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Light-Activated Triazabutadienes for the Modification of a Viral Surface

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Abstract

Chemical crosslinking is a versatile tool for the examination of biochemical interactions, in particular host-pathogen interactions. Herein we report a critical first step toward the goal of probing these interactions with the synthesis and use of a new heterobifunctional crosslinker containing a triazabutadiene scaffold. The triazabutadiene is stable to protein conjugation and liberates a reactive aryl diazonium species upon irradiation with 350 nm light. We highlight the use of this technology by modifying the surface of several proteins, including dengue virus.

Graphical Abstract

Aryl diazonium ions are masked in the form of triazabutadienes and appended onto proteins. The aryl diazonium ions are released in a light-dependent manner and go on to react with electron-rich aromatic moieties. This represents the first step toward a new strategy for catch-and-release pull down studies.

Keywords

bioconjugation; aryl diazonium ion; photochemistry; virus

Chemical crosslinkers enable the covalent trapping of protein interactions and are critical to improving our understanding of the cellular “interactome”^[1], mapping drug – target interactions^[2], and most relevant to our interests, the discovery and characterization of host-pathogen interactions^[3]. Of particular significance are crosslinkers that respond to environmental triggers, enabling a chemical “snapshot” of a key moment of interaction. The interactions that we seek to study occur between dengue virus (DENV) and its host(s).

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Supporting information for this article is given via a link at the end of the document.

DENV is the mosquito-borne causative agent of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS): diseases that afflict 390 million people annually.^[4] We seek to decorate the surface of viruses^[5] with chemical warheads,^[6] thus “arming” the virus for the long-term goal of interrogating interactions that occur during infection.

All four serotypes of DENV undergo an acid-induced conformational change that is critical for fusion to the late-endosomal membrane (Figure 1a).^[7] This pH change is something that we, as chemists, can target from a reactivity standpoint. While pH-sensitive reagents exist in the realm of chemical biology, none met our need for generating a species that would enable crosslinking. Recently we reported that triazabutadienes possess key characteristics that bring us closer to our goals of chemo-selective host-pathogen crosslinking experiments: they release aryl diazonium species in a physiologically relevant range of pH (Figure 1b).^[8] As such, this chemical functionality can be viewed as a protecting group for reactive aryl diazonium ions. We also reported that this functionality undergoes a photo-induced isomerization that enables release the aryl diazonium ion at higher pH.^[9] It is important to note that this light-dependent reactivity differs significantly from other photo-reactive groups in chemical biology. Whereas other functional groups generate reactive carbenes or radical species^[10] the photo-isomerized triazabutadiene simply accelerates formation of aryl diazonium ions by similar protonation-dependent mechanisms as the reaction that occurs in the dark.

Aryl diazonium ions selectively target electron-rich amino acid side chains, most notably tyrosine. The underlying chemistry of aryl diazonium ions interacting with proteins to form azobenzene products has been known for many years.^[11] However, Francis^[12] and others^[13] should be credited for revitalizing the chemistry in the modern chemical biology era. An added benefit of this chemistry comes in the context of pull-down studies: the azobenzene product that is formed can be selectively cleaved under reducing conditions if so desired.^[12a, 14] While it makes up a small fraction of the solvent exposed protein surface, tyrosine is abundant on the interfaces of protein-protein interactions, so called “hot-spots.”^[15] We hypothesize that this feature makes tyrosine an ideal residue to target for probing host-DENV interactions via chemical crosslinking. Herein we report the use of a triazabutadiene as a biocompatible protection strategy for aryl diazonium ions and its utility for modifying viral proteins (Figure 1c). This approach renders an entire protein (or even virus) as a directing group for the chemical warhead.

As mentioned above, we had previously established some of the underlying pH reactivity of triazabutadienes, but these studies were limited to the controlled setting of NMR tubes. In order to evaluate their efficacy of triazabutadienes conjugated directly to proteins we designed and synthesized a lysine-reactive triazabutadiene, **1**. The key step of this synthesis entailed the coupling of aryl azide **2**, made in two steps from *p*-aminobenzoic acid (**3**), with 1,3-bismesityl imidazolium salt **4**. The base-stable triazabutadiene functionality remained intact while ester **5** was saponified and subsequently coupled to *N*-hydroxysuccinimide to provide **1** in decent yield (Scheme 1). The 1,3-bismesityl system was slightly more stable as compared to the previously reported compounds,^[16] and while not water-soluble, **1** was

readily dissolved in DMSO. As expected, irradiation with light promoted rapid release of the aryl diazonium ion (Supporting information, Figure S1).

To test the bioconjugation capabilities of **1** on a protein surface we first studied a model protein, bovine serum albumin (BSA). BSA was reduced and alkylated^[17] and treated with **1** in minimal DMSO at pH 8.8 to curtail the anticipated acid-dependent release of the aryl diazonium ion. Mass spectral data confirmed the presence of our modification on BSA. To test reactivity of **1** on the surface of BSA the labeled protein was re-suspended in a solution buffered to pH 5 in the presence of a fluorescently labeled tyrosine analog (**6**) to serve as a surrogate for tyrosine on an interacting protein. A pH of 5 was chosen due to its similarity to the pH of the late endosome^[18] and our previous studies with triazabutadienes had shown that the aryl diazonium ion should be released within minutes.^{[8],[19]} Expecting liberation of the diazonium and subsequent conjugation to **6**, we were surprised that labeling of BSA was not observed at pH 5 after 2 hours of acidification, (labeling was surprisingly also not observed within the same time frame at pH 3) (see Supporting information, Figure S2). This result was attributed to the hydrophobic nature of the triazabutadiene scaffold that was chosen. If buried in the hydrophobic interior of the protein (or a pocket), access of the key N3 nitrogen atom to the aqueous environment could be occluded and thus protonation would be prevented (Figure 2a).^[8] If true, we hypothesized that a conformational change of the molecule would re-expose the N3 nitrogen and allow for aryl diazonium ion release. We recognize that this hydrophobic protection has advantages and disadvantages: an advantage is stability to neutral and acid pH; a disadvantage is that light would be required to liberate the aryl diazonium ion. Structural insights from our previous work proved instrumental in finding a solution to this problem.

Triazabutadienes undergo photoisomerization,^[20] and we reported that in addition to the massive conformational change from the *E* to *Z* isomerization (Figure 2b), there is a marked increase in basicity.^[9] We hypothesized then, that photo-isomerization of a previously occluded *E* isomer conjugated to a protein surface might expose the critical nitrogen atom to the aqueous environment. This isomer would enable protonation and aryl diazonium ion release at a pH that is neutral or even mildly basic. To test this hypothesis, triazabutadiene-conjugated BSA was subjected to irradiation with a 350 nm UV-LED light source in the presence of **6** at pH 8.8 (Figure 3a–b). We observed robust labeling of BSA by **6**, seeming to plateau within 1 min of irradiation. Strikingly, labeling was observable after only 10 seconds of exposure to light (Figure 3c, Supporting information Figure S3a). In addition to the desired reactivity, there were several other explanations for this data so we systematically ruled them out. Critically, fluorescent labeling was dependent on the modification of BSA with **1**, dismissing the possibility of non-specific photocrosslinking to **6**. Moreover, the labeling was dependent on the concentration of **1**, and the time of irradiation (Figure 3c, Supporting information Figure S3b). The modifications were confirmed by mass spectrometry experiments (Supporting information Figure S4). Benzene diazonium ions are known to undergo photochemical loss of nitrogen to generate benzene radicals, but we had previously shown that the benzene diazonium ions coming from triazabutadienes could be trapped with resorcinol in the presence of light.^[9] The specter of radical chemistry still loomed, so to test this we synthesized a fluorophore conjugated to a simple phenyl

substituent (**7**). True to our desired reactivity, this fluorophore did not significantly modify the BSA-bound diazonium ion (Figure 3a).

To provide further support for light-activated release of an aryl diazonium (as opposed to a much shorter-lived radical) we performed a pulse-chase experiment. Chemically modified BSA was irradiated with 350 nm light for 1 minute, followed by addition of **6** at a series of time points post-irradiation. We observed capture of **6** by BSA after exposure to light (Figure 3d). Fluorescence diminished to near background levels after 30 seconds and persisted for several minutes, indicating that the reactive diazonium ion formed on the protein surface is short-lived, and that the labeling was unlikely to be due to radical chemistry. This presents an additional advantage to aryl diazonium bioconjugation chemistry: the short existence of a tyrosine-reactive species should aid in minimizing spurious pull downs.

The hydrophobic protection of the surface-bound triazabutadiene is likely to be protein-dependent. To test the reactivity of the masked diazonium ion on a viral surface, DENV was purified^[21] and treated with **1**. Similar to BSA, we observed that labeled DENV failed to react under purely acidic conditions (see Supporting information, Figure S5).^[22] Labeled DENV at pH 8.8 was irradiated with 350 nm light in the presence of **6**. We observed capture of **6** on the envelope (E) protein of DENV after exposure to both **1** and light by SDS-PAGE, followed by fluorescence scanning. However, irradiation of labeled DENV for longer than 1 minute resulted in viral envelope (E) protein oligomerization and precipitation (see Supporting information, Figure S6). We postulate that this precipitation was caused by intra-viral reactivity between the surface-bound liberated aryl diazonium ion and residues on adjacent viral envelope proteins.

Most relevant to the application of this compound to the study of virus-host interactions is the maintenance of viral infectivity and structural integrity. Hypothesizing that the mechanism of protection of **1** from acidification on the surface of BSA was the hydrophobicity of the compound, we suspected that this reagent might be denaturing to the viral surface. To examine this effect, we evaluated the use of **1** with DENV at 10 μ M and 50 μ M (Figure 4b). Again, we observed light-induced, concentration dependent labeling of the E protein of DENV. However, we found that infectivity of the virus was markedly reduced after modification by **1**, as observed by TCID₅₀ assay (Figure 4c). In fact, the 50 percent dilution endpoint of DENV infectivity after incubation with 50 μ M **1** was not measurable. To observe morphological changes in the viral surface, we imaged DENV treated with 10 and 50 μ M **1** using transmission electron microscopy (TEM) (Figure 4d). TEM images of DENV labeled with 10 μ M **1** displayed more morphological irregularity than unmodified DENV. No DENV particles were observed after incubation with 50 μ M **1**. Infectivity and TEM results indicate that modification of DENV by **1** is denaturing and/or destabilizing to the viral capsid. Studies to synthesize variants of **1** to minimize these effects are ongoing.

In conclusion, we have established the utility of the triazabutadiene functionality in the context of chemical biology, and in doing so have developed a new class of protected heterobifunctional crosslinkers. This work represents the first demonstration of the use of a masked diazonium ion on a protein surface, and is a critical first step in our own progress

toward trapping and identifying host proteins that interact with DENV during the early stages of viral entry. We expect that others in the field will benefit from the installation and liberation of aryl diazonium ions at neutral pH with light. The added built-in feature of having a mildly-cleavable azobenzene bond between crosslinker and tyrosine should add tremendous value to those seeking downstream mass spectrometry applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Dengue virus (DENV) undergoes a pH dependent conformational change upon entry into host cells. (b) Triazabutadienes react with protic sources to release aryl diazonium ions that can react/crosslink with tyrosine side chains. (c) DENV (pdb: 1k4r) is labelled with a triazabutadiene to provide reactive aryl diazonium ions on the surface of the viral proteins.

Figure 2.

The hydrophobic triazabutadiene is thought to embed into the protein surface (a) and block the protonation of the key N3 nitrogen (in red). Upon photo-isomerization (to b) the N3 nitrogen atom is able to react with surrounding water.

Figure 3.

(a) A general scheme for the use of **1** for protein modification. After conjugation of **1** to bovine serum albumin (BSA), aryl diazonium is liberated with 350 nm light. The aryl diazonium species can be captured by tyrosine, or a resorcinol-conjugated fluorophore serving as a tyrosine analogue, compound **6**. (b) Fluorescent labeling of BSA is dependent on both light, and the presence of **1**. A control, **7**, did not react with BSA-TBD. (c) Extent of labeling increases with time of irradiation. Increased [**1**] on BSA resulted in increased labeling. (d) A “pulse-chase” experiment: BSA-TBD was exposed to 350 nm light for 1 minute in the absence of **6**. At various time points post-irradiation **6** was added to the solution.

Figure 4.

(a) DENV-3 modification by **1**. (b) Fluorescence of DENV irradiated with light in the presence of **6** was dependent on the concentration of **1**, as analyzed by SDS-PAGE/fluorescence and western blot. (c) The infectivity of DENV-3 decreased with each chemical perturbation. (d) TEM analysis of modified viral particles showing irregularities in viral shape after incubation with **1**. *DENV with 50 μ M **1** was not infective and not intact by TEM.

Scheme 1.
Synthesis of lysine-reactive triazabutadiene **1**