36),^{66–68} with the most provocative adduct (entry 37)⁶⁹ being a serendipitous byproduct formed during a copper-catalyzed azide–alkyne cycloaddition. To date, chemoselective side-chain modifications at serine (Ser) and threonine (Thr) are exceedingly rare, generally relying on proximity-driven¹⁹ or sequence-specific modifications; more broadly applicable methods represent an opportunity for invention.

NONCANONICAL AMINO ACIDS

Biosynthetically, Dha is often incorporated into peptides through the enzymatic dehydration of Ser;¹⁰⁴ targeted chemical approaches, in contrast, typically proceed through activation–elimination of the Cys thiol¹⁰⁵ or an analogous, and similarly facile, transformation at modified selenocysteine (Sec) residues.¹⁰⁶ The electrophilic, unsaturated side chain is frequently utilized as an acceptor in 1,4-conjugate additions (entries 39 and 40).^{71–75} In a recent example, both the Davis laboratory⁷⁵ and the collaborative team of Söll, Lee, and Park⁷⁴ independently reported a radical-based conjugate addition (entry 40) to Dha for the site-selective introduction of diverse non-native residues into several target proteins.

In addition to its role as a Dha precursor, Sec is often purposed as a handle for native chemical ligation.¹⁰⁷ Relatively few examples of covalent derivatization exist, primarily because of the low reduction potential and high polarizability of the selenol functional group.¹⁰⁸ Capitalizing on these otherwise unwieldy characteristics, Pentelute and Buchwald reported a copper-promoted umpolung approach to the arylation of Sec, which takes advantage of the electrophilicity of selenyl sulfides (entry 41).⁷⁶ While the reaction conditions were conclusively determined to favor Sec arylation, oxidation of unprotected Cys was unavoidable.

C-TERMINUS AND N-TERMINUS

Modification of peptide termini is an attractive approach, particularly in cases in which strict bioconjugate stoichiometry is required. The use of specialized linkers to reveal unique functionality on resin cleavage is a common approach to the alteration of C-terminal acids and has been the subject of prior reviews.^{109,110} Focusing instead on postassembly synthetic transformations, it is unsurprising that the aforementioned chemoselectivity challenges (see the discussion of Asp and Glu in Polar, Ionizable Side Chains) apply to α -carboxylic acids as well. Nevertheless, orthogonal protecting group strategies and judicious sequence choice have been employed to enable several decarboxylative functionalizations of α -acids,

including 1,4-additions (entry 44),⁷⁹ heteroarylations (entry 45),^{80,81} and borylations (entry 46).⁸²

Bioconjugation reactions that target α -amines have been the subject of much investigation for use in ligation and macrocyclization; for further insight into these invaluable processes, we direct readers to the many extensive reviews available.^{4–10} Alternative modifications at the N-terminus¹¹¹ often rely on participation from the associated side-chain functional group; reactions that engage ionizable residues (e.g., Cys) are common (entries 47 and 48),^{83–85} as are examples that employ aromatic (entry 56)^{94,95} and other nucleophilic side chains (e.g., Ser and Thr).⁸⁹ The formation of heterocycles is a common approach (entries 47–49, 51, 53, and 56)^{83–86,89,91,94,95} as is N-terminal oxidation and further diversification (entries 50 and 51).^{87–89}

BACKBONE MODIFICATION

As in small molecule chemistries, transition metals have enabled previously unimaginable bioconjugate transformations,^{112,113} an assertion particularly true in the burgeoning area of backbone modifications (Scheme 1).^{114–117} In 2016, White and co-workers reported an iron-catalyzed oxidative functionalization inspired by known nonribosomal peptide synthetase (NRPS) pathways.¹⁸ In an impressive display of versatility, the approach was used to synthesize 21 non-natural amino acids from four native residues, with no erosion of chirality observed. Notably, C–H oxidation of the tertiary carbon center of Leu and Val was also demonstrated.¹⁸

In another intriguing example, Ball and co-workers detailed a copper-mediated, His-directed amide N-functionalization in a rare example of selective backbone modification in the context of proteins.¹¹⁷ While relatively few examples of these methods currently exist, the profound effects such modifications have on peptide and protein structure and function^{118,119} will undoubtedly drive further innovation.

FUTURE OUTLOOK

The biological importance of modified peptides and proteins is a powerful impetus for the continued exploration of diverse amino acid-specific modifications. To this end, the operational simplicity and inherent flexibility of direct modifications of proteinogenic amino acids are undeniable. A high-level view of progress toward residue-specific peptide and protein modifications is outlined in Table 3 (Amino Acid Side-Chain Modification Report Card) and further detailed in the Supporting Information. Categorized by amino acid residue and type of chemical transformation, this graphical depiction illustrates recent and historical successes in the field, with the mild reaction conditions and exquisite chemoselectivity required for protein modifications serving as the ultimate evaluation parameters. Framed by these ideals, the amino acid landscape is marked by several remaining challenges, or rather, opportunities for invention: (1) robust aliphatic functionalization, (2) the transition from “proof of concept” (e.g., single amino acids or short, unadorned peptide sequences) to “widely applicable” (e.g., larger peptides, proteins), and (3) well-defined, site-selective modification in the presence of chemically equivalent residues. We anticipate that advances

in metal-catalyzed C–H functionalization will address a number of current limitations and continue to enlist both peptide and small molecule chemists, leading to an expanding library of interdisciplinary methods. Indeed, the requisite functional group selectivity and mild reaction conditions are enviable benchmarks for all new synthetic methods. Translational approaches to peptide and protein modifications therefore have the capacity to revolutionize the capabilities of modern organic synthesis while also delivering high-value biological targets to meet urgent societal needs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PEG	polyethylene glycol
Ab	antibody
Ar	aryl
Boc	<i>tert</i> -butoxycarbonyl
Alk	alkyl
2-pym	2-pyrimidine
PG	protecting group
Conj	conjugate
HetAr	heteroaryl
ee	enantiomeric excess
NHS	<i>N</i> -hydroxysuccinimide
HPLC	high-performance liquid chromatography
DHA	dehydroascorbate
PTM	post-translational modification
Fmoc	fluorenylmethoxycarbonyl
SPPS	solid-phase peptide synthesis
Bpin	boronic acid pinacol ester

dr	diastereomeric ratio
AA	amino acid
HOAt	1-hydroxy-7-azabenzotriazole
Nu	nucleophile
LDA	lithium diisopropyl amide
PMP	<i>p</i> -methoxyphenyl
TBHP	<i>tert</i> -butyl hydroperoxide
PDP	2-(((<i>R</i>)-2-[(<i>R</i>)-1-(pyridin-2-ylmethyl)-pyrrolidin-2-yl]pyrrolidin-1-yl)methyl)pyridine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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Scheme 1.

Table 1

ALIPHATIC SIDE-CHAINS

Ala

Gly

Ile

Leu

Pro

Val

While there are very few methods available for the derivatization of aliphatic residues, recent advances in C-H functionalization have enabled the direct modification of hydrocarbon side-chains in small peptide systems.¹⁶⁻¹⁸ Although not yet broadly applicable to complex peptides, these proof-of-principle demonstrations are expected to herald a new era of diverse peptide modifications.

POLAR, NON-IONIZABLE SIDE-CHAINS

Asn

Gln

Met

Aside from a few isolated examples, targeted modifications of Asn and Gln residues within peptides and proteins are exceedingly rare. However, the preponderance of enzyme-mediated post-translational modifications targeting these residues both justifies and inspires further examination of selective side-chain carboxamide functionalizations. Indeed, the first example of Asn and Gln selective modifications, described by Popp and Ball,¹⁹ exploited the enzyme-like specificity of a proximity-driven reaction with dirhodium metallopolymers. Further developments in the area will likely require similar molecular recognition strategies to achieve the desired chemoselectivity.

The Met side chain offers reactivity complementary to that of other nucleophilic, proteinogenic side-chains. As the only native residue that can be alkylated under acidic conditions, opportunities for Met-specific functionalization are abundant. In processes reminiscent of its biochemical roles, the methylthioether provides a platform for a number of modifications, including oxidation to the corresponding sulfoxide/sulfone or sulfimide and alkylation to the functional—in some cases, stable—sulfonium salt. Met may also act as a homoCys surrogate; selective alkylation followed by demethylation gives rise to a variety of functionalized homoCys products directly from native sequences.

entry	AA compatibility/comments	entry	AA compatibility/comments
1 	Epoxide alkylation (Ref. 20) C, H, K, R, α-amine (Pro) • wet, protic media • stable sulfonium; no dealkylation observed • stable to biorthogonal reaction conditions	3 	Reversible alkylation (Ref. 22) C, H, K, R, α-amine (Pro) • stable to exogenous nucleophiles, extreme pH/temperature • can be reversed; cystine disulfide bonds are stable to reaction conditions • installation of high-value tags
2 	Alkylation-demethylation (Ref. 21) C, H, K, R, α-amine (Pro) • provides stable, functionalized homoCys derivatives • lability of R < Me for efficient demethylation	4 	Sulfur imidation using oxaziridines (Ref. 23) protein • installation of high-value synthetic handles • high selectivity, mild conditions

AROMATIC SIDE-CHAINS

Phe

His

Tyr

Relatively few methods exist for the functionalization of Phe and His. This unmet challenge is particularly fascinating for His, given the prevalence of imidazole substitution reactions²⁴ and the privileged biological role of the His side-chain. Under physiological conditions, the protonation state of His is split between the imidazole and imidazolium forms, leading to the epithet, "Nature's proton shuttle".²⁵ and in metalloproteins, His serves as a common coordinating ligand—a property which may be fine-tuned through targeted modifications. New methods are needed to unlock the potential of both His and Phe as fully diversifiable residues in complex peptides.

The tyrosine side-chain may exist in either the phenol or phenolate form; while the latter is more reactive, a general desire to maintain near-physiological conditions in bioconjugate chemistry renders the phenolate form a minor contributor in most cases. Nevertheless, the electron rich aromatic ring participates in a number of transformations, including O- and C-alkylation and arylation through a variety of reaction pathways as well as diazonium couplings and ene-like reactions. Unsurprisingly, in cases of C-targeted modification, the vast majority of methods afford either mono- or bis-functionalization at the positions *ortho* to the hydroxyl group.

entry	AA compatibility/comments	entry	AA compatibility/comments
5 	Ene-like reaction with triazoline-diones (Ref. 26 and 27) H, K, R, S, W, protein • stable adduct formed • tolerates extreme pH, temperature • installation of high-value synthetic handles	8 	O-arylation via aniline coupling (Ref. 30) D/E, H, K, Q, R, S/T • enables efficient coupling of electron-rich aniline derivatives • limited to N,N-dialkyl anilines • free Cys may be oxidized • cannot readily differentiate between Tyr/Trp
6 	Coupling with polymeric diazonium salts (Ref. 28) E, H, K, Q, R, S/T, α-acid, α-amine • allows for ¹⁵ N isotopic labeling • conditions optimized for high Tyr selectivity	9 	Three-component Mannich-type reaction (Ref. 31 and 32) D/E, K, N/Q, R, S/T, W, Y, protein • method developed for attachment of pre-functionalized peptide building blocks • aniline of peptide coupling partner can be readily installed at C- or N-terminus
7 	Iodination-Suzuki cross-coupling (Ref. 29) M, S, α-acid, α-amine • proceeds through diiodinated intermediate • isolable or one-pot • para-iodination of Phe • aqueous media, mild temperatures	10 	Alkylation with n-allyl Pd complexes (Ref. 33 and 34) protein • aqueous media, room temperature reaction • facile installation of fluorescent tags

Trp

As one of only four aromatic side-chains, the Trp indole moiety offers unique opportunities for targeted functionalization. C-2 modifications have proven the most facile—and, accordingly, the most explored—avenue of derivatization. In reactions that seek to modify the C-2 position, however, N-H interference can be problematic, particularly in cases that utilize metallocarbene chemistry. The indole nucleus itself has been the subject of considerable investigation, translating into the variety of emerging Trp-based methods, including metal-mediated C-H functionalization reactions. Despite these advances, selective modifications to the benzo-component remain elusive.

entry	AA compatibility/comments	entry	AA compatibility/comments
11	Sulfenylation (Ref. 35) S, Y, (on-resin) <ul style="list-style-type: none"> demonstrated use in ligation high C-2 selectivity Boc protected indoles do not react orthogonal protection may allow selectivity in the presence of multiple Trp residues 	14	Metal-catalyzed C-H arylation (Ref. 39–41) <ul style="list-style-type: none"> Pd- and Ru-catalyzed reactions high functional group tolerance installation of high-value synthetic handles bifunctional arenes allow for ligation
12	Organoradical conjugation (Ref. 36) H, K, M, S, Y, disulfide, protein, mAb <ul style="list-style-type: none"> transition-metal free, aqueous media carbonyl can be further modified by oxime formation 	15	Rh-carbenoid labeling at mild pH (Ref. 19, 42, and 43) K, [N]/Q, R, S/T, protein <ul style="list-style-type: none"> mild temperature, pH, aqueous conditions both N-H insertion and C-2 alkylation products observed
13	Metal-catalyzed alkylation (Mn, Ref. 37; Au, Ref. 38) [W (unprotected)] <ul style="list-style-type: none"> R = SiR₃, Ar, Alk, alkenyl demonstrated use in macrocyclization high functional group tolerance 2-pyrim indole PG required (for Mn) 	16	Photoinduced cycloaddition of tetrazoles (Ref. 44) protein <ul style="list-style-type: none"> N-alkylated, bisadduct observed as a minor byproduct no reaction with His formation of fluorescent adduct

POLAR, IONIZABLE SIDE-CHAINS

Ser	Thr	<p>In contrast to many of the other underrepresented residues, both Thr and Ser possess a well-defined functional handle seemingly poised for site-selective modification. In practice, however, the superior nucleophilicity of other residues (e.g., Cys and Lys) has largely precluded chemoselective modifications of the side-chain hydroxyl group. Of the few existing methods, most are proximity-driven, or are highly sequence-dependent—relying on the local peptide environment to enhance the reactivity of the target hydroxyl side-chain. The realization of broadly applicable, side-chain specific functionalizations thus remains a significant challenge.</p>
Cys	<p>The Cys thiol is remarkably versatile, and readily participates in selective alkylation, conjugate addition, thiol-ene chemistry, elimination to form dehydroalanine, glycosylation, and metal-mediated functionalizations. The unique properties of the thiol render it susceptible to both radical and ionic processes, leading to an ever-expanding library of mild and selective modifications. The potent nucleophilicity of the side-chain enables the direct displacement of alkyl halides such as iodoacetamide, one of the first reagents employed for Cys alkylation. Although the thiol handle also makes for an exceptionally willing partner in conjugate additions, the formed adducts are often unstable, particularly in the case of maleimides; this has resulted in an active search for improved conditions and creative solutions to access stable conjugates of similar properties.</p>	

entry	AA compatibility/comments	entry	AA compatibility/comments
17	Strain-release alkylation (Ref. 45 and 46) H, K, N[O], [S], T, W, Y, α-acid, α-amine <ul style="list-style-type: none"> organic/aqueous media mild conditions, short reaction times installation of high-value small, strained ring system 	22	Conj. addition to 2-azidoacrylates (Ref. 52) S, R, protein <ul style="list-style-type: none"> dual functionalization facile reactivity with functionalized alkynes acceptor may possess high-value appendages, including fluorescents
18	Pd-catalyzed arylation (Ref. 47) D[E], H, N[O], R, S/T, Y <ul style="list-style-type: none"> stable, high-value products demonstrated use in stapling no N-terminal protection required Pd-complexes are easy to handle 	23	Thiol-Michael addition to carbonylacrylic reagents (Ref. 53) protein, Ab <ul style="list-style-type: none"> stable adduct formed simple, stoichiometric acceptors
19	Electrophilic trifluoromethylation (Ref. 48) D[E], K, T, W, α-acid, α-amine <ul style="list-style-type: none"> moderate yields low temperatures required 	24	3-arypropionitrile as a maleimide replacement (Ref. 54 and 55) Ab <ul style="list-style-type: none"> stable adduct formed installation of heterobifunctional handle acceptors are commercial
20	Aqueous cross-metathesis (Ref. 49) protein <ul style="list-style-type: none"> efficient allylation followed by aqueous cross-metathesis (CM) reactions 	25	Allenamide reagents for alkylation (Ref. 56) D[E], H, K, M, R, S, W, Y, protein <ul style="list-style-type: none"> short reaction time, high yielding unconjugated product observed stable adduct formed
21	Pd-catalyzed arylation/alkynylation (Ref. 50) N/Q, S, W, Y, α-acid, α-amine, Ab <ul style="list-style-type: none"> cross-coupling of either free Cys or one-pot disulfide reduction/coupling installation of HetAr, Ar, alkynyl, and alkenyl groups For an alternative approach to HetAr sulfides, see Ref. 51 	26	Disulfide desulfurization (Ref. 57) protein <ul style="list-style-type: none"> proceeds through desulfurization of disulfide-linked glycosyl amino acids protected or unprotected sugars retention of anomeric carbon epimerization of α-center
Asp/Glu	<p>Despite the vast array of carboxylic acid transformations available, site-selective modification of the Asp/Glu side-chain is often hampered by the presence of an unprotected C-terminal carboxylic acid. Common approaches include (a) orthogonal protection strategies and (b) reliance on the resin-linker as a masking group which reveals the desired C-terminal functionality only on cleavage. Along with optimization of transformations standard to carboxylic acids—including esterification, amidation, etc.—recent reports have demonstrated the unique ability of the acid to serve as a functional handle for metal-catalyzed decarboxylative cross-couplings. Such methods proceed through preactivation of the acid as a “redox-active ester,” which is capable of accepting an electron from a suitable metal complex.</p>		
entry	AA compatibility/comments	entry	AA compatibility/comments
27	Decarboxylative alkyl-alkyl cross-coupling (Ref. 58) (on-resin) <ul style="list-style-type: none"> dual functionalization of Asp/Glu installation of high-value synthetic handles 	28	Decarboxylative alkenylation (Ref. 59) [N]/[O], Y <ul style="list-style-type: none"> α-chirality maintained, no erosion of ee stereospecific olefin installation

Lys		Among the most popular targets for peptide modification, Lys has served as a convenient nucleophilic handle for the introduction of diverse functionalities. Current challenges include (a) differentiation between the ϵ -amine of Lys and the N-terminal α -amine and (b) prevention of unwanted side reactions with the notably more nucleophilic Cys. The former is typically circumvented through exploitation of the inherent differences in pK_a (ϵ -amine ≈ 10.5 vs. α -amine ≈ 8); the latter can be tuned through prudent selection of electrophiles and/or reaction pathways.	
entry	AA compatibility/comments	entry	AA compatibility/comments
29	<p>Pd-mediated arylation (Ref. 60) D/E, H, M, S, T, W, Y, α-acid</p> <ul style="list-style-type: none">demonstrated use in stapling, macrocyclizationbench-stable preformed Pd-complexes (in most cases)	32	<p>6π-azaelectrocyclization (Ref. 63) protein, mAb</p> <ul style="list-style-type: none">reversible, non-destructive labelingselective for surface-exposed Lys
30	<p>Orthophthaldehyde-amine condensation (Ref. 61) protein, α-amine (Pro)</p> <ul style="list-style-type: none">can be used for protein immobilizationcan be used for PEGylation (substitution at the benzo-moiety)	33	<p>Kinetically-controlled amidation with NHS esters (Ref. 64) protein</p> <ul style="list-style-type: none">peptide must be used in 2-fold excessinstallation of bifunctional handle
31	<p>Iminoboronate formation (Ref. 62) protein</p> <ul style="list-style-type: none">potentially reversiblereadily decomposes in the presence of glutathioneproceeds in aqueous buffer	34	<p>Amine capture with diazonium terephthalates (Ref. 65) protein</p> <ul style="list-style-type: none">stable adduct formedα-amine may reactmost accessible Lys reacts preferentially
Arg		Reactions that target Arg generally exploit the ability of the guanidine side-chain to serve as a bidentate nucleophile in condensation reactions with various dicarbonyl reagents. Aside from this well-explored mode of reactivity, Arg-specific methods are limited in scope, due in large part to the high pK_a of the guanidine unit and its low nucleophilicity at physiological pH. Modifications employing most electrophilic reagents thus result in preferential reactivity at other more nucleophilic sites. However, through careful choice of exogenous base, acylation reactions may be tuned to improve selectivity for the Arg side-chain. It is anticipated that further optimization will serve to expand the scope of Arg-specific modifications.	
entry	AA compatibility/comments	entry	AA compatibility/comments
35	<p>Acylation with activated esters (Ref. 66) E, H, S/T, W, Dha, α-amine</p> <ul style="list-style-type: none">installation of carboxyfluorescein, biotin, and diazirin labelschemically stable, HPLC isolationintolerant of Cys, free amines	36	<p>Condensation with glyoxal reagents (R = Me, Ref. 67; R = Ar, Ref. 68) protein</p> <ul style="list-style-type: none">R = Me, irreversible reaction leads to Arg selectivity (Lys, Cys react reversibly)R = Ar, reversible reactionenables traceless PEGylation
37	<p>Arg-dehydroascorbate adduct (Ref. 69) protein</p> <ul style="list-style-type: none">formed from DHA byproduct following copper-catalyzed azide-alkyne cycloaddition		
NON-CANONICAL AMINO ACIDS			
Dha		Sec	
Dha is one of the few inherently electrophilic residues, a characteristic that has been repeatedly exploited in the development of site-selective reactions. Commonly accessed through elimination of the Cys thiol, this unsaturated residue serves as a convenient precursor to a wide variety of unnatural amino acids through conjugate addition. Stereocontrol at the α -center remains, arguably, the most intriguing unmet challenge.		As compared to its sulfur congener, Sec has both a significantly lower pK_a (5.5) and reduction potential; despite these unique physicochemical properties, however, most methods for chemical modification tend to simply echo that of structural analogue Cys. While frequently employed in standard ligation and alkylation strategies, the so-called 21 st proteinogenic amino acid represents an underexplored area for unique bioconjugation approaches.	
entry	AA compatibility/comments	entry	AA compatibility/comments
38	<p>Cu-catalyzed hydroboration/silylation (Ref. 70) protein</p> <ul style="list-style-type: none">requires α-acid, α-amine protecting groupslimited to di- and tripeptides	41	<p>Cu-catalyzed, umpolung-based arylation (Ref. 76) D/E, H, K, N/Q, S/T, R, Y, α-amine</p> <ul style="list-style-type: none">no reducing agent, no O_2 exclusion requiredHigh functional group tolerance for aryl componentHetAr, -OH, -NO₂, Cl/F, CN, etc.
39	<p>Conj. Addition of thiols (Ref. 71–73) protein</p> <ul style="list-style-type: none">late-stage installation of PTM-lysine, serine mimicsmild reaction conditions	42	<p>Aqueous cross-metathesis (Ref. 77) protein</p> <ul style="list-style-type: none">rate of Sec-allyl > Cys-allyl in CM reactionrobust access to Sec-allyl via Dhainstallation of high-value synthetic handles
40	<p>Radical-based conj. addition (Ref. 74 and 75) protein</p> <ul style="list-style-type: none">compatible with 1°, 2°, 3° alkyl halidesinstallation of hydrophobic and charged/polar protic side-chains, including PTMs	43	<p>Alleneamide and heteroaryl functionalization (Ref. 78) AB</p> <ul style="list-style-type: none">alleneamides, heteroaryl sulfones, and iodoacetamides demonstratedalleneamides and heteroaryl sulfone adducts are stable to physiological conditions

Table 2

α -ACID FUNCTIONALIZATION			
entry	AA compatibility/comments	entry	AA compatibility/comments
44	<p>Decarboxylative 1,4-addition (Ref. 79)</p> <ul style="list-style-type: none"> demonstrated on α-carboxylic acid amenable to Fmoc-SPPS demonstrated use in macrocyclization loss of C-terminal α-chirality 	46	<p>Decarboxylative borylation (Ref. 82)</p> <ul style="list-style-type: none"> demonstrated on di-, tripeptides installation of high-value synthetic handles one example of side-chain acid (α-protected) observed <i>d.r.</i> dictated by temperature and substrate
45	<p>PET decarboxylative coupling (Ref. 80 and 81)</p> <ul style="list-style-type: none"> photoredox process limited to di- and tripeptides loss of C-terminal α-chirality 		
α -AMINE FUNCTIONALIZATION			
entry	AA compatibility/comments	entry	AA compatibility/comments
47	<p>2-cyanobenzothiazole condensation (Ref. 83 and 84)</p> <p>protein</p> <ul style="list-style-type: none"> limited to N-terminal Cys selective for 1,2- or 1,3-aminothiols demonstrated protein labeling on live cells 	52	<p>Ketene reactions (Ref. 90)</p> <p>protein</p> <ul style="list-style-type: none"> "moderate-to-high N-terminal selectivity" for most proteinogenic AAs installation of high-value appendages
48	<p>Thiazolidine boronate formation (Ref. 85)</p> <p>protein</p> <ul style="list-style-type: none"> limited to N-terminal Cys dative bond increases adduct stability selective for 1,2-aminothiols 	53	<p>Ascorbate-mediated oxidation-condensation of vinylboronic acids (Ref. 91)</p> <p>protein</p> <ul style="list-style-type: none"> aryl, alkyl-substituted vinylboronic acids tolerates D, V, M, W, C, H, S, Y, R at N-terminal position
49	<p>Imidazolidinone formation with 2-pyridinecarboxaldehydes (Ref. 86)</p> <p>protein</p> <ul style="list-style-type: none"> moderate yields for N-terminal Pro, Gly small amount of bis-functionalization observed in N-terminal Gly examples 	54	<p>Acylation with organometallic carboxylic acids (Ref. 92)</p> <p>(on-resin)</p> <ul style="list-style-type: none"> ferrocene and Reⁱ also investigated stoichiometric organometallic acid - near quantitative consumption; unused was recyclable
50	<p>N-terminal oxidation-oxime formation (Ref. 87 and 88)</p> <p>Ab</p> <ul style="list-style-type: none"> inexpensive transamination reagent (Rapoport's salt) Glu in the terminal or second position reacts most efficiently ineffective with Pro terminal 	55	<p>Oxidative coupling of o-aminophenols (Ref. 93)</p> <p>protein</p> <ul style="list-style-type: none"> Pro demonstrates highest compatibility Cys is modified under reaction conditions - reversible modification
51	<p>NaIO₄ oxidation-nitrone cycloaddition (Ref. 89)</p> <p>protein</p> <ul style="list-style-type: none"> limited to N-terminal Ser proceeds through nitrone intermediate stable adduct formed installation of high-value labels 	56	<p>Pictet-Spengler reactions (Ref. 94 and 95)</p> <p>D/E, H, [N]/Q, R, S/[T], W, Y</p> <ul style="list-style-type: none"> can be used for ligation or macrocyclization - in macrocycles, tetrahydro-β-carboline can be oxidized to introduce conformational rigidity - acidic reaction conditions

Table 3

^a

AMINO ACID SIDE-CHAIN MODIFICATION REPORT CARD																					
Residue	Method	Ala	Cys	Asp/Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn/Gln	Pro	Arg	Ser	Thr	Val	Trp	Tyr	Sec	Dha
(A)	Alkylation																				
(B)	Arylation																				
(C)	Acylation																				
(D)	Halogenation																				
(E)	Oxidation																				
(F)	1,4-Addition																				
(G)	Condensation																				
(H)	Cross-Coupling																				
(I)	Pericyclic Reaction																				
(J)	Photo Reaction																				
(K)	Radical Reaction																				
(L)	Transition-Metal Functionalization																				

Fully compatible

- protein or antibody substrates
- aqueous, mild conditions
- fully chemoselective
- multiple modes of reactivity
- reproducible (many examples)

Fully compatible

Compatible

Progress made

Preliminary

Unanswered challenge

Unanswered challenge

- no peptide examples
- methods unavailable or single amino acids only
- poised for innovation

^aSee the Supporting Information for details.