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Selective Modification of Tryptophan Residues in Peptides and Proteins Using a Biomimetic Electron Transfer Process

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Abstract

We report here a photochemical process for the selective modification of tryptophan (Trp) residues in peptides and small proteins using electron-responsive *N*-carbamoylpyridinium salts and UV–B light. Preliminary mechanistic experiments suggest that the photoconjugation process proceeds through photoinduced electron transfer (PET) between Trp and the pyridinium salt, followed by fragmentation of the pyridinium N–N bond and concomitant transfer of this group to Trp. The reaction displays excellent site selectivity for Trp and is tolerant to other, redox-active amino-acid residues. Moreover, the reaction proceeds in pure aqueous conditions without the requirement of organic cosolvents or photocatalysts, is enhanced by glutathione, and operates efficiently over a wide range of peptide concentrations (10–700 μM). The scope of the process was explored through the labeling of 6-Trp-containing peptides and proteins ranging from 1 to 14 kDa. We demonstrate the versatility of the *N*-carbamoylpyridinium salt both by tuning the electrochemical and photochemical properties of the pyridinium scaffold to enable challenging photoconjugation reactions and by using the carbamoyl moiety to tether a plethora of productive functional groups, including reactive handles, purification tags, and removable protecting groups.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c03039>.

Experimental details and procedures; mechanistic studies; NMR spectra ([PDF](#))

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The selective chemical manipulation of proteins has enabled the precision engineering of new properties and functions into biomolecular structure, in turn facilitating advancements in therapeutic development and chemical biology.¹ Given the vast diversity of biomolecular structure as well as the demand for increasingly complex applications of bioconjugation across multiple disciplines, the development of a diverse array of chemical bioconjugation methods with complementary site selectivities and capabilities remains a necessity.² In that regard, photochemically driven bioconjugation reactions, which are underexplored compared to thermal processes, can enable both spatiotemporal control of reactivity without the need for prefunctionalization with bio-orthogonal groups³ and access to site selectivity patterns that complement thermal processes.^{4a} Efforts to achieve photochemical labeling of biomolecular structure have primarily focused upon the use of photocatalytic approaches toward protein cross-linking^{4b,c} as well as the modification of cysteine,^{4d-f} tyrosine,^{4g,h} and C-termini.^{4i,j}

We propose here that a catalyst-free approach to photobioconjugation, wherein the inherent photolability of native protein structure is leveraged to induce protein labeling, could offer a complementary approach to catalytic techniques and enable access to mild labeling conditions (pure aqueous buffer, low [protein], short reaction times) as well as circumvent the challenge of achieving site selectivity between amino acids possessing similar oxidation potentials. To that end, we noted tryptophan's (Trp) propensity to participate in photoinduced electron transfer (PET).⁵ Trp possesses the largest molar absorptivity of the natural amino acids ($\epsilon \approx 4500, 500 \text{ M}^{-1} \text{ cm}^{-1}$, 290, 300 nm respectively) as well as the highest propensity to undergo photoionization ($\Phi_e \approx 0.008\text{--}0.08$).^{5a,b} As a result, Trp displays a rich varied profile of PET chemistry, participating in a wide variety of intraprotein PET events as both a reductive quencher and a photoreductant (Figure 1A).

Trp is a desirable bioconjugation target, as it is a rare (~1% natural abundance) amino acid. Thus, any method selective for Trp would allow for access to bespoke protein constructs with high precision, structural homogeneity, and reproducibility. Current strategies for Trp modification generally rely upon the generation of Trp-selective electrophiles such as metallocarbenoids,^{6a-c} nitroxides,^{6d} sulfonyl chlorides,^{6e} trifluoromethyl radicals,^{6f} transition metal complexes,^{6g} and oxidative strategies.^{6h} While elegant, indoles are only moderately nucleophilic⁷ and must compete with other biological nucleophiles. Exploiting Trp's inherent photolability could offer a complementary means of engaging Trp functionalization under more biologically compatible conditions. Recently, Shi reported a

method for photochemical modification of peptides that modified Trp, His, and C-termini through a unique, photoredox-mediated alkenylation process.⁸

Here, we propose to use a Katritzky-type⁹ *N*-substituted pyridinium salt (**1**) to couple PET and Trp labeling into a single process (Figure 1B). **1** features a cationic π -electron system that is readily reduced¹⁰ by SET. Variation of the substituents on the pyridinium ring, as well as the *N*-substituent, can lead to homolytic cleavage of the labile N–N bond, restoring pyridinium aromaticity and generating a reactive new radical species that can be harnessed for a bond forming reaction.¹¹ Using Trp as the single electron reductant to activate **1**, we propose to simultaneously generate a Trp^{•+} and a reactive *N*-centered radical that could recombine to result in a labeled protein with selective modification at Trp (Figure 1B), thereby enabling a labeling process that is kinetically viable and avoids photodegradation pathways typically associated with Trp photoionization.^{5c}

We sought to test this hypothesis in the context of a photolabile substrate, thereby necessitating the development of a robust process to achieve labeling. We chose Octreotide-OAc (**2**), a 1 kDa Somatostatin analogue that possesses a D-Trp residue and an established record of photolability,¹² as a substrate for our preliminary studies (Table 1). We commenced by irradiating a solution of **2** (100 μ M) and pyridinium salt **1a** (10 mM) in NH₄OAc buffer with UV-B light (UVP lamp, 302 nm, 8 W) for 30 min (Table 1, entry 1). We observed high conversion levels of **2**, but with only moderate conversion to the labeled peptide **2a** (34% by LC-MS, entry 1). Further optimization initially failed to improve upon this, with 7 mM of **1a** only showing formation of **2a** in 27% (entry 2). In these instances, we noted the formation of undefinable byproducts, which were interpreted as photodegradation of **2**. When the reaction was performed in the presence of 1 mM of glutathione (GSH), we observed complete conversion (>95%) to **2a** with a mono/di label ratio of >20/1 (entry 3). Given that GSH-disulfide was observed postreaction by LC/MS, we propose that GSH enhances the reaction by acting in its key biological function as a reactive oxygen species (ROS) scavenger.¹³ Notably, we did not observe any adducts of GSH with **2**, **2a**, or **1a** under these conditions. Tandem MS revealed that Trp is the exclusive site of modification in the reaction. These conditions also allowed the labeling of **2** at lower concentrations, with complete conversion when [**2**] = 10 μ M (entry 4). We next explored the role of buffer in the reaction, finding that the labeling reaction proceeded smoothly in 20 mM pH 7.4 phosphate (entry 5) and 20 mM NaOAc (entry 6), although the former condition required a slightly longer reaction time and resulted in a smaller mono/di labeling ratio (3:1). Tandem MS suggests the second label is also located on the Trp residue. Finally, no reaction took place in the absence of light (entry 7). **1a** displays both excellent aqueous solubility (>100 mM) and stability, with <10% decomposition over 48 h in pH 6.9 NH₄OAc buffer, millimolar M concentrations of buffered (pH 7.4) GSH, and human serum. We attribute this stability to the methylated 2, 4, and 6 positions of **1a**, which likely suppresses nucleophilic addition.

With these conditions in hand, we next explored the ability of **1** to deliver reporter handles to **2** (Figure 2). The carbamate group of **1a** provides a simple and convenient handle for derivatization, and we were pleased to observe that removable protecting groups (**2b**), purification tags (**2c**), and linking groups (**2d**) can easily be transferred using this chemistry (Figure 2A). Labeling of **2** could also be performed at higher concentrations to enable

milligram scale preparation; a 3.5 mg scale labeling of **2** ($[2] = 700 \mu\text{M}$) with **1a** yielded **2a** in an average of 68% isolated yield after HPLC purification. We additionally synthesized salt **1c**, which displays an amine group that can be used for further derivatization. Linking **1c** with an alkyne-containing NHS ester followed by photochemical labeling enabled us to transfer an alkyne to **2** in excellent conversion (Figure 2B). LC/MS traces of crude reaction mixtures show that the labeling process produces clean reaction profiles (Figure 2C).

We next explored the labeling of Trp residues on peptides and proteins possessing both differing topologies and redox/photolabile residues (Figure 3). Leuporelin (**3**), a 1.2 kDa peptide that possesses a single Trp, Tyr, and His residue, furnished singly modified conjugate **3a** in high conversion when irradiated with **1a**. MS-MS experiments confirmed Trp as the exclusive site of modification. **3** was also amenable to labeling on a 3 mg scale ($[3] = 500 \mu\text{M}$), furnishing **3a** with a single modification at Trp in a 73% average isolated yield. Additionally, antibiotic daptomycin (**4**) (1.6 kDa, 1 Trp, 1 kynurenine), Melittin (**5**) (2.7 kDa 1, Trp), and glucagon (**6**) (3.4 kDa 1 Trp, 1 His, 2 Tyr, 1 Met, carboxylate terminus) each underwent selective Trp labeling in good conversion. The concentrations of peptide used in each instance were varied in accordance with the solubility properties of each specific peptide, wherein **4–6** aggregate at concentrations $\sim 100 \mu\text{M}$.¹⁴

We next sought to gain a preliminary mechanistic understanding of the labeling process that could help explain the selectivity of this process for Trp (Figure 4). Temporal control experiments, consisting of successive exposures of the reaction mixture to UV-B light, clearly demonstrated that formation of **2a** only occurs in the presence of UV-B light (Figure 4A). Reactions performed either in the dark or with 365 nm UV-A irradiation showed no conversion. Spin-trap TEMPO and NaI, which is both an efficient quencher of Trp fluorescence¹⁵ and known to form charge transfer species with pyridinium species,¹⁶ also inhibited the reaction. A large excess of prenyl alcohol, which could serve to trap free *N*-centered radicals, had no effect on the reaction (Figure 4B). Stern–Volmer analysis of **2** with **1a** showed nonlinear (280 nm) and linear (295 nm) quenching (see Supporting Information); but the analysis of these curves is complicated by the overlap of absorption of Trp and **1c** (Figure 4C). Given that both **2** and **1a** have overlapping absorbance, it is possible that the labeling process could be initiated by photoexcitation of either/both Trp and **1a**. We confirmed the photoexcitation of Trp by observation of complete deuteration of the C4-position of D,L-Trp in D₂O under 302 nm irradiation^{5a} (Figure 4D). Given that **1a** does not absorb beyond 295 nm, we irradiated **2** and **1a** with light that was filtered using a 305 nm long-pass filter, and the reaction proceeded efficiently to completion, albeit at a slower rate (Figure 4B). We also estimated the quantum yield of the labeling of **2** under conditions that solely excite Trp, observing $\Phi_{\text{labeling}} = 0.08 \pm 0.02$. This value is in good agreement with the quantum yields for Trp-photoinitiation,^{5d} suggesting electron transfer from $[\text{Trp}]^*$ is a mechanistic component of the labeling process. NMR analysis of peptide conjugates **2a** and **3a** revealed that labeling occurs at the indole C2 position through C–N bond formation; no coupling at the methyl group was observed (Figure 4D).¹⁷ Taken together, we propose that PET is the key mechanistic process that drives the reaction (Figure 1B), though a clear understanding of the nature of the group transfer remains obscure. Trp selectivity can be partially rationalized by its large ϵ values compared to Tyr (~ 50 times larger) as well as

potentially by preorganization of Trp and **1** through precomplexation.¹⁸ A number of alternative mechanisms, including amination *via* a free *N*-centered radical^{11a} or a nitrenium¹⁹ species, are possible. However, the propensity of these two species to either rapidly rearrange^{17,19b} or aminate aromatic structures analogous to Phe and Tyr led us to disfavor these two possibilities.

Finally, we investigated the modification of lysozyme (**7**), a photolabile,²⁰ 14.3 kDa protein displaying 6Trp residues, 3Tyr residues, and a myriad of other redox active residues (Figure 5). While we observed labeling of lysozyme using **1a**, we also observed cysteine-glutathionylation (verified *via* TCEP reduction), which likely arises either through reduction of proximal disulfides by [Trp]^{•20a} or through thiyl radical exchange²¹ (Figure 5C). Seeking to avoid this undesired side reaction, we hypothesized that by altering the mechanism of the labeling process such that Trp → [**1**][•] became the dominant pathway, then degradation by intraprotein PET could be minimized. We therefore synthesized phenyl-substituted salt **1d**. **1d** red-shifts absorbance ($\lambda_{\text{max}} = 299$ nm, **1a** $\lambda_{\text{max}} = 272$ nm) (Figure 4C) and displays an irreversible reduction potential that is anodically shifted by nearly 200 mV compared to the irreversible reduction potential of **1a**. Using **1d**, labeling of Lysozyme could be achieved in >90% conversion in 15 min with a 6:1 mono/di labeled conjugate, and with glutathionylation being reduced to trace levels (Figure 5D). In these instances, we found it beneficial to use higher concentrations of GSH in order to fully suppress glutathionylation. Since **1d** possesses significant absorption beyond 320 nm, we irradiated **7** with **1d** using a 320 nm long-pass filter to suppress Trp excitation, leading to smooth conversion of labeled lysozyme of 94% conversion in 45 min (Figure 5E). In this instance, we observed that the labeling reaction proceeded to completion more rapidly using lower concentrations of GSH (3 mM). Digestion/MS² revealed Trp-62 to be the site of carbamylation. The doubly labeled conjugate was formed in insufficient quantities to establish the site of second label. While modification of **7** occurs in the active site, modified **7** still displays bacteriolytic activity, indicating that both the carbamate label and the reaction conditions do not denature the protein.

In summary, we have developed a photobioconjugation process that operates directly on native biological structure using *N*-carbamoyl pyridinium salts. The reaction proceeds with excellent selectivity for tryptophan, features short reaction times, and does not require catalysts or organic solvent. We demonstrate the versatility of the *N*-carbamoyl pyridinium salt scaffold by transferring relevant reporter handles to Trp residues and by adjusting the properties of the reagent to enable photochemical transformations on photolabile substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A Photochemical manifolds of Tryptophan in Biology

Photoreduction:
[Trp]^{*}-acceptor

Reductive quenching:
Trp-[Flavin]^{*}

B Coupling Trp's photolability with selective bond formation using *N*-substituted pyridinium salts (1)

Trp-containing Protein

Selective Labelling at Trp

$h\nu$

Wavelength/reagent dependant
activation of Trp through PET

Group Transfer

Figure 1.

Pyridinium salt reagents for Trp-selective protein modification by mimicking biological PET.

Figure 2.

(A) Transferring group scope. (B) Synthesis of alkyne-labeled **2** via derivatizable pyridinium salt **1c**. (C) LC/MS TIC traces of crude reaction mixtures. ^a 120 min reaction time. [GSH] = 5 mM, [**1a**] = 20 mM. ^b % conversion estimated by TIC, average of 2–3 experiments. Ratio of mono/di label >20:1 unless stated otherwise. ^c Isolated after HPLC purification, average of three experiments.

Figure 3.

Scope of the Trp photobioconjugation process. ^a % conversion was measured by TIC, average of 2 runs. ^b [1a] = 2 mM. ^c 150 min reaction, [GSH] = 5 mM, [1a] = 10 mM. ^d Isolated after HPLC purification, average of two experiments. ^e 20 mM 1a. ^f Tris(2-carboxyethyl)phosphine (TCEP) (1 mM) used as an additive.

Figure 4.

Mechanistic studies. (A) Temporal control experiments with **2** and **1a**. (B) Additive and light-perturbation experiments. (C) Absorption spectra of **2**, **1a**, and **1d**. (D) Mechanistic and NMR experiments performed on Trp analogs. ^a % conjugate observed by TIC. ^b Performed in a foiled reaction vessel that was irradiated. ^c UVP 365 nm lamp (8 W).

Figure 5.

(A) Photochemical labeling of **7** and mechanistic reversal of the labeling process using **1d**. Trp residues in **7** are highlighted in cyan. (B) Unmodified **7**. (C) **7** + **1a**. (D) **7** + **1d**, 15 min irradiation time at 302 nm. (E) **7** + **1d**, 45 min irradiation time with 320 nm long pass filter. (F) Confirmation of site selectivity by MS-MS. ^a Conversion estimated by TIC, average of 2 runs. ^b 7 mM **1a**, 1 mM GSH, 20 mM pH 6.9 NH₄OAc buffer. ^c 3 mM **1d**, 6 mM GSH, 20 mM pH 6.9 NH₄OAc buffer. ^d Ratio of +1 to +2 modifications. ^e 3 mM **1d**, 3 mM GSH, 20 mM pH 6.9 NH₄OAc buffer, 320 nm long-pass filter used.

Table 1.

Selected Optimization

 Octreotide (2) + 1a $\xrightarrow[\text{H}_2\text{O}]{h\nu (302 \text{ nm})}$ 2a									
Entry	[2] μ M	[1] mM	Buffer ^a	Additive	<i>t</i> (min)	Conversion of 2 ^b	%2a (mono/di) ^b		
1	100	10	NH ₄ OAc		30	>95%	34% (>20:1)		
2	100	7	NH ₄ OAc		30	>95%	27% (>20:1)		
3	100	7	NH ₄ OAc	1 mM GSH	30	>95%	95% (>20:1)		
4	10	7	NH ₄ OAc	1 mM GSH	30	>95%	95% (>20:1)		
5	100	7	Na ₂ HPO ₄	1 mM GSH	45	>95%	<95% (3:1)		
6	100	7	NaOAc	1 mM GSH	30	>95%	95% (>20:1)		
7 ^c	100	7	NH ₄ OAc	1 mM GSH	30	0%	0%		

^a [Buffer] = 20 mM. NH₄OAc: pH = 6.9. Na₂HPO₄: pH = 7.4. NaOAc: pH = 8.9.^b Estimated by LC-MS TIC, average of 2 runs.^c Reaction was performed in the dark.