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Photoinitiated release of an aziridinium ion precursor for the temporally-controlled alkylation of nucleophiles

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Abstract

A photo-activatable aziridinium precursor has been developed to investigate the possibility of a photo-initiated traditional nucleophilic reaction. The photolysis of a quaternary amine yields a tertiary amine and has allowed us to temporally-control aziridinium formation and subsequent alkylation of a colorimetric nucleophilic reporter molecule. We have also used this photo-initiated reaction to alkylate a sulfhydryl group. This new photo-initiated alkylation strategy is water-soluble and expands the toolkit of photo-activated crosslinkers for protein labeling research.

Keywords

Photochemistry; Alkylation; Ring closure; Nucleophilic addition; Bioconjugation

The development of novel and efficient methods aimed at the facile alkylation of biological substrates in a spatially- and temporally-precise manner has been a topic of much effort in the field of chemical biology.^{1–4} The ability to covalently deliver a low molecular weight contrast agent to a biological target allows for subcellular imaging and the study of dynamic protein motions on cells. In fact, new sub-diffraction resolution microscopy methods typically rely on the delivery and attachment, covalent or non-covalent, of fluorophores to proteins of interest.^{5–7} These fluorophores are usually delivered by one of two methods, genetic tagging of the protein of interest using a naturally fluorescent reporter protein such as GFP or by non-covalent labeling using the tried-and-true antibody method, sometimes in conjunction with a fluorescently-tagged secondary antibody. These methods, however, rely on genetic engineering of the target protein or on non-covalent interactions of fairly large antibodies for highlighting the location of the target biomolecules. More recently, hybrid chemical/genetic strategies such as the tetracycline/FIAsH and ReAsH or the tetraserine/RhoBo systems have been developed.^{8,9} These systems rely on a genetically engineered protein tag that selectively binds to a complementary fluorophore to label a protein of interest. In addition, enzyme catalyzed processes have been developed for orthogonal, selective chemical modification of expressed proteins including the use of BirA, LplA, and SrtA.¹⁰

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Recently, the use of small molecular probes for the delivery of fluorophores to a target of interest has become popular.^{11–13} These probes typically consist of a ligand for binding to the target protein, a fluorophore for visualization, and some form of reactive species, typically an inherent electrophilic moiety that forms a new covalent bond with the target upon binding to the protein. Quite often, the native nucleophile is the sulfhydryl of cysteine that is intrinsic to the target protein and the small molecule-based electrophile contains a maleimide for selective bioconjugation to this residue. While these probes offer many advantages in terms of overall size and their ability to be “traceless”, they all suffer from the fact that an electrophile must be delivered to the biological system and electrophiles are known to react with off-target biological nucleophiles.¹⁴ Here, we report the development of a small, photo-activated, electrophilic moiety that is compatible with aqueous buffer.

Another method for the delivery of a small molecule to a protein target is to employ photochemically-activated moieties appended to small molecules that may target proteins and then covalently modify the protein of interest for downstream analysis.^{15–17} There are three classes of commonly used photoreactive moieties that allow for photo-crosslinking to proteins. These are aryl azides, diazirines, and substituted benzophenones. Each of these functional groups is nominally chemically-inert under dark conditions but can be photo-activated to a reactive species by the application of high energy, ultraviolet light. Upon photo-activation, the azides and diazirine groups result in the production of either a nitrene-like intermediate or a carbene intermediate, respectively, while benzophenones depend on radical aryl ketyl intermediates for reactivity.^{18,19} However, the reactivity of the photo-activated products are difficult to tune and often react with the solubilizing media or buffer in the context of their normal use, thereby reducing their labeling efficiency. Thus, we focused on a method to photoinitiate the release of 2-chloro-*N,N*-dimethylethylamine, a molecule that is similar in reactivity to bis(2-chloroethyl)ethylamine (HN1), the nitrogen mustard alkylating agent that is known to form the promiscuous *N,N*-dialkylaziridinium electrophile under biologically-relevant conditions.^{20–23} To date, there have been a small number of reports of photo-activated, prodrugs of electrophilic moieties that are compatible with aqueous buffer systems.^{24,25}

The *N,N*-dialkylaziridinium ion formed from HN1 has been implicated in the alkylation of biological nucleophiles, most commonly the guanine of DNA after exposure to this nitrogen mustard.^{20,21} We reasoned that the formation of *N,N*-dimethylaziridinium from 2-chloro-*N,N*-dimethylethylamine might be initiated with light if we were to chemically mask the important lone pair of electrons on the tertiary amine of the latter in the form of a photo-releasable quaternary amine (Scheme 1). Upon investigation of potential photolabile protecting groups, we decided to employ a 4-methyl-7-methoxycoumarin chromophore for a number of reasons: 1) This chromophore undergoes heterolytic bond cleavage resulting in the release of a tertiary amine in neutral form with the lone pair of electrons made available for subsequent ring closure, 2) this chromophore can be activated by either ultraviolet light or, potentially important for future biological applications, two-photon infrared light which could allow for more precise spatial control of labeling and, 3) the quantum yield of release from this chromophore has been measured and reported to be highly efficient.²⁶ The *N,N*-dimethylaziridinium that is formed is expected to be relatively long lived in biological conditions. Reports on similar aziridinium species suggest that the half-life of the aziridinium in PBS at 37 °C is about 70 minutes.²⁷

The caged molecule **1** was synthesized by alkylating *N,N*-dimethylaminoethanol with 4-chloromethyl-7-methoxycoumarin (**2**) in 72% yield²⁸ followed by substitution of the hydroxyl with chloride using thionyl chloride to afford **1** in 81% yield (Scheme 2).²⁹ To investigate the photoinitiated release of the 2-chloro-*N,N*-dimethylethylamine and the subsequent cyclization to form an aziridinium ion, we employed a colorimetric assay to

determine the rate of putative aziridinium formation by monitoring nucleophile alkylation. 4-Nitrobenzylpyridine (NBP) has been employed as a detector of alkylation numerous times in past literature because *N*-alkylation and facile work-up produces a deep purple color that is easy to quantify using a UV/vis spectrophotometer.^{30–32} The pyridinium nitrogen is quarternized by the electrophile and the alkaline “stop solution,” which contains aqueous base, is added to the mixture. A benzylic proton is removed and the rings then become conjugated to produce a color that can be rapidly analyzed. We used this method of analysis to follow the evolution of *N,N*-dimethylaziridinium formation of the non-caged 2-chloro-*N,N*-dimethylethylamine, of the caged molecule (**1**), and of the caged molecule (**1**) after exposure to a short pulse of 365 nm light from an LED light source.

Our investigation began with the measurement of the aziridinium formation from 2-chloro-*N,N*-dimethylethylamine (hydrochloride) in aqueous buffer in the presence of NBP at 37 °C and pH 7.4.³³ We performed these reactions at this temperature due to our future plans to employ this photochemistry on live mammalian cells which are typically maintained at 37 °C. We followed the time course of NBP alkylation, presumably via formation of the aziridinium ion, by removing aliquots of the reaction mixture and incubating them with stop solution followed by ethyl acetate extraction of the colored product (Figure 1). The OD₅₃₅ of the ethyl acetate layer was then quantified. All data points were measured in triplicate and are from three different reaction runs. In parallel, we prepared an identical solution of the caged 2-chloro-*N,N*-dimethylethylamine (**1**) and allowed it to react with the NBP in the dark. We expected to see some background reactivity due to S_N2 displacement of the alkyl chloride on the caged molecule by the nucleophilic pyridine. To our delight, the caged molecule did not react at a detectable level, suggesting that the caging group and removal of neighboring group participation (i.e. quarternization of the tertiary amine) is sufficient to eliminate much of the electrophilic character of this alkyl chloride (Figure 2).

To investigate further this apparent lack of reactivity in the dark of **1**, we added 10 mol% of potassium iodide to encourage Finkelstein reaction conditions and facilitate S_N2 reactivity. Even after leaving this reaction for sixteen hours, only background reactivity similar to the solution of **1** that was maintained in the dark was discovered upon work-up and analysis (data not shown).

Upon photoinitiated release of 2-chloro-*N,N*-dimethylethylamine from the coumarin cage, NBP alkylation occurred efficiently and was quite similar in reaction rate to the uncaged 2-chloro-*N,N*-dimethylethylamine as determined by sampling over the course of the incubation. The photoinitiated release of 2-chloro-*N,N*-dimethylethylamine, we believe, allows the aziridinium formation to begin at a prescribed time and results in identical alkylation of the NBP reporter molecule.

Next, we investigated the selectivity of the photo-initiated release of 2-chloro-*N,N*-dimethylethylamine towards four amino acids that represent biologically-relevant potential nucleophiles.³⁴ For all experiments the *N*-terminus of the amino acids used were Fmoc-protected to discourage the alkylation of the free amine versus the side chain functional groups. As a positive control, 2-chloro-*N,N*-dimethylethylamine hydrochloride was incubated with Fmoc-serine, lysine, arginine, or cysteine for 2 hours at 37 °C and pH 7.4 in 2.5 mL of PBS/DMSO (4:1) solution. After two hours of incubation, 50 µL of the reaction mixture was diluted to 1 mL in methanol and analyzed by LC/MS. Under these conditions only cysteine was found to be alkylated (MW= 415.2). Likewise, caged **1** was incubated with of each Fmoc-protected amino acid under the same conditions in the dark. After two hours of incubation, 50 µL of the reaction mixture was diluted to 1 mL in methanol and analyzed by LC/MS. The mass corresponding to the alkylated amino acids were not found under these conditions. In addition, the mass corresponding to unreacted caged **1** was

present, indicating that the cage was not reactive to the buffer at a detectable level. However, under the same reaction conditions following exposure to 1 minute of 365 nm light, the mass corresponding to the alkylated amino acids was only found in the case of cysteine (MW = 415.2). This finding parallels the results from the positive control experiment with 2-chloro-*N,N*-dimethylethylamine.

In summary, we have developed a convenient method for the photoinitiated release of the pro-electrophilic moiety 2-chloro-*N,N*-dimethylethylamine that can form *N,N*-dimethylaziridinium in situ. While our data does not conclusively confirm the production of the aziridinium moiety, we have shown that our caged molecule is essentially unreactive before the release of light. In future studies, we plan to synthesize a similar molecule that contains a ligand and a propargyl group to allow for the electrophilic attachment to a protein of interest. This alkyne can then be used in a subsequent 3+2 click reaction for the attachment of an azido-based fluorophore or biotin. The present molecular system that is the subject of this manuscript is chemically-inert when kept in the dark and is stable in phosphate-buffered saline solution for >30 days at room temperature. The reactive species is only generated after light is applied to unmask the tertiary amine. The application of this technology to protein labeling studies could soon offer new tools to spatially limit protein modification for the purpose of protein tracking on cells and will add another versatile tool to the arsenal of spatially- and temporarily-controlled protein labeling bioorganic chemistry.

General information

All reagents were purchased through Fisher Scientific (Fair Lawn, NJ, USA). Merck silica gel (35–70 mesh) was used for flash chromatography. NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the standard for proton spectra and the carbon signal of the deuterated solvent as the internal standard for carbon spectra. Chemical shifts are reported in parts per million (ppm). When peak multiplicities are given, the following abbreviations are used: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Mass spectra were measured on a Waters ZQ device for LRMS while HRMS data was collected at the University of Massachusetts Mass Facility which is supported, in part, by the National Science Foundation. The colorimetric absorption measurements were made on an Evolution 100 UV/vis spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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28. *Experimental procedure for the synthesis of 2-Hydroxy-N-((7-methoxy-2-oxo-2H-chromen-4-yl)methyl)-N,N-dimethylethanaminium*: To a stirred solution of 4-chloromethyl-7-methoxycoumarin **2** (500 mg, 2.23 mmol), prepared according to a previous report³⁵ in methanol (15 mL) was added *N,N*-dimethylethanolamine (0.896 mL, 8.92 mmol). The reaction mixture was heated to reflux temperature and allowed to stir for 24 hours and then cooled to room temperature and the solvent was removed via rotary evaporation. The crude product was dissolved in a minimal amount of hot methanol and hexanes were added slowly until a solid precipitated. The solid was collected by filtration and was extensively rinsed with hexanes and then used without further purification (503 mg, 72%). ¹H NMR (400 MHz, D₂O) δ ppm= 7.78 (ds, *J*=9.09, 2.53 Hz, 1 H) 6.99 (dd *J*=9.09, 2.53 Hz, 1 H) 6.95 (d, *J*=2.53 Hz, 1 H) 6.58 (s, 1 H) 4.73 – 4.76 (m, 2 H) 4.03 – 4.09 (m, 2 H) 3.83 (s, 3 H) 3.59 – 3.64 (m, 2 H) 3.13 (s, 6 H) ¹³C NMR (400 MHz, D₂O) δ= 163.32, 162.62, 155.31, 142.70, 126.19, 119.26, 113.48, 112.22, 101.64, 67.30, 62.53, 56.03, 55.49, 51.46. HRMS (FAB+) Calc for C₁₅H₂₀NO₄ [M⁺]: 278.1387. Found: 278.1389.
29. *Experimental procedure for the synthesis of I*: The previously-produced alcohol (100 mg, 0.319 mmol) was added to thionyl chloride (2.0 mL, 27.6 mmol) and was stirred at room temperature for 16 hours. The volatiles were removed via rotary evaporation followed by high vacuum to leave a white solid that was recrystallized from isopropanol to yield a white solid (86 mg, 81%). ¹H NMR (400 MHz, D₂O) δ ppm= 7.79 (d, *J*=8.59 Hz, 1 H) 6.97 – 7.05 (m, 2 H) 6.58 (s, 1 H) 4.76 (s, 2 H) 4.00 – 4.07 (m, 2 H) 3.86 – 3.92 (m, 2 H) 3.84 (s, 3 H) 3.17 (s, 6 H) ¹³C NMR (400 MHz, D₂O) δ=

- 163.39, 162.47, 155.31, 142.26, 126.10, 119.36, 113.52, 112.10, 101.68, 66.18, 62.58, 56.05, 51.23, 35.38. HRMS (FAB+) Calc for $C_{15}H_{19}ClNO_3$ [M+]: 296.1048. Found: 296.1059.
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33. *Photolysis and N,N-dimethylaziridinium assay production and detection conditions*: All solutions were kept in the dark (aluminum foil wrapped) except during UV irradiation conditions. Solutions of 2-chloro-*N,N*-dimethylethylamine or caged molecule **1** were prepared by dissolving the hydrochloride salt of 2-chloro-*N,N*-dimethylethylamine or the chloride salt of **1** in PBS to a final concentration of 25 mM. Nitrobenzylpyridine solution was prepared in dry acetone to a final concentration of 25 mM. *N,N*-Dimethylaziridinium formation assays were performed by mixing 1.0 mL of PBS, 500 μ L of test solution containing either **1** or 2-chloro-*N,N*-dimethylethylamine, and 500 μ L of NBP solution and allowing them to incubate at 37 °C. Aliquots of the reaction mixture (100 μ L) were removed at predetermined times and were immediately quenched by the addition of 400 μ L ethyl acetate, 500 μ L of water, and 63 μ L of 1.0 M aqueous KOH. Mixtures were vortexed for 1 minute and centrifuged to efficiently separate the layers. The top, organic layer was removed via pipette and was either directly analyzed by UV absorption or, if highly colored, diluted 10X or 100X in order to produce OD₅₃₅ measurements between 0.0 and 1.0 AU. Each sample was measured after zeroing the UV/vis detector to pure ethyl acetate and each data point was measured in triplicate. Each data point used in the time course plot is defined by three separate reactions, aliquot removal, and workup. Photolysis of the protecting group was accomplished by irradiating the sample from the top of an open 1 dram vial with the light from a LedEngin High Power LED 365 nm source (Mouser Electronics, Mansfield, TX, USA) situated 5 mm from the top of the vial driven by 600 mA of current.
34. *Mass spectrometry analysis of amino acid labeling assay*: Stock solutions of the corresponding Fmoc-protected amino acids were dissolved in DMSO to a final concentration of 14 mM. Control experiments were conducted by dissolving the hydrochloride salt of 2-chloro-*N,N*-dimethylethylamine (0.07 mmol) in 200 μ L of PBS followed by the addition of 50 μ L of the amino acid DMSO solution for a final concentration of 28 and 2.8 mM, respectively. Each reaction was conducted at pH 7.4 and 37°C for 2 hours at which time a 50 μ L aliquot was removed, diluted to 1 mL in methanol, and analyzed by mass spectrometry. Similarly, caged **1** was dissolved in 200 μ L of PBS followed by the addition of 50 μ L of each amino acid DMSO solution and allowed to react for 2 hours at 37°C and pH 7.4 in the dark. The reaction mixture was then analyzed as previously described. Lastly, for the photolysis experiment, caged **1** was dissolved in 200 μ L of PBS followed by the addition of 50 μ L of each amino acid DMSO solution and warmed to 37 °C. The sample was irradiated for 1 minute from a LedEngin High Power LED 365 nm source and then allowed to react for 2 hours at 37°C and pH 7.4. The reaction mixture was analyzed as previously described.
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Figure 1.

Rate of NBP alkylation. Data points represent average of three independent experiments ($n=3$ for each experiment) of reaction between alkylating molecule and NBP following work-up and analysis. Lines are calculated as exponential associations. Green circles are for 2-chloro-*N,N*-dimethylethylamine, black boxes are for caged molecule **1** maintained in the dark, and purple triangles are for caged molecule **1** (at the same concentration and quantity as that used for the green circles) after exposure to 1 minute of 365 nm light as represented by purple bar at 60 minute mark.

Figure 2.

Photographs of alkylation reactions post-workup. a) 2-chloro-*N,N*-dimethylethylamine incubated with NBP at 30 minutes, b) caged molecule **1** irradiated at 365 nm for 1 minute and incubated with NBP for 30 minutes, and c) caged molecule **1** non-irradiated with NBP at 30 minutes. The top, color-containing layer is the ethyl acetate extract that is normally analyzed via UV/vis.

Scheme 1.

Photo-induced aziridinium formation and subsequent alkylation of the pyridine-based nucleophile, NBP.

Scheme 2.

Straightforward synthesis of caged *N,N*-dimethylaziridinium precursor **1**.