Communication

A vinylogous photocleavage strategy allows direct photocaging of backbone amide structure

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A vinylogous photocleavage strategy allows direct photocaging of backbone amide structure

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Supporting Information Placeholder

ABSTRACT: Side-chain modifications that respond to external stimuli provide a convenient approach to control macromolecular structure and function. Responsive modification of backbone amide structure represents a direct and powerful alternative to impact folding and function. Here, we describe a new photocaging method using histidine-directed backbone modification to selectively modify peptides and proteins at the amide N–H bond. A new vinylogous photocleavage method allows photorelease of the backbone modification and with it, restoration of function.

Protein secondary structure derives primarily from specific backbone amide interactions, including requisite hydrogen bonding that determines α-helix and β-sheet folds. Since folding determines function, modification of backbone structure is perhaps the most direct way to alter or control polypeptide function, biasing against or even disrupting natural folding altogether. In limited instances, nature uses modification of the backbone structure to control polypeptide function. For example, sulfenyl–amide S–N bond formation reversibly inactivates protein tyrosine phosphatase 1B,[1] and *N*-methylation in nonribosomal cyclic peptides alters conformation, proteolytic stability, and other attributes.[2] If chemical backbone modification could be made responsive to an external stimulus—such as light, redox potential, or metal ion concentration—the approach could offer a direct and predictable way to build responsive or switchable structure and function.

Photocaging backbone amide structure would be complementary to exiting photocaging approaches, including intein caging[3] and other side-chain caging approaches,[4–9] as well as whole photoresponsive protein domains.[10,11] In some cases, such as photocaged active-site residues, side-chain photocaging is an important and effective way to deliver photoswitchable structures.[12,13] But altering function with a side-chain photocaging agent can be roundabout, difficult to predict, and require empirical approaches.[14] Despite the rather obvious potential of directly photocaging a specific backbone amide bond, the approach has been little studied, largely because access to peptides or proteins with backbone-modified structures is extremely limited. Short backbone-modified peptides can sometimes be accessed by solid-phase synthesis with *N*-alkyl amino acids, which can potentially be integrated into larger sequences by native chemical ligation[14] or expressed protein ligation.[15] However, solid-phase synthesis with *N*-alkyl amino acids is plagued by low coupling efficiency, and standard HATU/HOBt coupling agents fail in these demanding applications.[5,16–18] Quite recently, a photocaged backbone approach was used for photocontrol of β-sheet formation, a result conveying both the significant potential of backbone photocontrol, but also the significant limitations of current methods.[19] Many modifications of interest, including *N*-arylation and *N*-alkenylation, are inaccessible by chemical synthesis. Furthermore, it is not yet possible to engineer the few examples of enzymatic backbone modification using general protein engineering tools. Ribosomal incorporation of *N*-alkyl amino acids using unnatural tRNA technology has also been demonstrated, but again, capabilities are severely limited.[20,21]

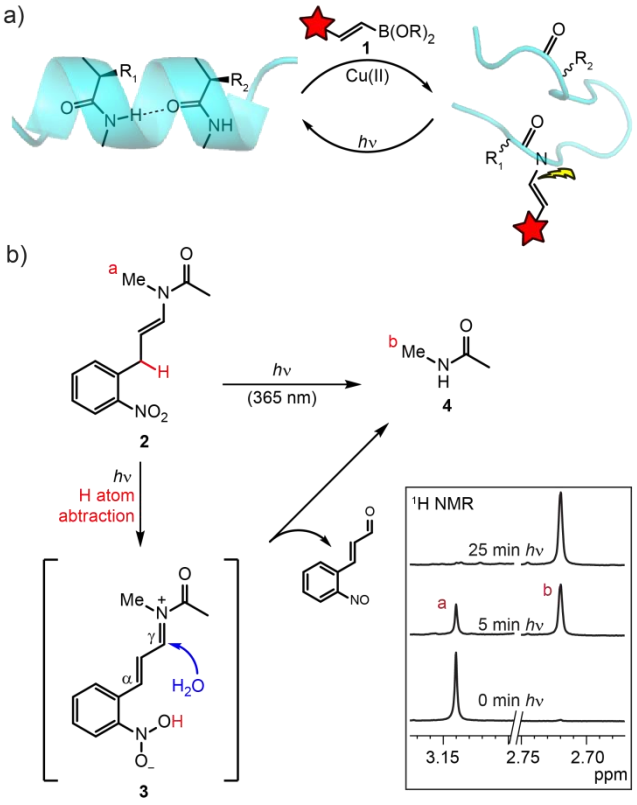


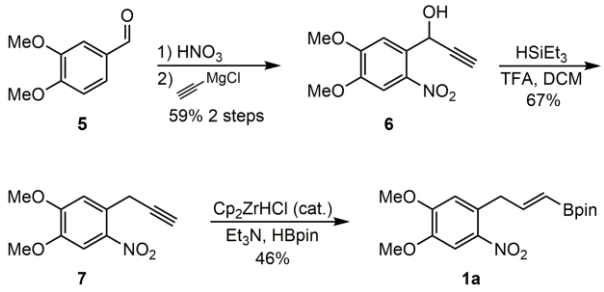
Figure 1. Vinylogous photocleavable modification of backbone N–H bonds. (a) Schematic depicting of structural perturbation with a photoremoveable reagent. (b) A model photocleavage reaction releasing *N*-methylacetamide (**4**) through the presumed intermediacy of C–H abstraction product **3**. (inset) ^{1}H NMR spectra demonstrating release of amide **4** upon photoirradiation, following peaks attributed to the *N*-methyl resonances (**a**) and (**b**) Conditions: irradiation at 365 nm of a 35-mM trimethylamine/trimethylamine HCl in 2:3 CD_3OD/D_2O soln.

This report describes a photocleavable backbone N–H modification that allows efficient photocaging of folding and function (Fig. 1a). Efforts to develop a backbone photocaging process stemmed from our recent discovery of predictable access to backbone N–H alkenylation and arylation with boronic acid reagents, directed by

a neighboring histidine residue in the i + 1 position.[22,23] We envisioned that a well-designed boronic acid could conceivably deliver a backbone modification capable of traceless photochemical cleavage. However, traceless photochemical cleavage, in biological contexts, typically involves cleavage of a C(sp^3)–X of a 2-nitrobenzyl derivative.[24] In contrast, histidine-directed backbone modification requires alkenyl- or aryl-boronic acids, delivering *N*-alkenyl or *N*-aryl polypeptide products, and thus necessitating photocleavage at a C(sp^2)–N bond. Despite extensive literature on photocleavage and photodeprotection,[24] there is limited precedent for C–X cleavage at sp^2 carbon atoms, typically involving cleavage of N–acyl bonds producing (formally) hydrolysis products.[25,26] In a new approach to this problem, we chose to examine a vinylogous[27–29] analogue of 2-nitrobenzyl cleavage (Fig. 1b). In this case, putative benzylic hydrogen atom abstraction would provide an extended conjugated system (**3**). We hypothesized that, among several possible pathways, nucleophilic attack by water on the γ-carbon of **3** would eventually release a "traceless" polypeptide product, together with an unsaturated aldehyde or decomposition product thereof. To explore this proposed pathway, a model vinylogous 2-nitrobenzyl amide (**2**) was prepared by analogy to a reported condensation.[30] Upon treatment with 365-nm light in water in the presence of triethylamine as a scavenger, the conversion of caged amide **2** to *N*-methylacetamide (**4**) was monitored by ^{1}H NMR, based on the *N*-methyl resonance shift from 3.16 ppm to 2.68 ppm upon photocleaveage.[31] The clean and efficient photocleavage observed provides a sufficient foundation for a vinylogous approach to sp^2 C–N bond cleavage in complex polypeptides.

In preparing an appropriate and effective boronic acid "photocaging" reagent, we incorporated a 3,4-dimethoxy (6-nitroveratryl) core for red-shifted absorption and improved photocleavage efficiency,[32] which ultimately led to the design of photocleavable reagent **1a** (Scheme 1). In the course of synthetic investigations, it was determined that the 2-propargylnitrobenzene structure, as in alkynes **6** and **7**, is sensitive to basic conditions, necessitating careful reaction sequencing, especially in more complex target structures (Scheme 2). In the end, boronate **1a** was effectively prepared by reduction of alcohol **6** under acidic conditions, followed by zirconium-catalyzed hydroboration.

Scheme 1. Synthesis of photocleavable reagent 1a.



We next evaluated the photocaging of a variety of peptides with boronic acid **1a** (Fig. 2). Upon treatment with boronic acid **1a** in the presence of a copper(II) salt, clean conversion of peptide to a backbone-modified product was observed for angiotensin I, a chymotrypsin substrate peptide (CSP), leuprolide, and a collagen mimetic peptide (CMP) (Fig 2 b-e, blue spectra). Gratifyingly, in each case the initial, uncaged peptide could be regenerated upon irradiation with 365-nm light to remove the modification (red spectra).

The CMP peptide allowed direct investigation of photocaged folding. The CMP sequence consists of repeating Gly-Pro-Hyp units (Hyp = hydroxyproline) and folds into a canonical polyproline type-II triple helix.[33] Interstrand glycine hydrogen-bonding interactions are essential for the formation of the triple helix, and substitutions of backbone N–H bonds at glycine completely disrupts folding. The CMP peptide has a single histidine residue, which enables backbone modification at the preceding glycine residue. Consistent with prior understanding, this histidine-containing peptide exhibits a characteristic polyproline type-II secondary structure, with a circular dichroism (CD) spectrum containing a maximum at 225 nm and a cooperative thermal unfolding event (m.p. = 42 °C, Fig. S5). After modification at Gly12 with boronate **1a**, folding and trimerization was completely disrupted: the CD spectra of the modified peptide lacked any feature at 225 nm (Fig. 2f). A CD thermal melt analysis contained no cooperative unfolding event (Fig 2g). After three minutes irradiation with 365-nm light, photocleavage of the modification was observed, and the peptide regained the positive CD signature at

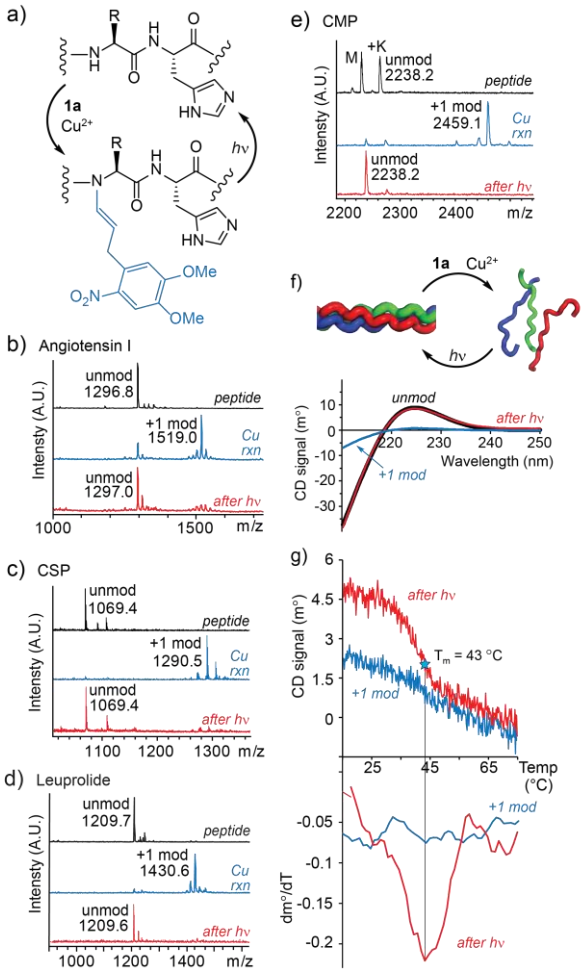


Figure 2. Backbone modification and photorelease of peptides. (a) Schematic depiction of modification and photocleavage reaction, (b-e) MALDI–MS analysis of various peptides before (top, black) and after (middle, blue) N–H modification with boronate **1a**, and subsequent analysis after photocleavage (bottom, red). (f) CMP modification-driven unfolding and photocleavage-driven refolding. CD analysis of unmodified (black) and modified CMP (sequence: Ac-$(POG)_4HOG(POG)_3$-NH_2) before (blue) and after (red) photocleavage with 365-nm light. (g) CD thermal melt (integrated, top and differential, bottom) of modified peptide before and after irradiation. Peptide modification conditions: 100 μM peptide, 1 mM boronate **1a**, 10 mM buffer (varied pH); angiotensin I: 500 μM $Cu(OAc)_2$, pH 8.5; CSP: 100 μM $Cu(OAc)_2$, pH 6.5; leuprolide: 500 μM $Cu(OAc)_2$, pH 8.5; CMP: 100 μM $Cu(OAc)_2$, pH 8.5. Size-exclusion filtration (700 MWCO) was employed to remove small molecules prior to CD analyses.

225 nm, consistent with triple-helix formation (Fig 2f). Furthermore, the unmasked CMP peptide exhibited a melting temperature of 43 ºC (Fig 2g), indistinguishable from as-synthesized peptide.

Modification of backbone structure is a direct approach to photocaged protease susceptibility of peptides, since proteolysis is quite sensitive to backbone N–H substitution near the cleavage site.[34] To test the potential for light-triggered peptide hydrolysis, we synthesized an α-chymotrypsin substrate peptide (CSP, sequence: Ac-SIINFGHKL-NH_2), based a prior study of enzyme selectivity.[35] Proteolysis with α-chymotrypsin cleaves this peptide between phenylalanine and glycine residues. Histidine-directed modification with boronate **1a** produced a peptide, photocaged at glycine. Next, mixtures of caged and uncaged peptide were treated with α-chymotrypsin (Fig. 3). Subsequent HPLC and MS analysis indicated that the unmodified peptide was entirely degraded, while the photocaged peptide remained intact. The α-chymotrypsin was deactivated by a pH jump, and the peptide was uncaged using 365-nm light, resulting in the formation of the parent CSP. If the mixture is irradiated without α-chymotrypsin deactivation, the CSP is consumed as it is formed, and thus no full-length CSP is observed after photoirradiation (Fig. S4).

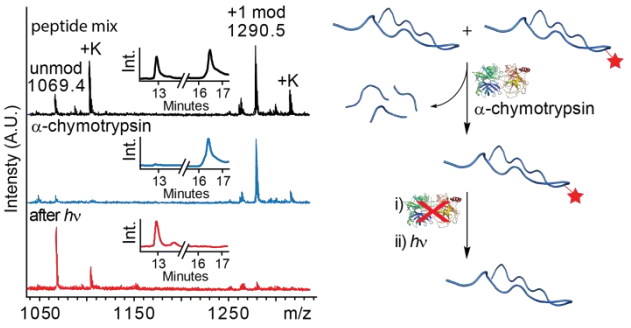


Figure 3. Photocaged control of a protease substrate, CSP. Protease activity was assessed by MALDI-MS and HPLC (inset). A mixture of modified and unmodified CSP (left, green trace), was treated with α-chymotrypsin, resulting in complete consumption of the uncaged CSP peptide (blue trace). After inactivation of α-chymotrypsin by a drop in pH, the parent CSP peptide was regenerated upon irradiation for 5 min at 365 nm. CSP modification conditions: 200 μM CSP, 200 μM $Cu(OAc)_2$, 2 mM boronate **1a**, 10 mM NMM buffer, pH 6.5.

Finally, we examined modification and uncaging of a model protein, soybean trypsin inhibitor (SBTI), which contains a single histidine residue (His 71) in an exposed loop region (Fig. 4a). To facilitate work with larger proteins, we found it useful to develop a bifunctional boronic acid reagent incorporating desthiobiotin[36,37] as a convenient handle for purification and analysis (Scheme 2). In designing this bifunctional probe, the core 6-nitroveratryl photoactive component was preserved. A convergent synthesis involving coupling of readily-available precursors **8** and **9** was carried out, allowing formation of the target structure **1b** through a reduction–hydroboration route analogous to that of photocaging agent **1a**. In this case Zr-catalyzed hydroboration failed, and so uncatalyzed hydroboration with catecholborane furnished the alkenylboronic acid **1b**, albeit with significant diminution in yield.

SBTI was modified with boronate **1b** at Gly70. Affinity purification produced modified protein of sufficient purity to test photocleavage, and single modification was established by anti-biotin western blot and by mass spectrometry. Gratifyingly, irradiation at 365 nm for 30 minutes led to complete removal of the photocaging agent, as judged by mass spectrometry and anti-biotin western blot (Fig. 4b).

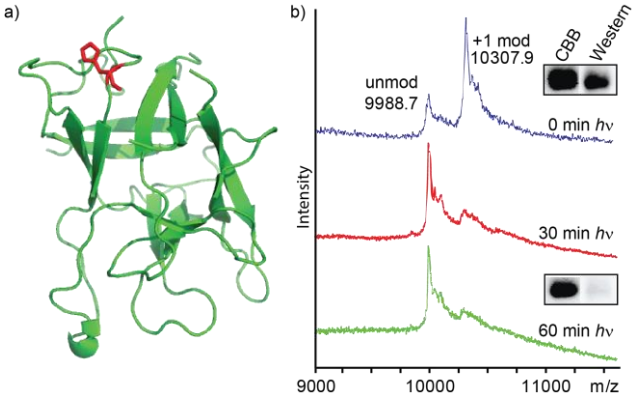
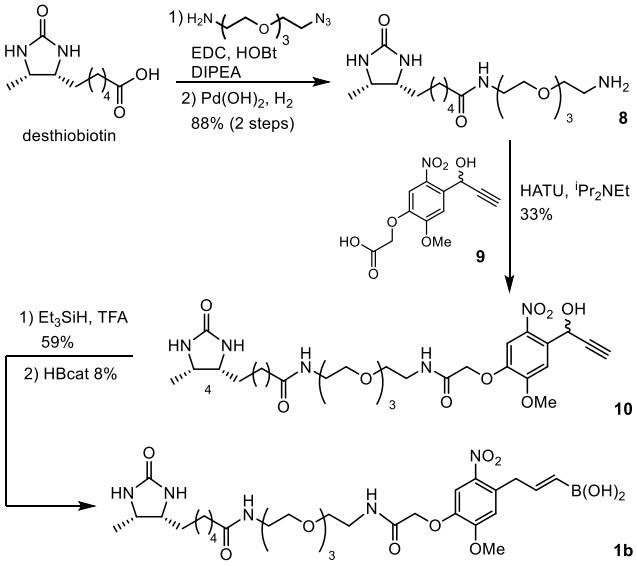


Figure 4. Photocleavable modification of soybean trypsin inhibitor (SBTI). (a) SBTI structure showing His71 (red). (b) MALDI analysis of modified STBI before and after irradiation. (inset) Anti-biotin western blot indicated loss of signal following photocleavage. Protein modification conditions: 20 μM SBTI, 500 μM $Cu(OAc)_2$, 500 μM boronate **1b**, 10 mM NMM buffer, pH 8. See supporting information for full blot.

Scheme 2. Synthesis of photocleavable reagent 1b.



A vinylogous 2-nitrobenzylboronic acid enables, for the first time, access to photocaged backbone N–H bonds by direct backbone modification of natural polypeptide structures. Furthermore, the *N*-vinylamide photocaging structure significantly perturbs the electronics of the amide bond, potentially allowing reversible perturbation of amide carbonyl basicity or of the kinetics of *cis-trans* amide interconversion. In contrast to side chain modifications, backbone modification presents a more direct, general, and easily designed approach to caging the function of a protein or polypeptide. Incorporation of a photocaging group is predictable, and can work on natural substrates without sequence engineering.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, characterization, and additional HPLC and CD spectra (PDF).

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Notes

The authors declare no competing financial interests.

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SYNOPSIS TOC