Phototriggering

DOI: 10.1002/ange.201000500

Tetrazine Phototriggers: Probes for Peptide Dynamics**

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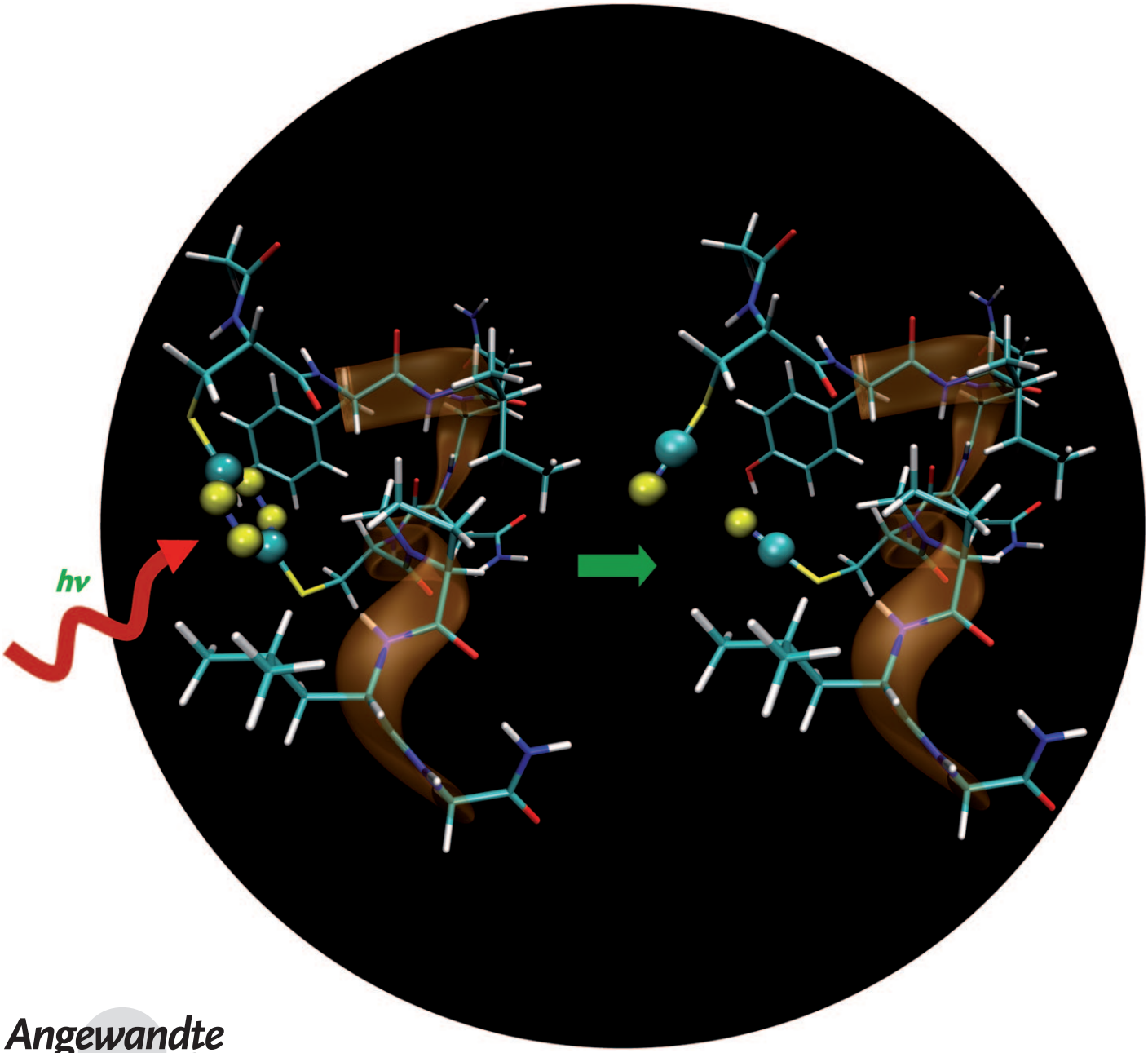
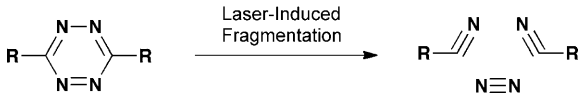


Ultrafast photochemical triggers hold the promise of providing information on the dynamics of peptide and protein folding.[1] Prior to photolysis, bonding to the trigger constrains the peptide to have a narrow structure distribution. Photochemical triggering releases the constraints and thus permits the molecule to evolve to a different equilibrium distribution. The structure evolution, even when ultrafast, can be followed by infrared probe or two-dimensional infrared spectroscopy. Fast phototriggering can thus reveal early kinetic events in protein dynamics by providing a means to explore the free-energy landscape of folding and misfolding. Several phototriggers have been developed for this purpose,[1,2] but there remain significant challenges. For example, disulfide bonds in peptides can be used as a phototriggers. Deep UV light severs the disulfide bond and releases the structural constraints; such experiments have been carried out in short helical peptides,[1a,b] cyclic peptides,[1d] and β hairpins.[1c] Although disulfide photolysis offers the capability of initiating ultrafast structure equilibration, limitations preclude the broad generality of this technique. Homolytic S–S bond scission reveals two reactive radicals that can undergo geminate recombination, as well as reactions with protein side chains. Moreover, the UV excitation required to dissociate the disulfide bond also excites the peptide backbone. Another example is azobenzene, which undergoes fast, reversible photoisomerization. When azobenzene is designed into a peptide, a light pulse can be used to cause the system to shift reversibly between significantly different equilibrium configurations.[3]

To optimize the knowledge gained on the early events in peptide/protein folding and unfolding, the triggering process should 1) be initiated on the picosecond timescale from a narrow structure distribution near equilibrium and be faster than the early events in conformational reorganization, such as single-bond rotations, hydrogen-bond formation, or helix nucleation;[4] 2) have a high photochemical yield; 3) produce inert products with negligible side reactions along the photolysis pathways; 4) be biocompatible for ease of incorporation into peptides and proteins; and 5) be photochemically accessible to nondamaging light-pulse frequencies. *s*-Tetrazine and its congeners are a class of compounds that fulfill most of these requirements. For example, tetrazine photoproducts are relatively inert, unobtrusive nitriles and molecular nitrogen (Scheme 1).[5] Furthermore, the $n\rightarrow\pi^*$ transition at 532 nm permits excitation in the visible region of the spectrum with a photoproduct yield, in the case of the parent *s*-tetrazine, close to unity in the vapor phase.[6] The quantum yields and light-induced pathways are environmentally sensitive,[7] and it is well-established that the photoreaction of *s*-tetrazine occurs on the ground state following internal conversion.[8]



Scheme 1. Photofragmentation of *s*-tetrazines.

With these observations as background, we report herein the potential of incorporating the *s*-tetrazine structural motif in peptides as an ultrafast photochemical trigger to initiate early events in peptide/protein folding. Although the overall photochemical yield of substituted *s*-tetrazines has been suggested to be lower in the condensed phase than in the gas phase,[5,9] the variation of yield with substitution has not been examined systematically. Our initial investigations therefore focused on the condensed-phase photophysical properties of the parent *s*-tetrazine along with dimethyl and diphenyl 3,6-substituted *s*-tetrazines as model compounds to guide the development of amino acid based systems.

The photochemical quantum yield for this series of 3,6-disubstituted tetrazines was measured in the condensed phase by nanosecond flash photolysis; the bleach signal of the parent molecules was observed at around 542 nm after a 1 ms delay, when all photochemical processes were complete. The photolysis yield per shot was determined from the ratio of ΔOD, the optical density of the bleach signal, and OD(0), the optical density before irradiation at 532 nm. The mechanism of the photodissociation of simple tetrazines involves a barrier crossing on the electronic ground state.[8] The parent molecule, *s*-tetrazine (R = H), dissociates on the subnanosecond timescale with a high photolysis yield (ca. 90%). As substituents are attached to the tetrazine, the number of modes accessible after internal conversion is greatly increased, and the photolysis yield is decreased as a result of vibrational-energy redistribution. For example, in the case of 3,6-dimethyl-*s*-tetrazine (R = Me), the yield is decreased to 18%. This trend continues for 3,6-diphenyl-*s*-tetrazine (R = Ph), for which the yield is further decreased to 1–2% owing to the involvement of the ring modes of the phenyl groups in the vibrational-energy distribution after internal conversion. Because of this overall reduction in the yield by substitution, photodissociation reactions with this mechanism are not useful for triggering relaxation in peptide systems.

A variety of heteroatom-substituted 3,6-tetrazines were evaluated in an effort to identify substituents that would lead to increased photoproduct yields. Pleasingly, acceptable photolysis yields of 24% at 355 nm and 12% at 410 nm were observed for 3,6-bisthiobenzyl-*s*-tetrazine (R = SBn) upon flash photolysis. The sulfenyl substitution causes the appearance of an additional band centered at 410 nm, which has been attributed to either a separate $n\rightarrow\pi^*$ transition[10] or charge transfer involving sulfur. This band is particularly useful because it presents the opportunity to initiate photolysis with a frequency-doubled Ti:sapphire laser pulse at approximately 400 nm that is not absorbed by a peptide or

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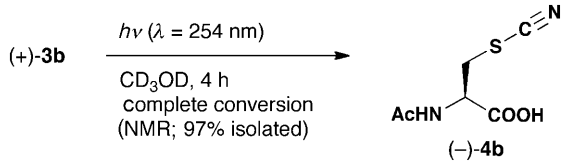
[**] This research was supported by the NIH; instrumentation was developed at the Research Resource (NIH P41RR001348 and RR001348S).

Supporting information for this article, including detailed synthetic procedures, full characterization data for all new compounds, and a description of the methods employed to obtain the photophysical data, is available on the WWW under http://dx.doi.org/10.1002/anie.201000500.

protein. Although the heavy-atom effect,[11] whereby the sulfur atoms slow down the energy flow out of the tetrazine ring, may offer an explanation for the effect of the sulfur atoms on the yield, it is considered more likely that new photochemical pathways are opened up by sulfur substitution. An absorption band observed in the transient spectrum of 3,6-bisthiobenzyl-*s*-tetrazine at around 510 nm for up to 1 μs, with a lifetime dependent on the oxygen concentration, is attributed to the triplet–triplet absorption.[12] Yuasa and Fukuzumi observed intersystem crossing in 3,6-diphenyl-*s*-tetrazine derivatives and demonstrated that the triplet state is photostable.[13] The photoproducts in the present examples are therefore believed to be formed through the singlet manifold, with triplet formation being a nonproductive pathway. The fluorescence lifetime for 3,6-bisthiobenzyl-*s*-tetrazine was determined to be 585 ps, which suggests photolysis on this timescale. Having identified a favorable tetrazine scaffold, the next step in designing a trigger suitable for incorporation within a peptide was to develop an amino acid based tetrazine derivative in which the tetrazine trigger was incorporated between two cysteine groups.

Previous synthetic studies demonstrated that the reaction of electrophilic dichloro-*s*-tetrazine (**1**)[14] with thiolates favors the symmetrical bissubstituted derivatives.[15] The key to reproducibly high yields of tetrazine (−)-**3a** (Scheme 2) was the exclusion of ambient light. This reaction can also be conducted under aqueous conditions by using 1,4-dioxane as a cosolvent to provide (+)-**3b**. The introduction of the amino acid based substituents did not significantly perturb the photochemical yield of the tetrazine fragmentation reaction: a photoproduct yield of 11% was observed upon flash photolysis of (−)-**3a**. A linear dependence of the photolysis yield on the irradiation intensity was found at lower energies (< 10 mJ), whereas the yield saturates at higher energies as a result of two photon-absorption processes that were not studied in detail.

We next sought to verify that the photofragmentation produced the anticipated thiocyanate product (−)-**4b** (Scheme 3). The steady-state photolysis was conducted with



Scheme 3. Photolysis of (+)-**3b**.

$\lambda = 254$ nm owing to the higher absorbance of (+)-**3b** at this wavelength. The photolysis of (+)-**3b** was monitored by ^{1}H and ^{13}C NMR spectroscopy (Figure 1), which indicated quantitative conversion into (−)-**4b**, as exemplified by the disappearance of the ^{13}C signal for the tetrazine ring with the concomitant appearance of the SCN resonance at $\delta_C = 113$ ppm.[16]

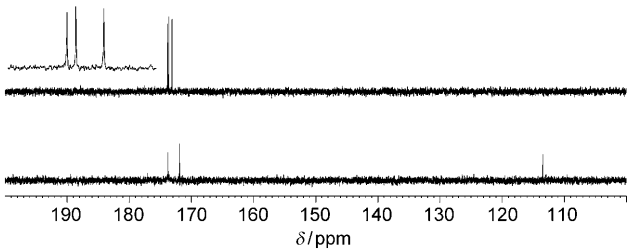
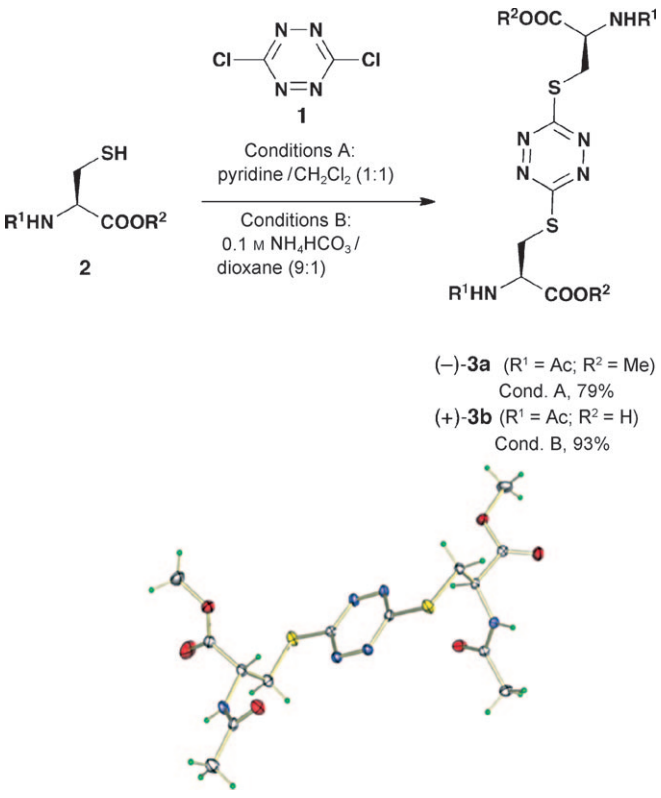


Figure 1. ^{13}C NMR ($\delta = 200$–100 ppm) spectroscopic monitoring of the steady-state photolysis of (+)-**3b** (20 mg mL^{-1}, CD_3OD, quartz NMR tube). Top: ^{13}C NMR spectrum of the starting material (+)-**3b**; bottom: ^{13}C NMR spectrum after irradiation for 4 h (complete conversion into (−)-**4b**).

The fluorescence of tetrazine (−)-**3a** decays mainly within a lifetime of 851 ps (with a few-percent component of 1.67 ns). To further investigate the timescale of photolysis, we performed femtosecond transient IR measurements; the results demonstrated that the ester carbonyl modes of (−)-**3a** are sensitive to photolysis. The transient difference spectrum obtained after a 1 ns time delay was similar to the difference spectrum obtained after complete photolysis from the stationary spectrum of (−)-**4b** (Figure 2). The IR transient absorption peaks at 1765 and 1745 cm^{-1} arise from the ester absorption after photolysis of the molecule, whereas the bleach signals at 1730 and 1755 cm^{-1} are due to the removal of parent molecules. These results demonstrate that the photolysis occurs within 1 ns.

The thiocyanate vibrational mode of the photoproduct was monitored at different delays between the optical pump



Scheme 2. Synthesis of cysteine-derived tetrazines and X-ray crystal structure of (−)-**3a**.

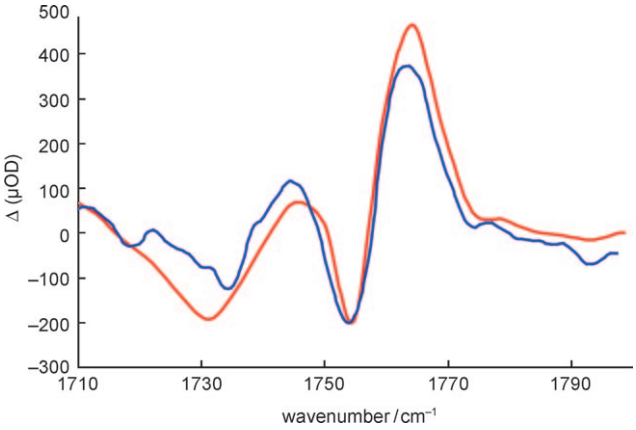


Figure 2. Carbonyl ester region of the femtosecond IR transient absorption spectrum of (−)-**3a** in CD_3OD (blue), and stationary FTIR spectrum of the photolysis product AcHN-Cys(SCN)-COOMe (red).

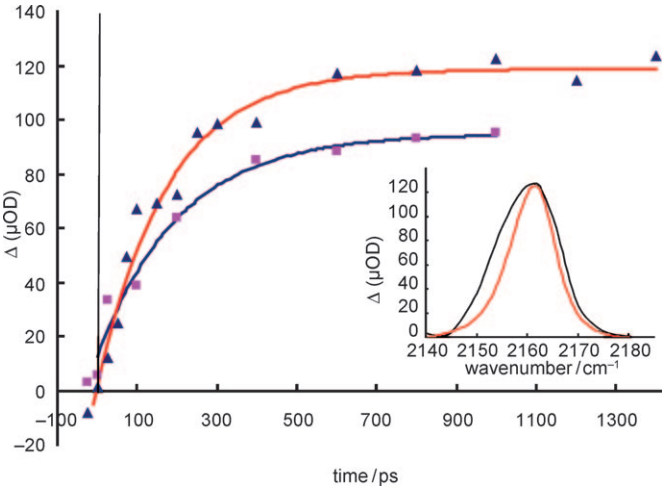


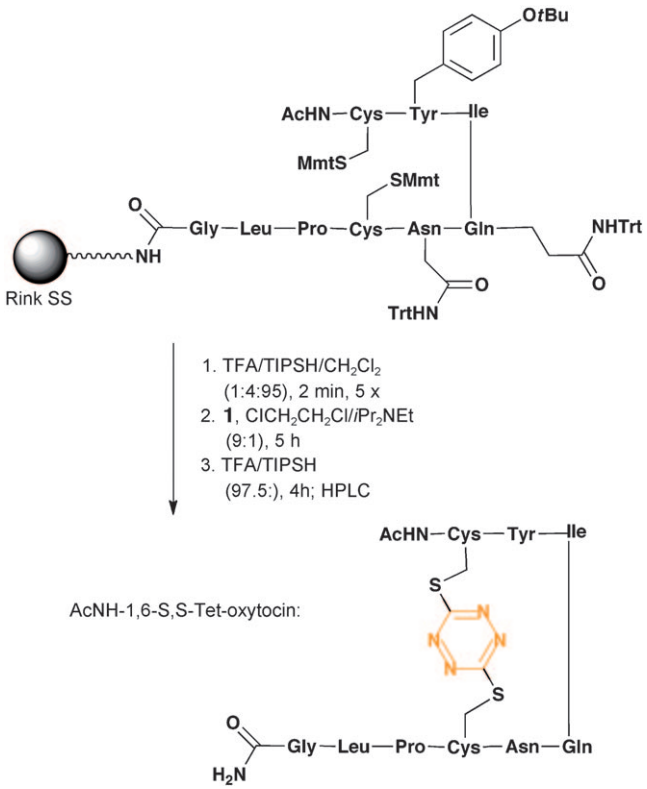
Figure 3. Time-dependence spectra of the SCN transient absorption following the photolysis of (−)-**3a** (triangles: raw data; red line: fit) and AcNH-1,6-S,S-Tet-oxytocin (squares: raw data; blue line: fit) in $CHCl_3$/MeOH (95:5 v/v). Inset: SCN region of the femtosecond IR transient absorption spectrum of (−)-**3a** at a delay of 1 ns (black), and FTIR absorption band of the SCN stretching frequency after steady-state irradiation of (−)-**3a** at 400 nm (red). The asymmetry of the transient absorption peak is not physically meaningful, but is caused by the detector.

and infrared probe. The transient absorption spectrum used to monitor the photolysis of (−)-**3a** in the SCN region contained one peak at 2163 cm^{-1} at 1 ns (inset of Figure 3, black curve). The peak corresponds to the 2163 cm^{-1} band observed in the FTIR spectrum of the compound after irradiation at 400 nm (inset of Figure 3, red curve). The thiocyanate signal was estimated from the number of photons and the photochemical yield of 166 μOD on the assumption that two identical thiocyanates with similar stationary lineshapes formed upon photolysis. The observed thiocyanate signal corresponded to 120 μOD at the 2163 cm^{-1} peak at a time delay of 1 ns for the photolysis of (−)-**3a**. However, the predicted and measured integrated absorption are in close agreement, which suggests that indeed two thiocyanates are formed. The thiocyanate transient at the peak maximum was fit to an exponential growth (Figure 3), and the time constant was determined to be 177 ps for (−)-**3a**. The rate constant corresponds to a rate of conversion that is fast relative to the S_1 lifetime, which suggests that the dissociation to produce the SCN groups occurs from a highly excited state.

Having validated that the tetrazine fragmentation reaction occurs within a few hundred picoseconds upon photolysis, we developed a method to insert the trigger within a cyclic peptide system. The peptide oxytocin was chosen as a readily available model system. Initial efforts to reduce the disulfide of the native peptide with immobilized tris(2-carboxyethyl)phosphane (TCEP), followed by treatment under the aqueous conditions for tetrazine insertion, proved irreproducible. To overcome these limitations, we developed a strategy for solid-phase peptide synthesis in which the orthogonal MMT (*para*-methoxytrityl) protecting group was used to mask the pivotal thiol groups (Scheme 4). Standard techniques were used to construct the peptide, with the N terminus capped as the acetamide. Removal of the Mmt groups liberated the thiol groups of the Cys residues within the resin-bound peptide. Subsequent treatment with **1** introduced the tetrazine bridge. The pseudodilution effect induced by immobilizing the peptide precludes the formation of tetrazine-bridged peptide dimers. Deprotection and cleavage from the resin under standard conditions, followed by purification,[17] then provided the target peptide: AcNH-1,6-S,S-Tet-oxytocin.

Detailed NMR spectroscopic studies support the location of the tetrazine ring between the two cysteine side chains. Assignment of the amide NH resonances and calculation of the $^3J_{NH,H^\alpha}$ values permitted simulation of the peptide backbone angles.[18] The position of the tetrazine bridge was further refined by analysis of the ^3J coupling constants between the well-resolved Cys(H^α) and Cys(H^β) resonances. This analysis permitted assignment of the tetrazine-bridged peptide structure as illustrated in Scheme 4. Details of this analysis and representative peptide structures for the lowest-energy conformational families are provided in the Supporting Information.

Having developed an effective method to insert a tetrazine trigger within a peptide construct, we next evaluated the photochemical properties of this system within the constrained cyclic peptide. The fluorescence decay of AcNH-1,6-S,S-Tet-oxytocin displays three components: a fast component at 200 ps (66 % of the amplitude) and two slower components at 1.09 ns (26 %) and 4.09 ns (8 %). The large contribution of the 1 ns component is likely to be from another conformer, as suggested by the NMR spectroscopic analysis. The rate of formation of the SCN stretching peak upon photolysis was again measured by femtosecond transient IR spectroscopy, and the thiocyanate transient was fit to a single exponential with a time constant of 207 ps for AcNH-1,6-S,S-Tet-oxytocin (Figure 3). The quantum yield of the model compound, (−)-**3a**, is dependent on the excitation wavelength. There is a twofold increase in SCN transient absorption upon excitation at 355 nm as compared to excitation 400 nm. Clearly, the photochemical transformation occurs from unrelaxed states following excitation.



Scheme 4. Solid-phase synthesis of AcNH-1,6-S,S-Tet-oxytocin. TFA = trifluoroacetic acid, TIPS = triisopropylsilyl, Trt = triphenylmethyl, MMT = *para*-methoxytrityl.

In summary, this study demonstrates that a disulfenyl tetrazine system can be readily incorporated within a bisthiolate peptide motif by treatment with **1**. The photolysis rate of this new phototrigger was measured directly by femtosecond transient absorption to occur on the picosecond timescale; thus, this method provides rapid access to non-equilibrium states. As far as we can ascertain, the two SCN groups are formed at the earliest measured times (ca. 25 ps). The photochemical yield upon flash photolysis is wavelength-dependent: 22% at 355 nm. The photolysis can be carried out to complete conversion by standard photochemical irradiation ($\lambda = 256$ nm). The principal advantages of the disulfenyl tetrazine triggering system are that the photolysis can be initiated with light of 330–400 nm or at lower yield with visible irradiation, which makes the tetrazine triggering system biocompatible. Upon incorporation into a cyclic peptide system, the photolysis rate and photolysis yield of the tetrazine trigger are similar to those of acyclic versions (e.g., (−)-**3a**). Thus, the mechanism of dissociation is not influenced by rigidity of the peptide system or the presence of other side-chain functionalities. Furthermore, site-selective $^{13}C{=}^{18}O$ replacements in the peptide backbone will permit FTIR or 2D IR tracking at the residue level[19] of the resulting peptide structure.

Received: January 28, 2010
Published online: April 9, 2010

Keywords: IR spectroscopy · peptides · photolysis · phototriggers · tetrazines

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