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Decarboxylative alkylation for site-selective bioconjugation of native proteins via oxidation potentials

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The advent of antibody–drug conjugates as pharmaceuticals has fuelled a need for reliable methods of site-selective protein modification that furnish homogeneous adducts. Although bioorthogonal methods that use engineered amino acids often provide an elegant solution to the question of selective functionalization, achieving homogeneity using native amino acids remains a challenge. Here, we explore visible-light-mediated single-electron transfer as a mechanism towards enabling site- and chemoselective bioconjugation. Specifically, we demonstrate the use of photoredox catalysis as a platform to selectivity wherein the discrepancy in oxidation potentials between internal versus C-terminal carboxylates can be exploited towards obtaining C-terminal functionalization exclusively. This oxidation potential-gated technology is amenable to endogenous peptides and has been successfully demonstrated on the protein insulin. As a fundamentally new approach to bioconjugation this methodology provides a blueprint toward the development of photoredox catalysis as a generic platform to target other redox-active side chains for native conjugation.

The last two decades have witnessed tremendous growth in research to address the challenge of site- and chemoselective protein modification[1]. Propelled by a high demand for technologies that furnish homogeneously modified protein adducts, chemical biologists have successfully delivered a number of robust methods that achieve site-selective protein functionalization via protein engineering and the incorporation of non-natural, bioorthogonal amino acids[2]. Chief among these methods are 'click' and Staudinger ligation strategies, where highly uniform products can be obtained by genetically encoding an azide reporter in a site- and number-specific fashion[3,4].

Pre-engineering of the protein scaffold (mutant proteins) has proven to be an indispensable technology for selective protein modification, but the bioconjugation strategies that harness the amino acids of wild-type proteins remain elusive, despite their great appeal. Traditionally, these native-modification methods mainly make use of 2 of the 20 canonical amino-acid residues—cysteine[5,6] and lysine—which incorporate heteroatom lone-pair nucleophiles. However, obtaining homogeneous products in which only a single residue at a single site has undergone reaction has proven challenging[7]. For example, selective modification of a specific lysine residue is difficult given their high abundance on protein surfaces[8]. One solution has been to target proteins where one lysine residue is more solvent-accessible to biotinylation than other Lys groups, as demonstrated by Chen and co-authors[9]. In a similar vein, this approach has been applied by Bader and colleagues for the selective lipidation of C-terminal cysteines[10]. More recently, elegant work from several groups has addressed this problem by extending the scope of natural amino acids employed in bioconjugation to N termini[11], tyrosine, tryptophan and methionine residues[12–19]. These methods take advantage of the inherently low abundance of these residues on protein surfaces, thereby achieving a higher degree of site selectivity.

Over the past several years, our laboratory has employed photoredox catalysis as a platform for activating native functional groups towards C–C and C–X bond formation. One such activation mode has focused on the use of naturally abundant carboxylic acids as latent carbon-centred radicals[20]. These transient intermediates, generated through single-electron transfer (SET) and subsequent CO_2 extrusion, have been shown to undergo successful coupling with a wide range of electrophilic partners including Michael acceptors[21], vinyl sulfones[22] and nickel complexes[23]. Recently, we questioned whether this technology could be applied to more complex architectures that incorporate multiple carboxylic acids, such as endogenous proteins (Fig. 1). Indeed, carboxylic acids are naturally present in proteins due to their incorporation in aspartate, glutamate and C-terminal residues. Despite the abundance of these carboxylic-acid-bearing residues, we reasoned that the innate difference in oxidation potentials between side-chain alkyl carboxylates (that is, aspartate and glutamate)[24] and C-terminal α-amino carboxylates[20] should permit a high degree of site selectivity, with decarboxylation–functionalization occurring at the more readily oxidized C terminus. It is also important to note that traditional methods for carboxylic acid bioconjugation typically fall within the realm of amide bond couplings and esterification with diazo compounds, two technologies that often suffer from indiscriminate regioselectivity[25–29]. In contrast, we reasoned that photoredox C-terminal decarboxylative functionalization might present a general strategy to target proteins in a site-selective manner, regardless of their intrinsic topological features. Moreover, we hypothesized that the presence of only one C-terminus position on most protein structures should effectively enable single site modification using only canonical amino-acid residues.

Based on previous studies conducted in our laboratory, we recognized that the stability of the resulting radical intermediate following decarboxylation is inherently linked to the carboxylate's ground-state oxidation potential[30]. Thus, we would expect internal

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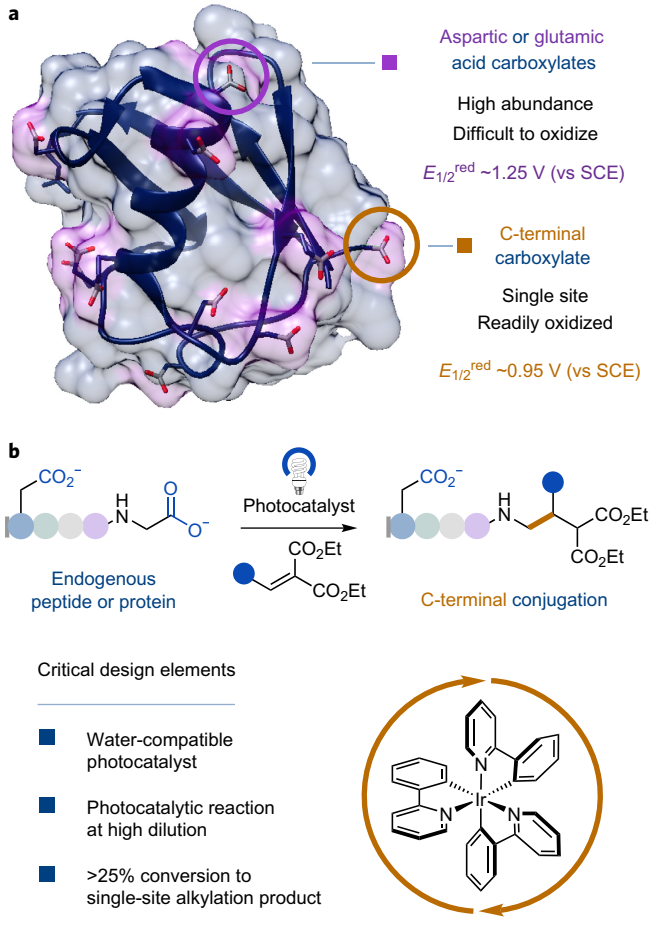


Figure 1 | Photoredox-catalysed decarboxylative functionalization as a novel electron transfer mechanism towards site- and chemoselective bioconjugation. a, Current methods targeting natural amino-acid motifs rely on the intrinsic availability of targeted residues on the protein surface to achieve homogeneously modified products. We postulated that photoredox-catalysed decarboxylation (a transformation that traditionally uses iridium-based photocatalysts) could capitalize on the inherent differences in oxidation potentials between the more abundant aspartic and glutamic acid residues versus the lone C-terminal carboxylate to obtain an exclusive C-terminal-modified product in which the net transformation involves a decarboxylative-alkylation at the more readily oxidized C terminus. **b**, For general applications of this technology, the ability to perform the reaction under biocompatible conditions (in water at high dilution) and obtain yields greater than 25% for a single modified product were defined as critical design elements (oxidation potentials are reported in acetonitrile as a solvent)[20,24]. $E_{1/2}^{red}$, half-wave reduction potential; SCE, standard calomel electrode; V, volt.

Asp and Glu residues to have higher barriers to oxidation relative to the C-terminal carboxylic acid due to formation of an unstabilized carbon-centred primary radical versus a heteroatom-stabilized α-amino radical at the protein terminus. In addition to these selectivity considerations, we recognized the need to develop reaction conditions compatible with biological substrates, namely using aqueous buffer, high dilution and mild temperatures. By virtue of operating through one-electron redox processes, photoredox catalysis presents a potentially valuable platform for selective reaction design under aqueous conditions. More specifically, one-electron transfer mechanisms are less susceptible to many of the challenges faced when translating cationic or anionic mechanisms to an aqueous environment (due to competitive trapping or attenuated nucleophilicities). As such, we recognized that a key element for the successful execution of these ideals would be the design of a water-compatible photocatalyst. Furthermore, we elected to use α,β-unsaturated carbonyls as the electrophile component, given their known ability to readily engage with carbon-centred radicals under photoredox conditions in the presence of water[31]. Although the cysteine thiol group is known to react with highly electrophilic Michael acceptors, we rationalized that the judicious selection of a less reactive α,β-unsaturated carbonyl would allow for selective entrapment of the relatively high-energy C-terminal radical. Finally, to be competitive with existing methodologies that achieve single-site modification with naturally occurring residues (for example, Tyr, Trp and N termini)[13,14,16,17,32], we recognized that synthetic efficiencies in the range of 25% conversion or greater would render this system a useful bioconjugation technology.

Results and discussion

As a model system, we selected diethyl ethylidenemalonate **5** as the Michael acceptor and the N-terminal acetylated tetramer Ac-AGFP-OH as a representative short peptide sequence[21]. Initial evaluations using $Ir[dF(CF_3)ppy]_2(dtbbpy)^+$ ($dF(CF_3)ppy$ = 2-(2,4-difluorophenyl)-5-(trifluoromethyl)pyridine, dtbbpy = 4,4′-di-*tert*-butyl-2,2′-bipyridine) as the photocatalyst with 10 mM pH 7 buffer and 5% vol/vol glycerol (1 mM peptide) proved to be less than fruitful. Moreover, all attempts to use common photocatalysts that are water-soluble (ruthenium-based catalysts, organic dyes) likewise proved ineffective (<20% yield; see Supplementary Table 1, page 5). We next began to consider strategies that employ biocatalytic cofactors that are known to readily operate in water. More specifically, given that flavins have been shown to mediate acetate decarboxylation (albeit by a two-electron pathway), we hypothesized that these cofactors might function as suitable photocatalysts for SET in aqueous bioconjugation processes[33]. Gratifyingly, a survey of catalytic flavins (Supplementary Table 2, page 6) revealed that 30 mol% of riboflavin tetrabutyrate (photocatalyst **1a**, Fig. 2) was capable of producing the decarboxylative conjugate addition adduct in 79% yield (Table 1, entry 3). It should be noted that sparging of the reaction mixtures with nitrogen before irradiation was essential for the success of the reaction (reactions that were not sparged generally gave diminished yields).

Mechanistically, we propose that the flavin photocatalyst **1** is initially promoted to its singlet excited state by excitation with a 34 W blue light and undergoes subsequent intersystem crossing (quantum yield of $\Phi_{ISC} = 0.38$ for riboflavin in water at pH 7)[34]. The resulting triplet-excited photocatalyst **2** is a strong single-electron oxidant ($E_{1/2}^{red} = 1.5$ V versus saturated calomel electrode (SCE) in water)[35] and should undergo facile SET with C-terminal carboxylate **3** ($E_{1/2}^{red} = 1.3$ V versus SCE for Ac-AGFP-OH in water) (see Supplementary Section D for experimental details). Subsequent loss of CO_2 from **3** furnishes α-amino radical **4**, a species that is stabilized by the adjacent nitrogen. This transient intermediate then undergoes open-shell addition into Michael acceptor **5** to provide an α-acyl radical **6**, which, upon reduction by the photocatalyst, would generate the corresponding enolate[36]. Subsequent protonation would provide the bioconjugation product **7,** while the α-acyl radical reduction step would regenerate the ground state of photocatalyst **1** to complete the catalytic cycle.

With these SET bioconjugative decarboxylative conditions in hand, we next evaluated the functional group tolerance of this technology with various peptides that incorporate the amino-acid residues most commonly found on the surface of proteins (that is, we selected residues that are found with ≥2% surface abundance, based on crystallographic data)[8]. Using solid-phase peptide synthesis, we systematically altered the N-terminal residue of the parent Ac-XGFP-OH template to afford 14 peptides that incorporate these most abundant amino acids. To our delight, a significant

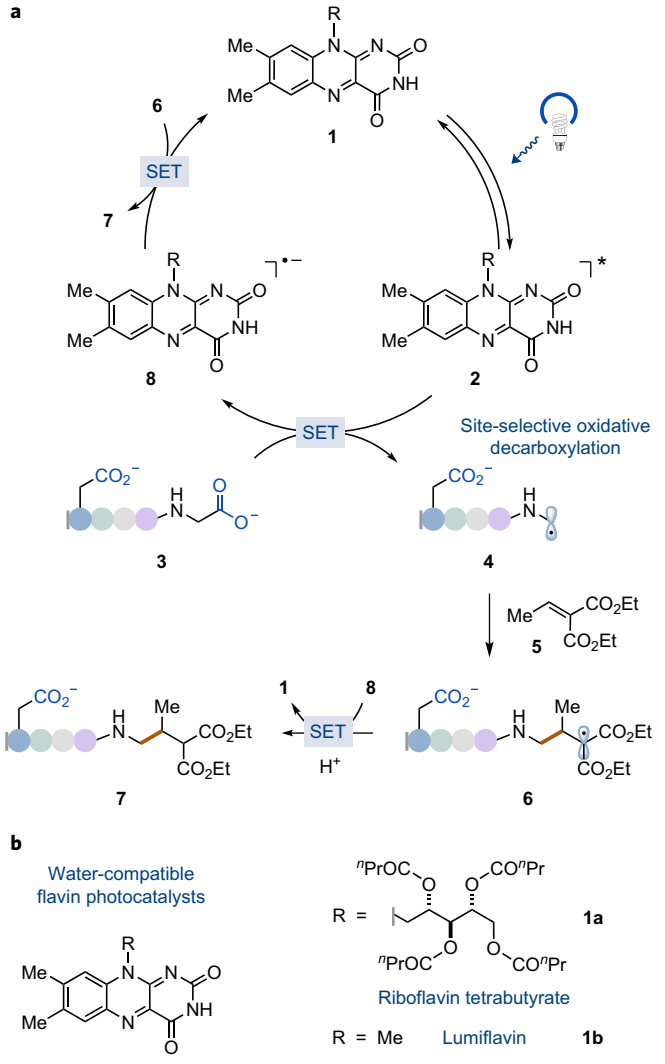
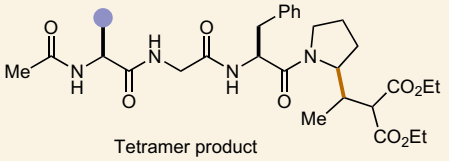


Figure 2 | Proposed mechanism for the C-terminal-selective photoredox decarboxylative conjugate addition. a, The mechanism is proposed to proceed via one-electron oxidation of **3** by the excited photocatalyst **2** to furnish α-amino radical **4** after decarboxylation. Addition into Michael acceptor **5** provides adduct **6**, which is reduced by reduced photocatalyst **8** and protonated to give C-terminal-modified product **7**. **b**, After optimization studies, the most effective photocatalysts are riboflavin tetrabutyrate (**1a**) and lumiflavin (**1b**). ^{n}Pr, *n*-propyl; SET, single-electron transfer.

number of these peptide sequences were able to participate in the desired decarboxylative conjugation at pH 7 to furnish the desired adducts in yields between 71 and 79% (Table 1, entries 1, 3–7 and 9–13, third column).

However, certain residues performed less efficiently at pH 7, namely Lys (entry 2), Tyr (entry 8) and His (entry 14). In these cases, we rationalized the decreased yields could be attributed to deleterious oxidation of these residues over the C-terminal carboxylate. With respect to Lys and His, we hypothesized that decreasing the pH of the buffer should ameliorate oxidative side reactivity by increasing the equilibrium concentrations of ammonium and imidazolium, respectively, as these species should be resistant to oxidation[37]. Indeed, lowering the pH to 3.5 using a caesium formate buffer resulted in 65% yield for the Lys derivative (Table 1, entry 2, fourth column) and 70% yield for the His derivative (entry 14). Gratifyingly, use of pH 3.5 buffer universally improved the yields for all tetrapeptide sequences that we evaluated, delivering conjugation adducts in yields ranging from 87 to 95% (entries 1, 3–7 and 9–13). With respect to the Tyr-containing system, although it is known that Tyr oxidation is less facile at lower pH[38,39], initial trials using riboflavin tetrabutyrate (**1a**) gave poor yields. To address this issue, we next examined lumiflavin (Fig. 2a, photocatalyst **1b**), a less oxidizing flavin photocatalyst[40]. From a structural perspective, the absence of the electron-withdrawing ribityl side chain renders the isoalloxazine carbonyls as stronger hydrogen-bond acceptors with water, resulting in greater stabilization of the charge separated excited state relative to the ground state[41]. This phenomenon serves to render lumiflavin less oxidizing as an excited-state photocatalyst. Indeed, application of lumiflavin with our Tyr-bearing peptide did in fact raise the efficiency of this process to 23% (entry 8). It should be noted that amino acids that are not abundant in proteins, such as Trp, Cys and Met, were also surveyed in this study (not shown in Table 1). In all cases, selective alkylation was also achieved, albeit with slightly longer reaction times or using modified conditions (see Supplementary Information page 25 for more details). Given the low surface abundance of these residues in proteins, we do not expect that such modifications will be generally required (or relevant) for the implementation of this bioconjugation technology in a general sense. Perhaps most importantly, tetramers incorporating Asp and Glu (both of which exhibit primary carboxylic acids) undergo decarboxylation exclusively at the C-terminal position. (C-terminal functionalization of all products was established by MS/MS analysis, Supplementary Section C.) Indeed, these substrates exhibit perfect selectivity for C-terminal conjugation and high yields at pH 7 (77 and 75%, entries 4 and 7, respectively) and pH 3.5 (93 and 91%, entries 4 and 7, respectively). Moreover, regardless of pH, we did not observe any coupling products arising from heteroconjugate additions of nucleophilic residues such as Lys, Ser, Thr and His. Given that these types of heteroatom functionalization protocol

Table 1 | Survey of functional group tolerance for the C-terminal selective photoredox decarboxylative conjugate addition.



Tetramer product

Entry	Side chain	Yield pH 7.0, 8 h (%)	Yield pH 3.5, 6 h (%)	Surface abundance (%)
1	Ser	75	87	8.9
2	Lys	52	65	8.9
3	Ala	79	92	7.9
4	Asp	77	93	7.4
5	Thr	73	90	7.1
6	Asn	76	91	6.3
7	Glu	75	91	6.2
8	Tyr	8[a]	23[a]	4.8
9	Val	76	95	4.6
10	Gln	71	94	4.5
11	Leu	74	95	4.3
12	Arg	71	87	4.0
13	Ile	77	90	3.0
14	His	11	70	2.2

The scope of decarboxylative functionalization proves quite general with respect to the various functional groups present in the canonical amino acids at room temperature, under dilute conditions (1 mM peptide) and physiological pH (phosphate buffer, pH 7, 10 mM). Entries are sorted by decreasing surface abundance. Importantly, no reaction was detected at the Asp or Glu site in tetramers containing these competing acid functionalities. Enhanced reactivity is observed using a caesium formate buffer (pH 3, 10 mM). Conditions: tetrapeptide (1 mM), photocatalyst **1a** (30 mol%), diethyl ethylidenemalonate (**5**), 95:5 buffer:glycerol, 34 W light-emitting diode. [a]30 mol% photocatalyst **1b** instead of **1a**. Yields are reported as % conversion, as determined from reverse-phase HPLC, and are an average of three independent trials.

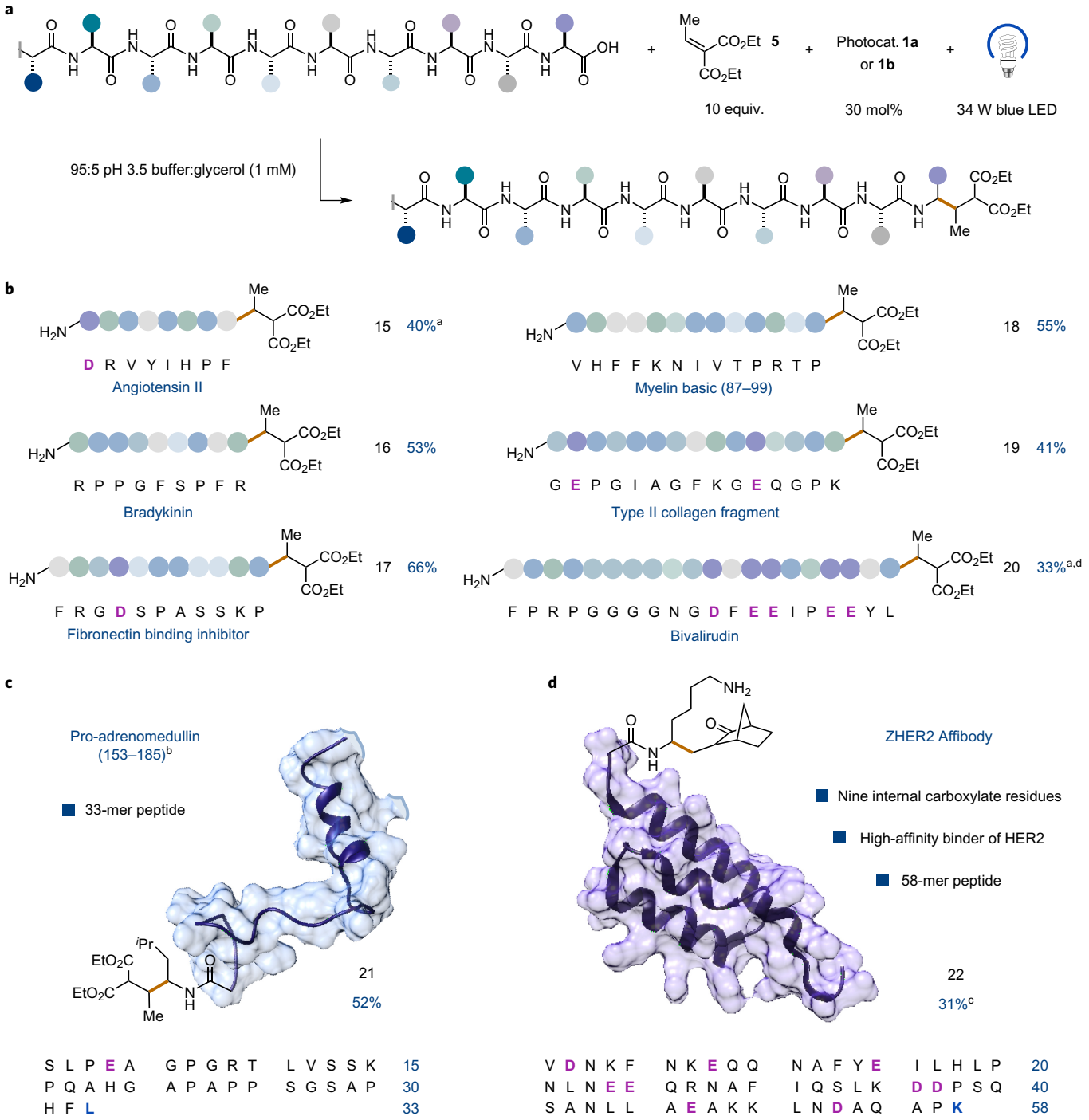


Figure 3 | Scope of the photoredox decarboxylative conjugate addition applied to endogenous peptides. A variety of fully unprotected peptides from 8-mers to 58-mers can be site-selectively modified at room temperature, in caesium formate buffer (pH 3.5, 10 mM) in this transformation. **a**, Generalized reaction scheme. **b**–**d**, Substrate scope. All peptides are commercially available. In the case of the fibronectin-binding inhibitor peptide (entry 17), the N-terminal Phe residue was added only for ease of analysis when initially developing the quantitative HPLC assay. Notably, longer peptides with nascent secondary structure (**c**) and peptides bearing high ratios of internal Asp and Glu residues (**d**) furnish C-terminal-modified products exclusively. For entries 15–19 and 21–22, yields are reported as a % conversion as determined from reverse-phase HPLC and are an average of three independent trials. [a]Using photocatalyst **1b**. [b]Representative structure derived from the crystal structure of the full-length protein preproadrenomedullin. [c]3 equiv. photocatalyst **1b** and 3-methylene-2-norbornanone as the Michael acceptor. [d]Yield reported as a combined % conversion after re-subjection of recovered starting material (Supplementary Information page 37).

represent a common strategy in bioconjugation chemistry, we feel that this outcome reveals both the kinetic and thermodynamic benefits of employing electron-transfer and open-shell mechanisms in lieu of closed-shell, nucleophile trapping protocols[42].

Having assessed the functional group tolerance of our reaction on a large, representative group of tetrapeptides, we next decided to examine the applicability of this photocatalytic methodology for endogenous peptides, including several with biological activity.

At the outset, we selected commercial peptides ranging in size from 8 to 10 amino acids. Among those examined were a cardiovascular regulator, angiotensin II, and an inflammation inhibitor, bradykinin. These peptides underwent C-terminal decarboxylative alkylation with the desired monofunctionalization selectivity in yields of 40 and 53%, respectively (Fig. 3b, entries 15 and 16). Moving forward, we next examined mid-range, endogenous peptides consisting of 11–15 amino-acid residues. As highlighted in Fig. 3b, myelin basic protein (87–99), type II collagen and fibronectin-binding inhibitor peptide (modified only by appending an N-terminal Phe for ease of spectroscopic analysis while developing a quantitative HPLC assay) were all found to give good yields under our optimized reaction conditions (41–66%, Fig. 3b, entries 17–19). It should be noted that in some cases, reactions of macropeptides afforded other unidentifiable side products, albeit in unappreciable quantities. As a testament to the levels of site selectivity of our new bioconjugation protocol, we also demonstrated a successful alkylation of bivalirudin (Angiomax), an antithrombotic icosapeptide. Despite an initially lower yield of 28%, we observed exclusive C-terminal functionalization in the presence of five additional carboxylic acids. HPLC analysis of the crude reaction mixture revealed the remaining mass balance to be unreacted starting material. As such, a second round of photocatalytic activation following re-isolation of the unmodified peptide increased the overall yield to 33% (Fig. 3b, entry 20). We hypothesize that the reaction halts at 28% conversion (with no further loss of starting material) due to photocatalyst deactivation as evidenced by UV–vis time-course studies (Supplementary Section E).

Having demonstrated the selectivity of our reaction for the terminal alkylation of linear peptides (4–20 amino acids), we next decided to examine sequences with known secondary structure. For this purpose, we selected commercially available pro-adrenomedullin (fragment 153–185) and the ZHER2 affibody derived from immunoglobulin-binding protein A. To our delight, pro-adrenomedullin selectively delivered the C-terminal modified product in 52% yield (Fig. 3c, entry 21) using our standard conditions. To our surprise, with the ZHER2 affibody, use of the diethyl ethylidenemalonate Michael acceptor resulted in the formation of the desired adduct along with side-chain conjugate addition adducts via a competing Lys-addition pathway. At this stage, we rationalized that use of α,β-unsaturated carbonyls that are less susceptible to $2e^-$ nucleophile pathways than diethyl ethylidenemalonate, yet are still able to participate in radical additions, might overcome this issue. Indeed, the implementation of the strained, monocarbonyl-containing compound, 3-methylene-2-norbornanone, an established radicalphile under photoredox conditions[43], eliminated any undesired side reactivity, furnishing the modified product in 31% yield with intact α-helical structure (Fig. 3d, entry 22; see Supplementary Section F for circular dichroism studies). In this case, recovered starting material accounted for the remaining mass balance.

A true test of any bioconjugation technology lies in its capacity to perform site-selective modification of peptides that exhibit tertiary structure. However, the structural linkages that confer these higher-order architectures (for example, disulfide bridges) can themselves be susceptible to chemical modification[44], thereby often changing the structure–function relationship of any given higher-order proteins. To test the viability of this new photoredox protocol in this context, we selected insulin as a suitable molecular platform to examine the chemical selectivity of electron transfer and open-shell mechanisms with a molecule that contains a variety of functional groups (Fig. 4). Structurally, insulin is composed of two parent chains linked by two disulfide bridges, with a third disulfide bond in the A-chain backbone. Insulin also contains four tyrosines, residues that proved particularly challenging during our assessment of tetrameric peptides. Furthermore, and unlike the peptide substrates described thus far, insulin bears two C-terminal carboxylic acids in addition to having two Glu residues on each of the A and B chains. In the event, subjecting native insulin to our optimized reaction conditions (with 3-methylene-2-norbornanone as the electrophile) resulted in formation of a monoalkylated adduct in 44% isolated yield wherein modification occurred exclusively at the A-chain C terminus. A minor adduct (less than 5%) was detected in which both the A- and B-chain C termini were functionalized (see Supplementary Information pages 43 and 143 for details). Importantly, product analysis revealed that all three disulfide linkages remained intact and no side-chain decarboxylative or heteroatom conjugate addition was detected. The selectivity for A-chain monoalkylation is particularly noteworthy as current technologies generally offer selective functionalization of insulin's B chain through covalent modification at His10 (ref. 45), Tyr26 (ref. 46) and Lys29 (ref. 47). This photoredox methodology therefore offers a new technology that not only selectively targets a specific C-terminal carboxylic acid, but also modifies the light chain, a structural component that heretofore has not been susceptible to bioconjugation. We speculate that the observed selectivity for A-chain modification could arise from either (1) inherent differences in oxidation potentials between the C termini wherein A-chain oxidation is favoured or (2) adsorption of the photocatalyst to a lipophilic region on the surface of the protein proximate to the A-chain C terminus. Moreover, expanding the scope of Michael acceptors to norbornanones that bear bioorthogonal tags (that is,

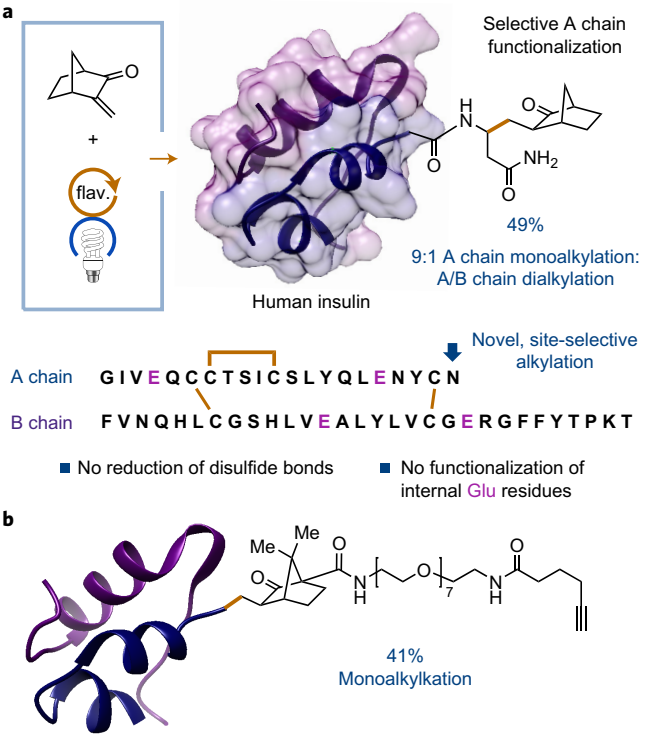


Figure 4 | Photoredox-mediated decarboxylative functionalization of human insulin. a, Human insulin was functionalized at room temperature using our decarboxylative photoredox methodology to furnish highly selective mono-alkylation at the C terminus of the A chain, exclusively. **b**, Functionalization of human insulin with a Michael acceptor incorporating a bio-ambient alkyne. Reaction conditions: 1 equiv. insulin (500 nmol), 10 equiv. of the respective Michael acceptor, 3 equiv. photocatalyst **1b**, 95:5 pH 3.5 caesium formate buffer:glycerol (1 mM), 34 W blue light-emitting diode, 8 h. Yield is reported as % conversion as determined from reverse-phase HPLC. Current work exploring azide- and biotin-bearing Michael acceptors is ongoing.

an alkyne) has also demonstrated promising results (Supplementary Information pages 44 and 144). We fully expect this Michael acceptor will be able to incorporate other bioorthogonal handles (biotin and azides), and work is ongoing in this area.

In conclusion, we present a photoredox bioconjugation strategy that selectively targets C-terminal carboxylic acids in lieu of other functional groups found in protein structures. To our knowledge, this transformation represents an unprecedented approach to site-selective bioconjugation that can provide facile access to homogeneous alkylation adducts by virtue of the inherent presence of only one C-terminal carboxylate in most peptide and protein structures. Work is ongoing to apply this decarboxylative bioconjugation strategy to the selective functionalization of a wide range of proteins, enzymes and antibodies with biologically active conjugates. Moreover, the development of photoredox catalysis as a generic platform for site-selective bioconjugation will be investigated in the context of targeting other redox-active side chains.

Data availability. All relevant data are provided in the Supplementary Information or are available from the authors upon request.

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Author contributions

S.B., C.L. and D.K.K. performed and analysed the experiments. S.B., C.L., D.K.K. and D.W.C.M. designed the experiments and prepared this manuscript. J.X.Q., Y.Z., M.A.P. and W.R.E. provided discussions. J.X.Q. assisted with peptide synthesis.

Additional information

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Competing financial interests

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