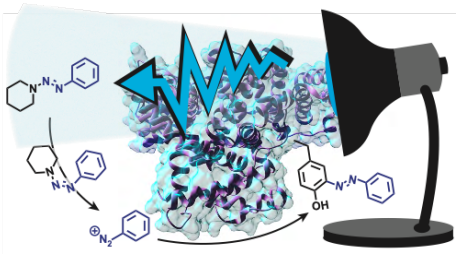
Protein modification via mild photochemical isomerization of triazenes to release aryl diazonium ions

Garrett J. Davis, Julia A. Townsend, Madeline G. Morrow, Mohamed Hamie, Abigail J. Shepard, Chih-Chieh Hsieh, Michael T. Marty, and John C. Jewett*

Department of Chemistry and Biochemistry, University of Arizona, Building 41, Room 104, 1306 East University Boulevard, Tucson, Arizona 85721, USA

Supporting Information Placeholder

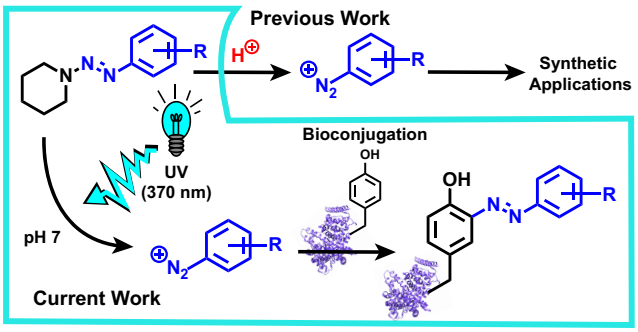


ABSTRACT: This work describes the development of phenyl diazenyl piperidine triazene derivatives that can be activated to release aryl diazonium ions for labeling of proteins using light. These probes show marked bench stability at room temperature and can be photoisomerized via low-intensity UVA irradiation at physiological pH. Upon isomerization the triazenes are rendered more basic and readily protonate to release reactive aryl diazonium ions. It was discovered that the intensity and duration of the UV light was essential to the observed diazonium ion reactivity in competition with the traditionally observed photolytic radical pathways. The combination of their synthetic efficiency, coupled with their overall stability makes the triazene an attractive candidate for use in bioconjugation applications. Bioorthogonal handles on the triazenes are used to demonstrate the ease by which proteins can be modified.

The use of aryl diazonium ion chemistry for protein modification has a long and diverse history dating back over a hundred years to Pauly's work in 1905.[1] In the last two decades, aryl diazonium ions have undergone a renaissance within the realm of bioconjugate chemistry with numerous protein modification applications described. These include PEGylation, introduction of orthogonal and bioorthogonal handles, fluorescent tagging, and further chemo-selective reactions targeting the azobenzene formed.[2,3,4,5] Aryl diazonium ions have long been known to react with various electron-rich aromatic residues via electrophilic aromatic substitution chemistry. Upon reaction, residues such as tyrosine, histidine, and to a lesser extent tryptophan provide stable azobenzene adducts.[6,7,8] Though their selectivity is highly advantageous, other challenges remain and have stifled their widespread adoption for bioconjugation. Because of their reactive nature, in situ generation of the aryl diazonium ion is typically required, which adds a step of uncertainty to the workflow.[9] Though a select few aryl diazonium ions can be isolated as stable salts, their bench-life is often limited, and they remain prone to degradation. Finally, storage of large amounts of diazonium salt can pose danger due to their shock sensitivity and explosive potential.[10] To overcome these challenges, described herein we present the synthesis, characterization, and implementation of a newly established photo-basic system that releases aryl diazonium ions to modify proteins.

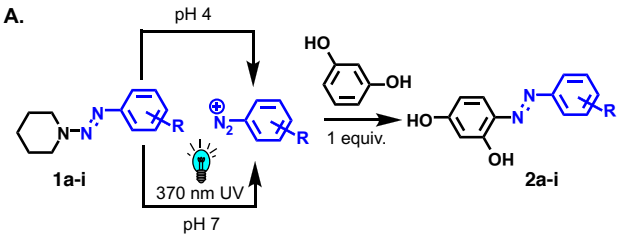
Previous work within our lab has focused on applications of a unique class of π-conjugated triazenes known as triazabutadienes (TBDs) as masked aryl diazonium ions for bioconjugation applications.[11,12] These scaffolds have been shown to be markedly bench stable and their reactivity can be tuned to release aryl diazonium ions within various conditions, most commonly with the introduction of mild acid, or irradiation with UV light.[13] They also have many unique advantages including the ability to be functionalized with orthogonal handles, such as alkynes, or fluorophores for imaging applications.[14,5] The limitation that remains with TBD chemistry is the lack of synthetic ease. Herein we report our work with dialkyl triazenes made simply via treatment of secondary amines, such as piperidine, with aryl diazonium ions (**Scheme 1**).

Dialkyl triazenes and related scaffolds have long garnered attention for wide variety of applications ranging from unique antivirals and anticancer properties, to applications in polymer chemistry. [15,16,17] However, our attention focused on their structural similarities with TBDs, recognizing that they could be used as a bench stable source of aryl diazonium ions.



Scheme 1. Phenyl diazenyl piperidine triggered release of aryl diazonium

A.



B.

Triazene #	R group	Hammett value	Abs. max (nm)	% yield of 2a-i pH 4	% yield of 2a-i pH 7 + UV
1a	*p*-NO_2	+0.78	380	0	0
1b	*p*-CN	+0.66	334	0	0
1c	*p*-CF_3	+0.54	315	0	0
1d	*p*-$CONHCH_3$	+0.36	332	0	0
1e	*p*-Br	+0.23	322	5	30
1f	*m*-OCH_3	+0.15	315	13	36
1g	H	—	313	20	40
1h	*p*-CH_3	-0.17	326	31	46
1i	*p*-OCH_3	-0.27	324	56	74

Figure 1. (A) Diazonium ion reactivity with resorcinol can be triggered by release within pH 4 media, or at pH 7 via 370 nm UV irradiation. Samples were incubated at pH 4, or at pH 7 and irradiated for 3 h prior to being extracted with CH_2Cl_2. (B) Percent yields of azo-adducts (**2a-i**) derived from triazenes with varying substituent electronics treated with either pH 4 citrate buffer, or 370 nm UV at pH 7. Note that in the absence of UV irradiation at pH 7 there was no reaction observed over the 3 h time course.

Triazenes have been shown to liberate aryl diazonium ions in strongly acidic conditions and this feature has made them useful for numerous synthetic applications, including azide synthesis, palladium cross-coupling strategies, Sandmeyer chemistry, Balz-Schiemann fluorination, and various other metalation reactions.[10,18] These triazenes have considerably higher thermal stability than their diazonium salt counterparts and are comparatively shock insensitive, thus diminishing explosive dangers of handling and storage.[19] With these benefits in mind, we aimed to evaluate triazenes as bench-stable, masked aryl diazonium ions that could be environmentally triggered to label proteins via azo-modification (**Scheme 1**).

Prior to experimentation with triazenes in a bioconjugation setting, we sought to understand their fundamental reactivity and potential limitations. To that end, we first synthesized a small library of triazenes using anilines with different electronic properties. Aniline starting materials were treated with standard diazotization conditions using sodium nitrite and HCl. Following diazotization, the solution was added to excess piperidine in an alkaline borate buffer solution. The resulting triazenes precipitated from solution allowing for filtration of pure products (**1a-i**). In general, we found that anilines with electron withdrawing groups (more positive Hammett values) afforded higher yields of their respective triazene (for example, *p*-NO_2 analog **1a**, **Figure 1**), consistent with the expectation of increased electrophilicity of the diazonium (see **Supporting Information Figure S1**).[20]

Next, we sought to determine whether these triazenes could be triggered to release aryl diazonium ions in a physiologically-relevant manner. We trapped the resulting diazonium ions with resorcinol to form a range of azobenzene analogs. Knowing that triazenes are often deprotected in acidic conditions, we challenged the simple benzene scaffold, **1g**, with resorcinol in acidic conditions (0.1 M pH 4 citrate buffer). Treatment of **1g** with 1 equivalent of resorcinol in pH 4 citrate buffer for 3 hours provided a modest 20% yield of the respective azo-adduct (**2g**), confirming that diazonium could be released, albeit slowly, at a near-physiological pH (**Figure 1b**). We followed this up by challenging several other triazene analogs to determine whether electronics of the aryl substituents could dictate reactivity. Indeed, we found that the highly donating analogs such as *p*-methyl (**1h**) and *p*-methoxy (**1o**) triazene produced higher yields of azo-adduct (31% and 56% respectively) than **1g** in the same amount of time. Conversely, triazenes with more withdrawing substituents such as *m*-OCH_3 and *p*-Br had diminished yields. Interestingly, the *p*-nitro (**1a**), *p*-nitrile (**1b**), *p*-CF_3 (**1c**), and the *p*-$CONHCH_3$ (**1d**) analogs failed to produce any of the corresponding azo-adducts (**Figure 1b**). These data are consistent with the expectation that the electron deficient triazenes are less basic, and as such, less prone to deprotection via a protic mechanism.

Having noted that these triazenes could release aryl diazonium ions at near physiological pH, we postulated that we could employ photochemistry to render them reactive in more alkaline environments. In recent years photochemistry has become increasingly popular within bioconjugate strategies and protocols allowing for accelerated reaction rates and controlled accessibility to highly reactive intermediates *in situ*.[21,22] Importantly, previous work by our lab and others has shown that similar TBDs scaffolds undergo isomerization about the N=N bond in the presence of 365 nm UV light and these isomerized compounds are more basic.[13,23,24] This increased basicity allows for protonation and release of their diazonium cargo in neutral or basic solutions. According to previous reports, 1-phenyl diazenyl piperidine analogs and similar 1-aryl-3,3-dialkyl triazenes also undergo isomerization upon irradiation with similar wavelengths of UV to their TBD counterparts, but these studies did not speak to their basicity.[25,26] Other triazenes have been used for various UV-related applications including photoresists and UV sensitive polymers where the triazenes are ablated by high energy light to form benzene radicals.[27,28] Wanting to avoid radical formation, we hypothesized that using mild irradiation we could isomerize the triazenes to render them more basic and release aryl diazonium ions, while avoiding the homolytic cleavage pathway.

To test our hypothesis, we again assessed the series of triazenes with varying electronic properties, this time by irradiating them with a low-wattage 370 nm UV LED light to determine if irradiation could liberate the aryl diazonium ions at pH 7. After 3 hours of irradiation in the presence of resorcinol to trap the resulting ions, products were extracted with dichloromethane and analyzed via NMR. The resulting azo-adducts, if present, were purified to provide isolated yields. Comparing yields of respective azo-adducts, we found that electron-rich triazenes were once again favored for product formation and that the yields were higher than with low pH treatment across the triazenes tested (**Figure 1B**). The highest yielding reactions were again derived from the *p*-OMe (**1i**, σ = -0.27) analog. Interestingly, moderately withdrawing substituents such as *p*-Br (**1e**) saw an increase in yield from 5% to 30%. Akin to the pH 4 studies, analogs with more withdrawing substituents than *p*-Br did not produce azo-adducts, again highlighting that electronics dictate the basicity. We hypothesize that these compounds

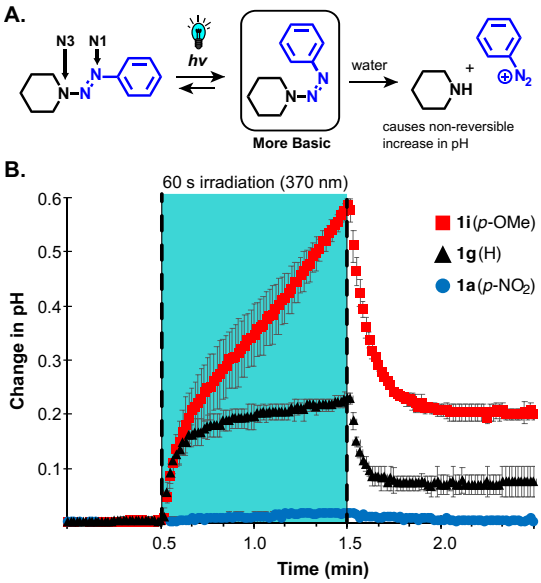


Figure 2. (**A**) Scheme of UV driven isomerization of the triazene scaffold promoting protonation at the N3 position and leading to diazonium release. (**B**) Measurements of pH change upon irradiation of various triazene scaffolds (**1a**: *p*-nitro, **1g**: H, and **1i**: *p*-OMe).

can still become more basic, but are not sufficiently basic to protonate. Notably, **1a** has an absorption maximum best suited to the 370 nm light source (**Figure 1**), but did not liberate the corresponding diazonium ion. While many have previously reported the use of UV to perform photolysis of these triazene scaffolds, to our knowledge, UV irradiation has not been previously shown to liberate diazonium ions from similar triazenes for a further application.[29] Interested if this photochemistry was unique to piperidine-based triazenes, we assessed the reactivity for a range of cyclic amine precursors (see **Supporting Information Figure S2**). We observed a correlation between the yield of the resulting azobenzene and the basicity of amine precursor. As the amines became more basic, the yields for the diazonium ion release improved. This opens up possibilities for tunability in future applications.

Having established that UV irradiation of triazenes in water releases aryl diazonium ions, we sought to understand the mechanism of release. Previous studies with similar triazenes in strong acids have indicated that protonation at the N3 nitrogen atom leads to diazonium ion release (**Figure 2**).[30] This is mitigated by the fact that the N1 nitrogen atom is more basic. The protonation mechanism is consistent with our synthetic data (**Figure 1**). We hypothesize that isomerization of the N1-N2 bond renders the triazene more basic.[23] Although the pH effects of UV isomerization have not been formally evaluated for triazenes of this type, the isomerization and subsequent increase in basicity of the molecule would enable diazonium ion release (**Figure 2A**). To test this mechanistic possibility, we examined pH changes within triazene solutions upon irradiation. In the event, triazenes **1a**, **1g**, and **1i** were dissolved in unbuffered water and the pH was measured continuously before, during, and after irradiation with 370 nm light. Irradiation of the *p*-NO_2 analog (**1a**) for 1 minute did not elicit significant pH change. However, irradiation of the benzene analog (**1g**) and the *p*-OMe analog (**1i**) both showed notable increases of pH during the time of irradiation (**Figure 2B**). The relative change in pH observed was larger for the more electron-rich triazene, with the change observed for **1i** being over double that of **1g**. Furthermore, after irradiation ended there was an immediate drop in pH consistent with a thermal reversion of the scaffold to the less basic starting *trans*-species. The pH did not return to baseline, which is consistent with some of the triazene reacting to release piperidine along with the diazonium ion. The high pK_a of piperidine increases the pH of the solution above initial levels. The shape of the pH curve of **1i** is likely due to a rapid increase from isomerization overlapping with an increase from the release of the piperidine. We hypothesized that the lack of pH increase for **1a** could have been the result of poor spectral overlap between its absorbance and the light source, but indeed **1a** has the best overlap of the three compounds (see **Supporting Information Figure S3**).

After providing evidence to support the isomerization mechanism, we wanted to observe the direct conversion of triazene to the respective azo-adduct by means of HNMR. To ensure solubility of all species, we dissolved *p*-methyl triazene, **1h**, in deuterated methanol along with one equivalent of resorcinol (**Figure 3A**). The sample was irradiated with a 370 nm LED and monitored by NMR at intervals up to 9 h of total irradiation time. While the *cis*-triazene was not observed in solution because of the thermal isomerization back to *trans*, there was a clear but slow conversion of **1h** to piperidine and azobenzene **2h** (**Supporting Information Figure S4**). The slow conversion is consistent with methanol being less acidic than water. Given the clean conversion to **2h**, the concentration of **1h** was determined for each time point by integration of the benzylic methyl peaks of both **1h** and **2h** and those concentrations were plotted as a function of total time in the NMR tube (**Figure 3B**). Importantly, the reaction did not proceed in the absence of UV light. When concentration of **1h** was plotted against irradiation time only, it showed the gradual loss of **1h** over time in a manner resembling a first-order reaction (**Figure 3B**).[31]

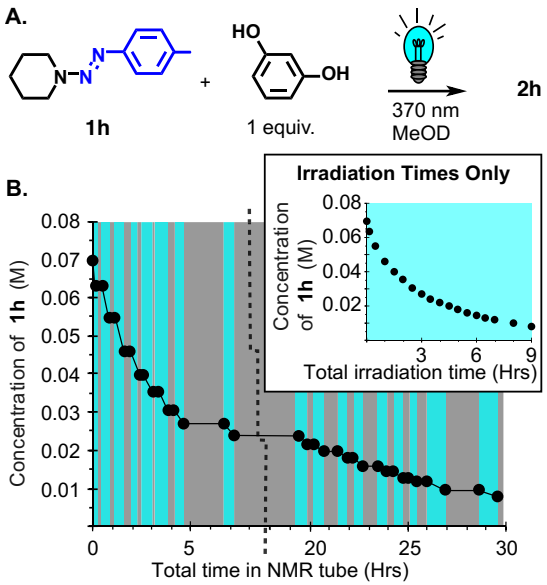


Figure 3. (**A**) General reaction scheme for **1h** irradiated in the presence of resorcinol. (**B**) Concentration of **1h** across total incubation time where UV irradiation time is highlighted in blue and incubation void of light is highlighted in grey. Inset plot of **1h** concentration over irradiation time.

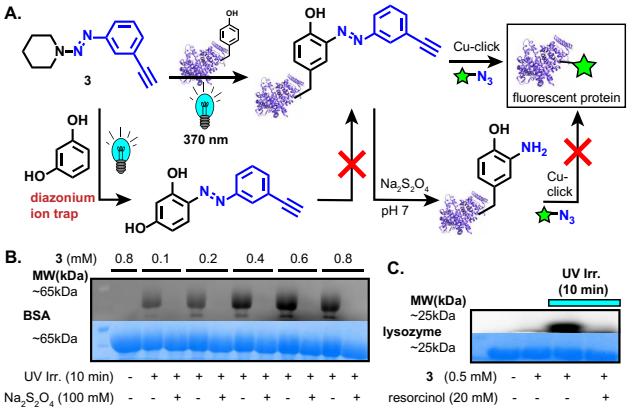


Figure 4. (**A**) Scheme showing the UV triggered labeling of BSA by **3**, as compared to competition with resorcinol, which prevents protein labeling. Following protein modification, reduction with sodium dithionite forms an aminophenol, which is unreactive to the subsequent click conjugation with the fluorescent azide. (**B**) SDS-PAGE gel analyzing BSA labeled by increasing concentration of **3** and UV irradiation compared to samples subsequently treated with 100 mM sodium dithionite. (**C**) SDS-PAGE gel showing treatment of lysozyme with **3** under UV irradiation, with and without the presence of resorcinol.

Moving beyond small molecules, we assessed the ability for the piperidyl triazenes to be useful tools for bioconjugation applications. First, we synthesized an ethynyl triazene capable of modifying proteins with alkynes for subsequent click reactions. Alkynes have become a popular tool within bioconjugate chemistry due to their efficiency and bioorthogonality.[32,33,32] To synthesize a suitable ethynyl triazene, we chose to use 3-ethynyl aniline as the alkyne is only moderately withdrawing. Furthermore, others have already vetted the use of ethynyl aryl diazonium ions for protein labeling and orthogonal modification.[34,35,36] Starting with 3-ethynyl aniline, triazene **3** was synthesized in 83% yield (**Supporting Information Figure S5**). With **3** in hand, we next irradiated resorcinol in the presence of equivalent amounts of 3 to determine if it was capable of labeling. Indeed, we isolated 33% of the respective azo-adduct **4** following 3 hours of irradiation (See **Supporting Information Figure S6**).

To assess protein labeling, we utilized bovine serum albumin (BSA), a protein containing many solvent-exposed tyrosine residues. After confirming that **3** was able to label BSA after pre-exposure to pH 1 to release the diazonium ion (see **Supporting Information Figure S7**) we set out to examine the use of light. BSA was again treated with **3** and irradiated for intervals up to 30 minutes. Following 'click' treatment with a fluorescent azide, we noted marked labeling of BSA in as little as 5 minutes of irradiation (see **Supporting Information Figure S8**). Within 10 minutes we observed robust labeling and moved forward with later experiments using that amount of time (**Figure 4A**). Given the possibility of radical intermediates and their potential photocatalyzed reactivity, we sought to test if the labeling was a result of azobenzene formation.[37,38] To accomplish this we irradiated BSA in the presence of increasing concentrations of **3** (0.1 mM to 0.8 mM) and subsequently treated the samples with sodium dithionite, a reducing agent known to cleave azo-bonds.[39] Proteins that had been modified by aryl radicals would be expected to still maintain the alkyne handle, whereas the cleavage of the azobenzene removes the bioorthogonal handle. Following purification and buffer exchange via G-10 SEC resin, samples were subjected to click conditions with AlexaFluor™ 488 azide, in the presence of copper sulfate and a ligand, tris-hydroxypropyltriazolylmethylamine (THPTA).[40] SDS-analysis showed that treatment with sodium dithionite removed fluorescence across all treatment concentrations, supporting the existence of azo-modifications (**Figure 4B**). To further support labeling by the diazonium ion, we treated another tyrosine-containing protein, lysozyme, with 0.5 mM **3** and compared that to an analogous sample containing a heavy excess of resorcinol. As expected, we saw band fluorescence following irradiation of 0.5 mM **3** for 10 minutes, while the presence of resorcinol outcompeted the protein and greatly diminished protein labeling (**Figure 4C**).

In order to establish optimal timing of irradiation for protein labeling efficiency we selected a small, tyrosine-containing protein MSP1D1T2(-) (MSP) that we have previously shown to work well for aryl diazonium ion chemistry and is well suited to native mass spectroscopy (MS).[41,42] We started by running a gel experiment where MSP was incubated with 0.5 mM **3** and irradiated for intervals between 0 and 10 minutes followed by a Cu-click reaction to add a fluorescent label. Interestingly SDS-PAGE showed that fluorescence intensity increases for irradiation up to 3 minutes and then it steadily declines (**Figure 5A**). We then turned to native MS to provide us with more quantitative insight to this phenomenon. Indeed, we found that MSP treated with 0.5 mM **3** and irradiation for 1 minute showed 4 mods, while a max of 5 modifications was observed at 3 minutes irradiation. The number of mods decreased for samples irradiated longer than 3 minutes, consistent with our gel output (see **Supporting Information Figure S9**).

Curious about the labeling trend in **Figure 5A**, we hypothesized that UV irradiation may be liberating the diazonium ion, but rapidly destroying it. To test this, we performed a comparative study of diazonium ion stability with and without the presence of UV irradiation (**Figure 5B**). To accomplish this goal, we liberated the diazonium ion by treating **3** to a low pH solution for 10 minutes and subsequently bringing it to pH 7 in the absence of protein. To separate vials was added MSP at 0, 5, 10, and 30 minutes post neutralization, serving to trap remaining active diazonium in solution. SDS-PAGE analysis illustrated that diazonium remained reactive up to 10 minutes following addition to pH 7 buffer, but after 30 minutes, the amount of labeling was significantly reduced (**Figure 5B**). Following a similar protocol, we liberated the aryl diazonium ion from **3** with acid at pH 1 and then subjected it to UV irradiation prior to adding protein. SDS-PAGE analysis showed that 5-minutes of irradiation markedly reduced the fluorescent labeling, while protein labeling after 10-minutes of irradiation is barely visible.[43] Based on these results, we hypothesize that UV irradiation is indeed expediting diazonium degradation, likely through a radical intermediate, thus limiting the amount available to perform tyrosine modifications, which is slower than the photolytic degradation at pH 7 (**Figure 5C**). Note that even with a photodegradation mechanism working against labeling, a direct comparison of samples irradiated for 3 minutes at pH 7 showed significantly more labeling than those where **3** was treated with pH 1 for 10 minutes prior to labeling at pH 7 (see **Supporting Information Figure S10**).

To complete our studies, we tested the range of pH for which UV irradiation could be employed to initiate protein labeling. Samples of 25 μM MSP protein were diluted in buffers from pH 5 to 10. Each sample was then treated with 0.5 mM of **3** and

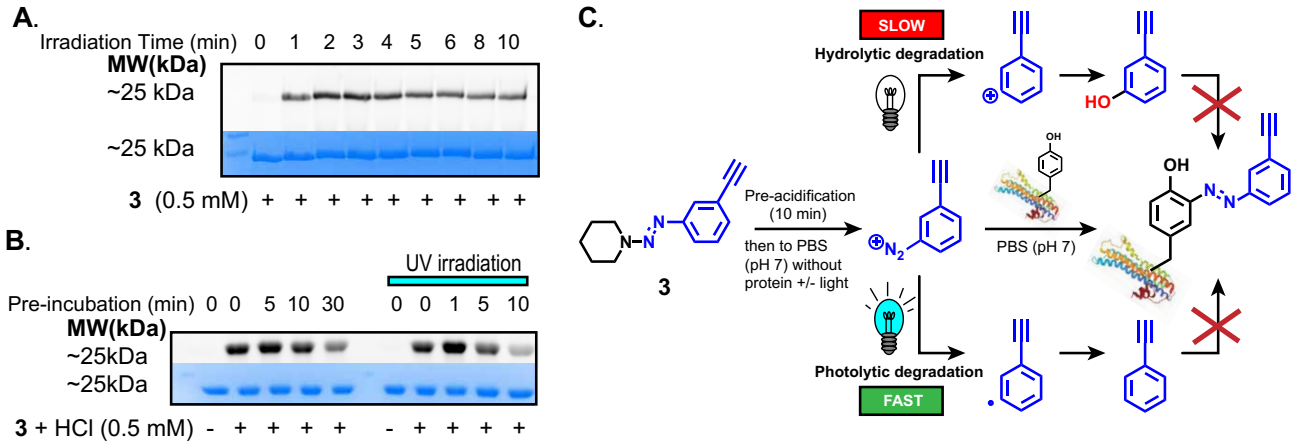


Figure 5. (A) Pre-acidified **3** was incubated for 10 min before being diluted in PBS buffer (pH 7). Samples were incubated with and without UV irradiation for time points 0-30 minutes prior to addition to protein samples. Samples were evaluated by SDS-PAGE following treatment with Alexa Fluor azide (488 nm) under copper click conditions. (B) Hypothesized mechanism of diazonium degradation due to hydrolysis at pH 7, or via UV irradiation at pH 7. (C) Time point assessment of MSP labeled by **3** via UV irradiation (0-10 min).

split into two aliquots. One of the aliquots was irradiated while the other was incubated void of light. Based on the experiments above, samples were irradiated for 3 minutes to minimize photochemical diazonium ion degradation. While protein labeling was not observed in the absence of UV light, labeling in the presence of UV light was highest at pH 7-8, negligible at pH 5, and minimal at pH 9 (see **Supporting Information Figure S11**). The reduction of signal at pH 9 was expected due to the lower likelihood of N3 protonation at elevated pH, whereas the loss of fluorescence at pH 5 was likely due to the decreased nucleophilicity of tyrosine at low pH. Overall, the effective pH range for which irradiation can be employed is likely diazonium ion specific and dependent on the relative electrophilicity of the aryl diazonium ion and nucleophilicity of residue being targeted.

Herein we have shown that 1-phenyldiazenyl piperidine triazenes can be readily synthesized and used to modify aromatic nucleophiles, including those on protein surfaces via UV promoted diazonium ion release. Furthermore, we provided evidence that UV irradiation allows for protein modification via an isomerization mechanism analogous to similar systems. While we have learned much about the nuance of these triazene systems, ongoing work has shown the increasing potential with this system to provide unique tunability and utility that could be leveraged for development of cross-linking agents, fluorogenic probes, and novel modes of protein purification and orthogonal labeling. Furthermore, the abundance of various piperidine analogs, as well as other secondary amines, provides immense opportunity to expand upon our scaffolds to develop more complex structures for a wide variety of bioconjugate applications.

ASSOCIATED CONTENT

Supporting Information: A supporting information is provided, free of charge at: [placeholder].
Synthetic and biochemical protocols including compound characterization, native mass spectrometry, SDS-PAGE analysis, and apparatus setup are provided.

AUTHOR INFORMATION

Corresponding Author

* John C. Jewett
jjewett@email.arizona.edu

Author Contributions

Garrett J. Davis – *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Julia A. Townsend - *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Madeline G. Morrow – *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Mohamed Hamie– *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Abigail J. Shepard - *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Chih-Chieh Hsieh - *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*
Michael T. Marty - *Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States.*

Notes

The authors declare no competing financial interest.

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