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Design, Synthesis, and Photochemical Validation of Peptide Linchpins Containing the *S,S*-Tetrazine Phototrigger

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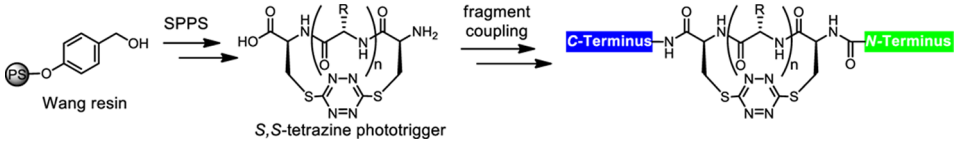
ABSTRACT



The design, solid-phase synthesis, and photochemical validation of diverse peptide linchpins, containing the *S,S*-tetrazine phototrigger, have been achieved. Steady state irradiation or femtosecond laser pulses confirm their rapid photofragmentation. Attachment of peptides to the *C*- and *N*-termini will provide access to diverse constrained peptide constructs that hold the promise of providing information about early peptide/protein conformational dynamics upon photochemical release.

Understanding the early kinetic events that govern conformational dynamics in peptides and proteins provides an avenue for the development of models of folding and, in turn, the misfolding of proteins.[1] To explore these early events, ultrafast photochemical triggers are required to initiate conformational transitions that occur on the pico- to nanosecond time scale.[2] With the appropriate design, incorporation of a phototriggering moiety would confine a peptide/protein construct within a narrow distribution of conformers. Photolysis would then permit release of the geometric constraint with precise temporal control for interrogation with now available state-of-the-art spectroscopic techniques, such as two-dimensional infrared spectroscopy (2D-IR),[3] to monitor the conformational evolution on the pico- to nanosecond time scale, as the peptide/protein relaxes to a new equilibrium conformation.[4]

The first example of a photochemical tactic to explore the dynamics of peptide and protein folding was introduced by Hochtrasser and DeGrado, exploiting the photolysis of a disulfide bond.[5] While effective in releasing a peptide system, homolysis of the disulfide bond required high energy (λ = 270 nm). The photochemical event revealed two reactive sulfur-centered radicals that could either undergo rapid geminate recombination or react with the protein side chains. In related work, Chan and coworkers developed 3′-methoxy and 3′,5′-dimethoxybenzoins that are both water-soluble and undergo rapid photolysis with a time constant of 5 ps.[6] The analytical methods employed however did not permit temporal resolution of the conformational dynamics that occur on the ultrafast time scale, but instead focused on global

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changes (ms) of the peptide conformation. Photochemical switches have also been employed, for example azobenzenes, to enable fast, reversible photoisomerization to permit conformational reorganization.[7] This constraint however significantly limited the accessible conformational space available to the peptide or protein.

Recently, we introduced and validated the feasibility of incorporating the *S*,*S*-tetrazine phototrigger system between two cysteine residues.[8] Photolysis of the *S*,*S*-tetrazine construct proceeded rapidly with a ps time constant to furnish thiocyanate photoproducts, in conjunction with loss of dinitrogen (Figure 1).

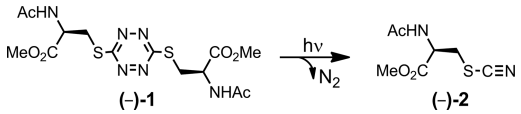

Figure 1. The photolysis of *S*,*S*-tetrazine (−)-**1**, under a variety of solvents and light sources, yields thiocyanate (−)-**2** with loss of dinitrogen.

The *S*,*S*-tetrazine scaffold possesses a number of advantages when compared to other nonreversible trigger systems: (1) flash photolysis can be conducted with near-UV (λ = 355 or 410 nm) laser pulses, which would not lead to damage of the peptide; (2) acceptable photolysis yields are observed; and (3) the thiocyanate photoproducts are relatively inert and provide a potentially useful IR probe. In addition to identifying the *S*,*S*-tetrazine phototrigger as a suitable candidate, we successfully replaced the native disulfide bond of the peptide oxytocin with the tetrazine moiety as a model system.[9] Subsequent photophysical analysis of the derived cyclic peptide revealed that the peptide backbone and the side chains do not interfere with the photofragmentation reaction, highlighting the biocompatibility of the *S*,*S*-tetrazine phototrigger. With these experiments as background, we next sought to broaden the scope of the *S*,*S*-tetrazine trigger with the development of a general protocol to incorporate the trigger within a peptide system having two cysteine residues as desired along a peptide chain.

Herein we describe the design, synthesis, and photophysical validation of a series of peptide linchpins containing the *S*,*S*-tetrazine phototrigger. In addition, we demonstrate the feasibility of employing these linchpins in solution phase fragment coupling, which should permit the ready construction of more complex, relevant peptide and protein systems.

Standard Fmoc-based Solid-Phase Peptide Synthesis (SPPS; Figure 2), with the requisite cysteine side chains protected as *para*-monomethoxytrityl (Mmt) thioethers, was employed to construct the target peptide linchpins employing either *N*-methyl indole aminomethyl or Wang resins. Selective removal of the Mmt cysteine side-chain protecting groups, followed by insertion of the *S*,*S*-tetrazine ring by treatment with 3,6-dichlorotetrazine under mildly basic conditions, and then removal of the peptide from the resin successfully furnished tripeptides **4a**−**c**, with an overall yield of 15−30%. Pleasingly, steady-state irradiation of linchpins **4a** and **c** at a wavelength of 355 nm led solely to the formation of the anticipated bis-thiocyanate peptide photoproducts **5a** and **b**. The photolysis was monitored by LC-MS and ^{1}H, ^{13}C NMR. The rapid disappearance of the ^{13}C signals for the tetrazine ring carbons ($\delta_c \approx 170$ ppm), with concomitant appearance of the SCN resonance at $\delta_c \approx 113$ ppm proved particularly diagnostic [see Supporting Information (SI)].

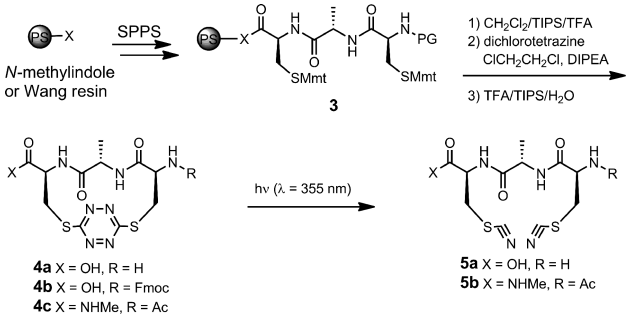

Figure 2. SPPS was used to access resin bound tripeptides **3**; selective deprotection, tetrazine insertion, standard cleavage conditions, and subsequent photolysis yielded peptides **4** and **5** respectively.

With the goal of incorporating the *S*,*S*-tetrazine linchpins within larger peptide/protein scaffolds, the compatibility of various peptide constituents present during the solid-phase peptide synthesis, in conjunction with the effect of proximal side chains on the photochemical behavior of the *S*,*S*-tetrazine phototrigger, was explored. To this end, tripeptide tetrazines **4d**−**j** were prepared (Figure 3). Although **4k** could be successfully constructed and released from the solid support, the conditions to release the pbf-protected Arg side chain led to decomposition, presumably due to the incompatibility of the guanidinium functionality with the tetrazine system.[10] Indeed, treatment of simple *S*,*S*-tetrazine (−)-**1** with basic guanidine led to similar decomposition. Importantly, tripeptide linchpins **4d**−**i** underwent clean steady-state photolysis,[11] demonstrating that the amino side chains do not

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(10) *S*,*S*-Tetrazine **1** was stable to guanidine hydrochloride; however basic guanidine resulted in a complex mixture. For further details refer to Supporting Information.

(11) **4d**−**i** were photolyzed under standard conditions to give single compounds by ^{1}H NMR.

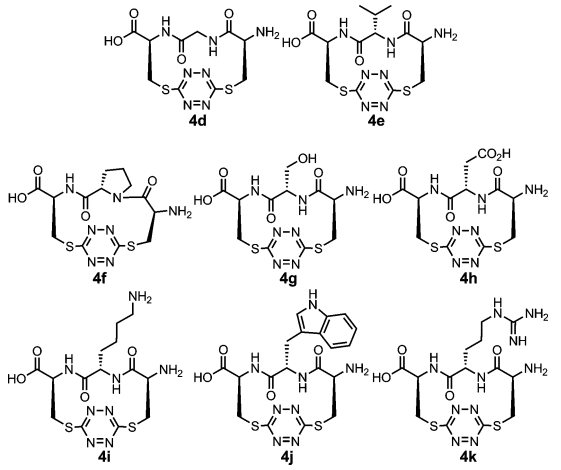

Figure 3. Tetrazine tripeptides synthesized in 7−30% overall yield with the exception of **4k** which undergoes decomposition upon purification.

perturb the photolysis of the electron-deficient *S*,*S*-tetrazine chromophore.

To monitor structural transitions on a fast time scale employing 2D-IR, differentiation of the amide I IR stretching modes of the diverse peptide carbonyls would be required.[12] To this end, tripeptides **4l,m** and **7** were prepared (Figure 4), wherein the alanine and/or cysteine amino acid carbonyls were labeled with ^{13}C or corresponding $^{13}C/^{18}O$, the latter prepared by $H_2{}^{18}O$ exchange[13] of the ^{13}C-amino acid (see SI). Tripeptides **4l,m** were then prepared according to the previously developed solid-phase conditions employing the isotopically labeled amino acids. A modification to this protocol was required to construct tripeptide **7**, as the ^{18}O enrichment procedure proved incompatible with the *para*-monomethoxytrityl sulfur-protected cysteine. Pleasingly employing the thio-*tert*-butyl sulfur protected amino acid during the ^{18}O exchange[14] followed by solid-phase peptide synthesis proved effective. Sequential removal of the cysteine protecting groups with tributylphospine and TFA prior to insertion of the tetrazine moiety permitted preparation of tripeptide **7**.

IR analysis of the isotopically labeled tripeptides revealed that the $^{13}C/^{18}O$ and ^{13}C amino acid amide carbonyl stretching bands are shifted by ~60 and ~30 cm^{-1}, respectively, with the doubly labeled peak appearing at ~1590 cm^{-1}, well separated from the main amide I band (Figure 5). The stretching frequency of the singly labeled carbonyl, however, remained somewhat buried in the main band.

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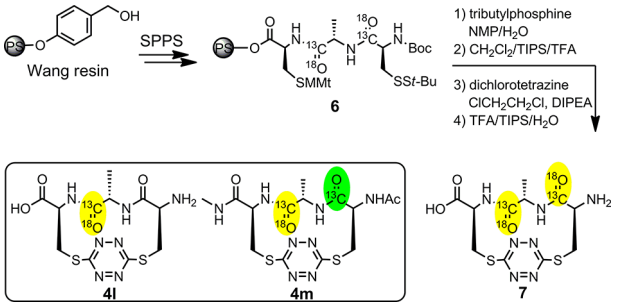

Figure 4. Isotopically labeled *S*,*S*-tetrazine containing tripeptide units with doubly labeled carbonyls highlighted in yellow and singly labeled in green.

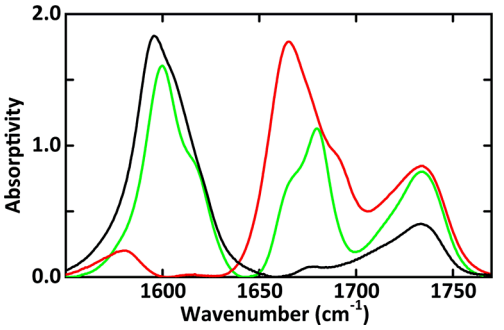

Figure 5. Infrared spectra (1550−1770 cm^{-1}) of **4a** (red), **4l** (green), and **7** (black) recorded in CD_3OD.

Following the successful preparation of the **4a**−**m** series of tetrazine tripeptide linchpins, designated as (*i*, *i*+2) with regard to separation of Cys residues, we next sought to study larger macrocyclic congeners that inscribe the tetrazine ring. Standard Fmoc-based solid-phase peptide synthesis was employed to construct the (*i*, *i*+3) and (*i*, *i*+4) target peptides **9a,b** and **9c** respectively (Figure 6). With these model linchpins in hand, the photofragmentation reaction was again investigated. Nanosecond flash photolysis[15] experiments revealed a dependence of the photochemical yield on the size of the macrocyclic peptide constrained by the *S*,*S*-tetrazine. Peptide **4c**, with an (*i*, *i*+2) relationship between the Cys residues, afford the highest photoproduct yield upon flash photolysis (>25%). The (*i*, *i*+3) relationship in **9b** led to a decrease in photoproduct to >15%; a further decrease to ~10% was observed for the (*i*, *i*+4) peptide **9c**. The observed photochemical yield dependence is likely related to the conformational strain exerted by the peptides on the *S*,*S*-tetrazine ring. Importantly the photochemical yields are sufficient that population changes are observable by 2D IR, while adequate intact linchpin is maintained to enable spectroscopic data to be accumulated over an extended period of time.

(15) Papp, S.; Vanderkooi, J. M.; Owen, C. S.; Holtom, G. R.; Phillips, C. M. *Biophys. J.* **1990**, *50*, 177–186.

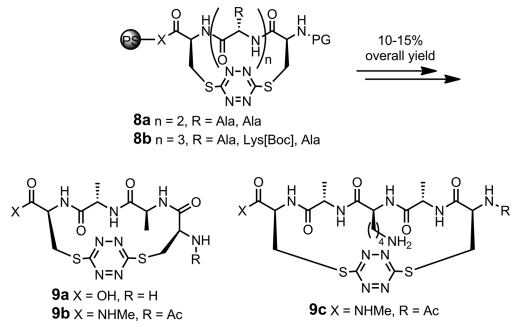

Figure 6. The *S*,*S*-tetrazine incorporated at different positions along the peptide backbone to furnish **9a,b** (*i*, *i*+3) and **9c** (*i*, *i*+4) macrocylcic peptides.

Building upon the successful synthesis and development of the (*i*, *i*+2) peptide linchpins, we focused attention next on constructing larger peptide constructs containing the *S*,*S*-tetrazine with an (*i*, *i*+2) linchpin relationship between the Cys residues. The solid-phase conditions employed earlier to incorporate the *S*,*S*-tetrazine chromophore unfortunately proved unworkable when ≥8 amino acid peptides were immobilized on the solid support, despite employing a wide variety of reaction paradigms, such as including denaturants, elevated temperatures, and using 3,6-dibromotetrazine[16] (Figure 7). We also explored early incorporation of the tetrazine unit **4b**; repeated iterative treatment with piperidine employed in the solid-phase peptide synthesis tactic however led to decomposition of the *S*,*S*-tetrazine.[17,18] We, thus, chose to develop and deploy the *S*,*S*-tetrazine linchpins employing a fragment union tactic, whereby readily prepared peptide residues would be attached to the *C*- and *N*-termini of the tetrazine linchpins to access more complex peptides and proteins.

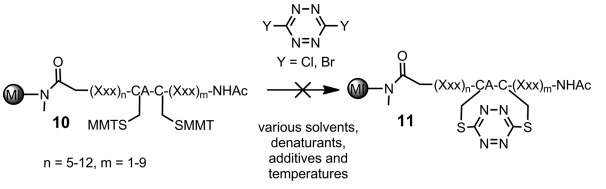

Figure 7. Attempted synthesis of lengthy peptides containing the *S*,*S*-tetrazine on solid support.

To prepare a tetrazine linchpin suitable for solution phase fragment union, the *N*-terminus of tripeptide **4a** inscribing the *S*,*S*-tetrazine was protected as the *tert*-butyl carbamate. With peptide linchpin **12** in hand, the *C*-terminus was then coupled to phenylalanine benzylamide employing standard Boc-based peptide solution-phase synthesis to furnish **13**. Following removal of the Boc protecting group (cf. **14**), the *N*-terminus was then coupled to *N*-α-Boc-*N*-ε-Cbz lysine to furnish **15** (Figure 8). Overall the yields were good (*ca.* 43% over 4 steps).

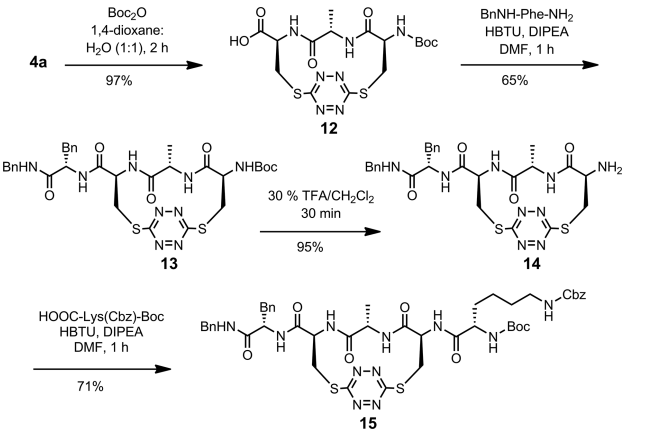

Figure 8. Use of a chromophore containing (*i*, *i*+2) peptide linchpin in a solution phase fragment condensation approach.

In summary, an effective synthetic protocol has been designed and validated to access a variety of peptide linchpins containing the *S*,*S*-tetrazine phototrigger. With the appropriate Boc protected tetrazine linchpins available from the corresponding (*i*, *i*+2), (*i*, *i*+3), and (*i*, *i*+4) peptide linchpins, access to a wide variety of peptide or protein scaffolds that contain the *S*,*S*-tetrazine photochemical trigger should be feasible. These constrained peptides with an (*i*, *i*+2), (*i*, *i*+3), and (*i*, *i*+4) relationship between the Cys residues present an avenue for irreversibly constraining a variety of common secondary peptide structural motifs, and ultimately the tertiary and quaternary structures of more complex proteins. Importantly, a variety of spectroscopic methods, such as IR probe and 2D IR spectroscopy, are now available to track conformational transitions on the pico- to nanosecond time scale, as the designed peptides, at some known displacement from the equilibrium state, relax to new equilibrium conformations upon the triggering event.

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Supporting Information Available. Details of the initial photophysical studies, and the preparation and spectroscopic characterization for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

(16) For synthesis of 3,6-dibromotetrazine refer to Supporting Information.

(17) Alternative protocols to remove the Fmoc protecting group including morpholine, triaminoethylamine, DBU, and TBAF were unsuccessful.

(18) Evidence for displacement of bis-(alkylthio)-tetrazine: Panek, J. S.; Zhu, B. *Tetrahedron Lett.* **1996**, *45*, 8151–8154.

The authors declare no competing financial interest.