Published in final edited form as:

Chembiochem. 2011 July 25; 12(11): 1712–1723. doi:10.1002/cbic.201100204.

Bis-azobenzene cross-linkers for photo-control of peptide structure

Dr. Subhas Samanta and

Department of Chemistry, University of Toronto, 80 St. George St., Toronto, ON, M5S 3H6, Canada

Prof. G. Andrew Woolley

Department of Chemistry, University of Toronto, 80 St. George St., Toronto, ON, M5S 3H6, Canada, awoolley@chem.utoronto.ca, telephone: (416) 978-0675, fax: (416) 978-8775

Abstract

Cross-linkers that undergo large changes in length upon photoisomerization may produce large conformational changes, and thereby functional changes, in biomolecules. We have designed and synthesized extended and rigid bis-azobenzene cross-linkers, 4,4'-bis(4-(2-chloroacetamido)phenyl)diazanyl biphenyl (BPDB) and the water-soluble sulfonated analog 4,4'-bis(4-(2-chloroacetamido)phenyl)diazanyl biphenyl-2,2'-disulfonate (BPDBS). These photo-switches can produce a minimum ~ 5 Å and a maximum ~ 23 Å end-to-end distance change upon trans/cis isomerization. They have high absorption coefficients (45-60,000 M⁻¹cm⁻¹) and can produce up to ~80% cis isomers under favorable conditions. The photo-switching behavior of BPDBS cross-linked peptides was found to be highly dependent on the cross-linker attachment site. Upon UV (365 nm) irradiation, a significant decrease in α -helix content was observed for peptides that were cross-linked with BPDBS via Cys residues at i, i+19, and i, i+21 positions. In contrast, a large increase in α -helix content was exhibited by i, i+11 cross-linked peptides. BPDBS thus constitutes a particularly bright and effective photoswitch for biomolecule photo-control.

Keywords

azobenzene; peptide; conformation; photo; control

Intramolecular cross-linking of peptides and proteins can stabilize secondary structures, enhance protease resistance, and improve cell penetration ability. ^[1, 2] These features of cross-linked (“stapled”) peptides have encouraged their development as peptide-based drugs, for targeting cancer, HIV, and other diseases. ^[2, 3] When the cross-linker contains an azobenzene moiety, the conformation of the peptide or protein can be altered reversibly using light as an external stimulus. ^[4, 5] Azobenzene-based photo-switchable cross-linkers have been used to photo-control peptide affinity for protein and DNA targets, to control protein folding, and to control enzyme activity. ^[6, 7]

The trans and cis isomers of azobenzene differ substantially in ground state stability with the trans isomer being ~10-12 kcal/mol more stable. ^[8] This large difference in stability can, in principle, drive large changes in the conformations of target biomolecules. For such changes to be realized however, the conformational change of the azobenzene unit must be effectively coupled to peptide or protein folding/unfolding coordinates. Most studies have used the end-to-end distance change that occurs upon azobenzene isomerization as the

means to drive conformational transitions. Consequently, azobenzene derivatives that show large end-to-end distance changes are desirable as switches in a variety of systems.

BSBCA (3,3'-bis(sulfonato)-4,4'-bis(chloroacetamide)azobenzene) (Scheme 1), a water soluble diamidoazobenzene derivative,^[9] has been used to cross-link peptides through Cys residues spaced at $i, i+4$; $i, i+7$ and $i, i+11$ positions.^[5] The first two cross-linking sites lead to an increase in α -helix content upon irradiation, while cross-linking at the $i, i+11$ position shows decreased α -helix content in the irradiated state.^[10-12] For the BSBCA cross-linker, the end-to-end distance change upon photoisomerization is ca. 3 Å.

We recently reported a long and rigid *para*-substituted phenylene ethynylene azobenzene DDPBA (Scheme1), which undergoes a ~13 Å end-to-end distance change upon photoisomerization, however, this derivative gave only ~15% *cis* isomer in the irradiated state under standard experimental conditions.^[13] Hecht and colleagues^[14] reported a *meta*-substituted phenylene ethynylene azobenzene that exhibited better photoswitching properties but *meta*-substitution (vs. *para*-substitution) leads to a much larger range of predicted end-to-end distances for the *trans* isomer, complicating application as a photoswitch for peptides and proteins. Moroder and colleagues designed an extended azobenzene derivative containing alkyne moieties at the *para* positions^[15] and a related derivative was reported by Kuil et al^[16]. This switch was successful at photo-controlling conformations of collagen-like peptides and SH2 domain binding peptides despite have a flexible methylene unit as part of the cross-linker.^[15, 16] Standaert and Park extended azobenzene by incorporating biphenyl groups. Molecular modeling revealed a difference of 13 Å between the end-to-end distances of *trans* and *cis* isomers of a derivative designated mpAbc,^[17] however this has yet to be applied as a conformational switch in biomolecules.

In an attempt to couple a high yield of photoisomerization to a relatively large end-to-end distance change, we have now designed two bis-azobenzene derivatives: 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl (BPDB) and the sulfonated analog 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl-2,2'-disulfonate (BPDBS) (Scheme 1). Cyclic bis-azo compounds have been studied previously as components of photoswitchable ion chelating systems and cyclic supramolecules.^[18] Linear bis-azo compounds similar to the ones reported here with amido substituents at the *para* positions have been reported in the patent literature as coloring agents (*e.g.* Vulcan Fast Yellow, Pigment Yellow 5K^[19]). In addition, Matějeka & Dušák reported photochromic polymers containing amide substituted linear bis-azo compounds.^[20] Also, Cisnetti et al reported bis-azo compounds where the central benzene ring was shared between a pair of azobenzene units.^[21] Photoisomerization of an azo unit was found to depend on the isomeric state of the other azo unit, particularly in the case of the *para* substituted derivative. To our knowledge there are no reported applications of bis-azo compounds as conformational switches for biomolecules.

The presence of two azobenzene chromophores means that upon UV irradiation either one or both azo units may isomerize - a feature we expected might complicate the interpretation of the conformational response of stapled peptides or proteins. On the other hand, these bis-azobenzenes were expected to exhibit high molar extinction coefficients, and we hoped, might produce large percentages of *cis*-isomers upon irradiation. Moreover, their rigidity is expected to lead to substantial end-to-end distance changes even if only one of the two azobenzene chromophores isomerizes (see below). We report here the synthesis and characterization of these bis-azobenzene photo-switches and their effects on helical peptide structures when incorporated via Cys residues spaced at $i, i+21$; $i, i+19$; $i, i+14$ and $i, i+11$ positions.

Results and Discussion

Cross-linker design

The structures of the designed bis-azobenzenes are shown in Scheme 1. The all-trans forms of both BPDB and BPDBS have a length nearly double that of the well-studied cross-linker BSBCA. The two azobenzene chromophores are linked by a biphenyl unit and the ends are functionalized with an α -chloroacetamido group for covalent attachment of these cross-linkers to peptides via Cys residues. These features are intended to introduce the minimum number of single bonds between the azo bonds that isomerize and the peptide backbone in order to maximize the degree of conformational coupling. The compounds are symmetrical so that only one chemical species is formed upon cross-linking. The incorporation of two sulfonate groups in BPDBS *meta* to the azo moieties not only increases the water solubility of the cross-linker without directly altering the absorption spectrum, but also inhibits coplanarity of the two aromatic rings of core biphenyl moiety so that each azobenzene chromophore is expected to behave independently. The biphenyl torsion angle in a crystal of the bis-azo dye Congo red varies between zero and 25° [22], whereas Mal et al reported *ortho*-sulfonated *para*-amino-substituted biphenyl compounds in which this angle is near 90° . [23]

We used molecular dynamics simulations to estimate the end-to-end distances of *trans-trans*, *trans-cis* and *cis-cis* isomers of the BPDB cross-linker. Simplified models for *trans-trans*, *trans-cis* and *cis-cis* conformers attached to peptides *via* Cys residues were built in which the β -position of the cysteine side-chain was replaced by a methyl group (Fig. 1). The thermodynamically most stable *trans-trans* form exhibited an end-to-end (sulfur-to-sulfur) distance of ca. 27-30 Å. In the *trans-cis* form, this distance varied between 15-25 Å. Rotation around the single bonds linking the azo units to the biphenyl core in the *cis-cis* isomers can produce two conformational families (*cis-cis(l)*, *cis-cis(s)*) with very different end-to-end distances so that, overall, the *cis-cis* form shows a very broad S-S distance distribution in the range of 4.5-25 Å. The most probable end-to-end distances for *trans-trans* and *trans-cis* isomers were approximately 28.5 Å and 21 Å, respectively, while approximately 20 Å and 6 Å were observed for *cis-cis (l)* and *cis-cis (s)* isomers, respectively.

The conformational properties of these bis-azobenzene cross-linkers are thus significantly more complicated than the single azobenzene cross-linkers used previously, a feature that complicates prediction of conformational effects on target biomolecules. Nevertheless, substantial end-to-end distance changes are possible upon isomerization with these compounds, and even the *trans-trans* to *trans-cis* transition can produce a larger end-to-end distance change than the BSBCA cross-linker.

Synthesis of bis-azobenzene cross-linkers

To investigate the photoswitching behavior of both sulfonated and non-sulfonated cross-linkers, we prepared BPDB(r) and BPDBS(r) as reference compounds. The synthetic protocol for the preparation of BPDB and BPDBS as well as BPDB(r) and BPDBS(r) is shown in Scheme 2. Benzidine derivatives, **2a** and **2b**, were first tetrazotized using NaNO_2 and dilute HCl. The tetrazonium solutions were then reacted with the sodium salt of α -anilinophenylmethane sulfonate (**3**), prepared by the reaction of aniline with formaldehyde in presence of NaHSO_3 . [24] Hydrolysis of the resulting coupled products using NaOH led to 4,4'-bis((4-aminophenyl) diazenylbiphenyl (**1a**) and 4,4'-bis(4-aminophenyl)diazenylbiphenyl-2,2'-disulfonate (**1b**), respectively. The chloroacetamide derivatives BPDB and BPDBS were prepared from **1a** and **1b** using chloroacetic anhydride in the presence of pyridine-diethyl ether, and chloroacetyl chloride in the presence of

aqueous Na_2CO_3 . Similarly, the acetyl derivatives BPDB (r) and BPDBS (r) were generated from their precursor amines **1a** and **1b** by reaction with acetic anhydride in the presence of pyridine, and aqueous Na_2CO_3 solution, respectively.

UV-Vis spectra and photo-switching of bis-azobenzene cross-linkers

UV-Vis absorption spectroscopy was used to characterize the properties of the reference bis-azobenzene cross-linkers. BPDB(r) showed a strong absorption maximum near 400 nm in DMSO, whereas BPDBS(r) showed a maximum near 380 nm in DMSO and 360 nm in 10 mM sodium phosphate buffer, pH 7.0. (BPDB(r) is not soluble in water). These absorptions are typical of $\pi-\pi^*$ transitions in amide-substituted *trans*-azobenzenes. [25] The molar absorption coefficient was determined to be $60,000 \text{ M}^{-1}\text{cm}^{-1}$ for BPDB at 400 nm in DMSO, $52,000 \text{ M}^{-1}\text{cm}^{-1}$ for BPDBS at 380 nm in DMSO, and $45,000 \text{ M}^{-1}\text{cm}^{-1}$ for BPDBS at 360 nm in sodium phosphate buffer, pH 7.0. These values are substantially higher than that for BSBCA ($27,000 \text{ M}^{-1} \text{ cm}^{-1}$). [26] The 20 nm blue shift in the absorption maximum and the lower molar extinction coefficient of BPDBS(r) compared to BPDB(r), suggest a lack of electronic communication between the two azo chromophores in BPDBS(r) consistent with steric interactions between the two sulfonate groups at 2 and 2' positions inhibiting coplanarity of the two phenyl rings in the biphenyl core.

When BPDB(r) in DMSO was irradiated with 400 nm light, a photostationary state was obtained in which the absorbance at 400 nm decreased by ~52% (Fig. 2(A)). When BPDBS(r) was irradiated with light of 380 nm (in DMSO) or 365 nm (in 10 mM phosphate buffer, pH 7.0), the absorption maxima decreased by approximately 70% and 83% respectively. New absorption bands with maximum near 450 and 330 nm in DMSO and near 435 and 320 nm in phosphate buffer appeared (Figure 2(B)). We expect that the UV-Vis spectra of *trans-cis* isomers are similar to those of mixtures of *trans-trans* and *cis-cis* isomers so that it is not possible to deconvolute these spectra of irradiated solutions into spectra of individual isomers. Nevertheless, a decrease of more than 50% of the absorption intensity of the *trans-trans* isomer suggests the appearance of some fraction of *cis-cis* isomer along with the *trans-cis* isomer. The thermal decay of irradiated BPDBS(r) in phosphate buffer (pH 7.0) was measured by following the recovery of absorbance at 365 nm as a function of time. Mono-exponential fits of the decay curves gave half-lives of approximately 43 min at 20°C and 11 min at 37°C (Table 1). These half-lives are nearly equal to that exhibited by BSBCA (35 min at 25°C and 12 min at 37°C) [26], and are shorter than that observed for DDPBA (75 min at 20°C and 10 min at 40°C) [13].

NMR analysis of bis-azobenzene cross-linkers

To test whether *cis-cis* isomers of the bis-azobenzene cross-linkers form upon irradiation, we carried out ^1H NMR for BPDB(r) in $\text{DMSO}-d_6$, and BPDBS(r) in D_2O and $\text{DMSO}-d_6$ (Figure 3). Irradiated solutions of both cross-linkers showed four acetamido methyl signals, which are denoted by Me_{tt} , Me_{tc} , Me_{ct} and Me_{cc} in Figure 3. The Me_{cc} and Me_{tt} signals arise from the two homotopic methyl groups of *cis-cis* and *trans-trans* bis-azobenzenes respectively, whereas the *trans-cis* isomer produces two distinct methyl signals of equal intensity - Me_{tc} due to the methyl group nearer the *trans* azo group and Me_{ct} due to the methyl group nearer the *cis* azo group (Fig. 3). Only a small amount of the *cis-cis* isomer (Me_{cc}) was formed upon irradiation of BPDB(r) (Fig. 3(B)), while irradiation of BPDBS(r) produced a significant amount of the *cis-cis* isomer, particularly in D_2O where a ratio of *trans-trans* : *trans-cis* : *cis-cis* of 1 : 3.2 : 3.3 was reached (Fig. 3(D)). This result is consistent with the UV-Vis data presented above (Fig. 2) indicating that the largest fraction of *cis* isomers is formed with BPDBS in aqueous solution. Irradiation of the concentrated NMR sample leads to less efficient production of *cis* isomers than in diluted solutions used for recording UV-Vis spectra (Fig. 2) so that irradiation times are longer. We note that although

the two azo double bonds are indistinguishable in the reference compounds, they are distinct when part of a cross-linked peptide or protein. Thus a *trans/cis* cross-linked peptide may differ in stability from a *cis/trans* cross-linked peptide or protein (see below).

Owing to the substantially better switching behavior exhibited by the sulfonated derivative, together with its water solubility, we opted to use this compound to test effects of photoisomerization on the conformations of attached peptides.

Design and analysis of bis-azobenzene cross-linked peptides

Using predicted end-to-end distance distributions of the bis-azobenzene cross-linkers (Fig. 1) as preliminary tool, we designed and synthesized four peptide sequences designated FZ21, SS19, SS14, and SS11 where each peptide contained two Cys residues for installing the cross-linker (Figure 4). All of the peptides contain the general sequence Ac-(EAAAR)₃-A-NH₂ that is known to exist in a monomeric and predominantly helical form in aqueous solution. [10, 27] The spacing between Cys residues when the peptides are in a helical conformation is intended to match the end-to-end distance of the *trans-trans*, the *trans-cis/cis-trans* or the *cis-cis(I)* isomers. FZ21 with Cys residues spaced at *i* and *i*+21 positions, which was previously analyzed with the DDPBA cross-linker, [13] has a range of S-S distances from ~29 to 35 Å and was expected to accommodate the dark adapted *trans-trans* cross-linker although the optimal length of *trans-trans* BPDBS is at the extreme short end of this range. A better fit was predicted between the *trans-trans* isomer of the cross-linker and the *i*, *i*+19 peptide SS19 (S-S range 26.5 to 32.2 Å). In peptide SS14, the distance between S atoms of Cys residues spaced at *i* and *i*+14 positions is 19.2-24.5 Å when the peptide is in an ideal α -helical form. This range was expected to be compatible with the end-to-end distances of *trans-cis/cis-trans* and partly with the *cis-cis (I)* forms of the cross-linkers. The SS11 peptide was expected to be compatible with all *cis*-containing forms of the cross-linker (*trans-cis/cis-trans* and *cis-cis (I)*). Also included on the histogram (Fig. 4B) are S-S distance ranges for helical peptides with *i*, *i*+7 and *i*, *i*+4 Cys spacings. Peptides with these Cys spacings cross-linked with the BSBCA linker were studied in detail previously. [10, 11, 13, 28] While the S-S distance ranges of these peptides would be compatible with *cis-cis* forms of the bis-azo BPDBS cross-linker, the Cys residues are so close in the linear sequence that even if the unfolded peptide were fully extended, the S-S distance would be too short to accommodate an undistorted *trans-trans* form of the cross-linker. [10, 28] We note that this limitation would not be present in a folded protein where two Cys residues could be close together in three dimensions but far apart in the linear sequence.

Each of these peptides was reacted with the cross-linker BPDBS to generate the cross-linked peptides FZ21-BPDBS, SS19-BPDBS, SS14-BPDBS, and SS11-BPDBS. All the cross-linked peptides were HPLC purified and characterized by MALDI/ESI mass spectrometry. The absence of free Cys residues after cross-linking was checked with Ellman's reagent. [29]

Photoisomerization of bis-azobenzene cross-linked peptides

All BPDBS cross-linked peptides exhibited dark-adapted absorption spectra with maxima near 370 nm, and a shoulder in the range of 420-520 nm. SS19-BPDBS and FZ21-BPDBS had absorption maxima that were slightly red-shifted compared to SS11-BPDBS and SS14-BPDBS. Irradiation of all BPDBS cross-linked peptides with 365 nm light led to a significant decrease in absorbance near 370 nm but the magnitude of the decrease varied from peptide to peptide. Irradiation of FZ21-BPDBS produced a 49% reduction, irradiation of SS19-BPDBS produced a 40% reduction, irradiation of SS14-BPDBS produced a 59% reduction, and irradiation of SS11-BPDBS produced a 66% reduction (Fig. 5A) at 20°C. The magnitude of the absorbance change was temperature dependent with all the BPDBS cross-linked peptides showing larger absorbance changes at higher temperatures indicating a

larger fraction of cis isomers was produced. However, the magnitude of this effect varied from peptide to peptide. For instance, irradiation of SS19-BPDBS produced a significantly larger fraction of cis isomers at higher temperatures (Fig 5B) whereas irradiation of SS11-BPDBS produced a large fraction of cis isomers even at 4°C (estimated to be ~80% based on comparison with spectra for pure cis and trans isomers of BSBCA [28]) and this increased only slightly at higher temperatures (Fig. 5C). These results indicate that there are significant effects of peptide structure on the quantum yields for trans/cis photoisomerization of these cross-linkers.

The thermal reversion of all cross-linked peptides was followed by UV-Vis spectroscopy. Isosbestic points at approximately 249, 305 and 449 nm were observed (Fig. 5D shows the thermal relaxation of SS11-BPDBS as a representative case). As noted above, the *trans-cis* and *cis-trans* cross-linked peptides are distinct species. Thus, assuming only cis-to-trans isomerization can occur thermally, the process of thermal reversion may be represented as shown in Scheme 3.

The absorbance at 370 nm is given by:

$$A_{370\text{nm}} = \epsilon_{\text{trans-trans}}[\text{trans-trans}] + \epsilon_{\text{trans-cis}}[\text{trans-cis}] + \epsilon_{\text{cis-trans}}[\text{cis-trans}] + \epsilon_{\text{cis-cis}}[\text{cis-cis}]$$

Where $\epsilon_{\text{trans-trans}}$ is assumed to be 50,000 M⁻¹cm⁻¹ for BPDBS cross-linked peptides and $\epsilon_{\text{cis-cis}}$ can be estimated to be ~4000 M⁻¹cm⁻¹ based on the BSBCA case [28] and $\epsilon_{\text{trans-cis}}$ and $\epsilon_{\text{cis-trans}}$ are each approximately 25,000 M⁻¹cm⁻¹. Numerical simulations using Tenua 2.1 (<http://bililite.com/tenua>) [30] indicate that such a system can exhibit a variety of behaviors depending on the relative values of the rate constants k_1 , k_2 , k_3 , k_4 . However, if all rate constants are approximately equal or if $k_3 \approx k_4 > k_1 \approx k_2$, an approximately monoexponential increase in absorbance at 370 nm is expected. If instead, $k_1 \approx k_2 > k_3 \approx k_4$, or $k_1 \approx k_2, k_3 \approx k_4$, a biexponential increase is expected. Both behaviors were observed for different peptides at different temperatures (Fig. 6).

To simplify comparison with other cross-linkers, thermal reversion curves were fitted as monoexponential processes. All BPDBS cross-linked peptides showed half-lives for thermal reversion between 10-30 min at 20°C and 3-8 min at 37°C (Table 1). These thermal relaxation rates are faster than observed for DDPBA cross-linked peptides ($\tau_{1/2}$ = 102-159 min at 20°C and 37-54 min at 30°C) [13] and also for BSBCA cross-linked FK11 peptides ($\tau_{1/2}$ = 35 min at 25°C and 12 min at 37°C). [26] All cross-linked peptides showed faster thermal relaxation rates than the free cross-linker BPDBS(r) indicating that the peptide reduces the activation energy barrier by stabilizing the transition state for thermal reversion and/or destabilizing the cis isomers to varying extents depending on the cross-linker attachment sites.

Effects of photoisomerization on peptide conformations

The secondary structures of all the cross-linked peptides in dark-adapted and irradiated states were investigated using circular dichroism (CD) spectroscopy. Spectra for all cross-linked peptides before and after irradiation at different temperatures are presented in Figure 7. Helical contents were estimated as described in the experimental section.

The calculated end-to-end distance (S-S) of the *trans-trans* BPDBS isomer varies between 27-30 Å (Fig. 1). If FZ21 adopts an ideal α -helical structure, the S-S distances range from ~29 to 35 Å whereas the SS19 peptide has an S-S range of 26.5 to 32.2 Å. The dark-adapted BPDBS cross-linked FZ21 and SS19 peptides both show high helical content (Fig. 7, A,B), with that of SS19-BPDBS being somewhat higher consistent with a better match between

the *trans-trans* form of the cross-linker and the helix. The small mismatch in the FZ21-BPDBS case perhaps causes a reduction in helix content since in order to bridge the $i, i+21$ spacing, the BPDBS cross-linker, like the DDPBA cross-linker,^[13] must pack very closely to the FZ21 peptide surface and may thereby distort the helix geometry via steric interactions.

Irradiation of FZ21-BPDBS and SS19-BPDBS led to a decrease in α -helix content consistent with the prediction that irradiated states of the cross-linkers would be even less compatible with an α -helix structure. The decrease in helix content upon irradiation was temperature dependent; higher temperatures produced greater decreases in helix content (Figure 7A,B, Table 2), consistent with the observation that higher fractions of *cis* content were produced at higher temperatures (Fig. 5). Higher temperatures also decrease the lifetime of *cis* states (Table 1) so that recording full CD spectra becomes difficult. Instead, Figure 8 shows the CD change at 222 nm as a function of time after irradiation of SS19-BPDBS to produce *cis* isomers. Helix content is clearly diminished upon irradiation and then restored on a timescale reflecting thermal isomerization to that *trans* state.

Based on predicted end-to-end distance distributions (Fig. 4) we expected that the SS14-BPDBS peptide would show relatively low helix content in the dark (*trans-trans* form) but increased helicity upon irradiation to produce *trans-cis/cis-trans* isomers. The helix content of the dark-adapted SS14-BPDBS peptide was higher than expected and the changes in CD spectra upon irradiation were very small (Fig. 7C, Table 2). The small change may be due to the formation of *cis-cis* isomers which may be too short to be compatible with ideal helical structure.

The SS11 peptide contains Cys residues at i and $i+11$ positions and was expected to accommodate the *trans-cis/cis-trans* and *cis-cis* (l) cross-linker isomers. The observed helix content of the dark-adapted state of SS11-BPDBS, was <10 %, consistent with a poor match to the dark-adapted *trans-trans* isomer. Upon irradiation with 365 nm light, a large increase in the helix content of SS11-BPDBS was observed (>50%)(Fig. 7D, Table 2).

Moreover, unlike the other cross-linked peptides, irradiated SS11-BPDBS exhibited a larger change in helix content at lower temperatures, although a somewhat lower percentage of *cis* isomers is formed at lower temperatures (Figure 5C). This may be due to stabilization of α -helix structure as the temperature is lowered.

Stability of the cross-linked peptides in a reducing cellular environment

The cellular environment is reducing in nature due to the presence of small tripeptide glutathione in its reduced form in 1-10 mM concentration.^[31] We therefore tested the photo-switching behavior of BPDBS cross-linked peptides in phosphate buffer pH 7.0 at 37°C in the presence of 10 mM reduced glutathione. To mimic the conditions for using such peptides in cells in culture,^[7] cross-linked peptide was incubated with glutathione in the dark for 24 hours at 37°C. Photo-switching remained unaffected (data not shown), which suggests that BPDBS is inert to glutathione, and hence BPDBS-linked peptides should be functional in typical intracellular environments.

Summary

We have designed and synthesized biphenyl based diamidobisazobenzene cross-linkers BPDB and BPDBS, and examined their photoswitching behaviors both as free cross-linkers and when attached to a series of peptides. Compared to previously studied mono-azo cross-linkers BSBCA and DDPBA, these bis-azo species offer certain advantages for photo-control. First, the end-to-end distance change upon *trans*-to-*cis* isomerization is substantially

larger for the bis-azo species than the mono-azo BSBCA compound. Second, the fraction of *cis* isomers produced upon irradiation for the bis-azo species, particularly the sulfonated BPDBS version, is substantially greater than for DDPBA, reaching ~80 % in favourable cases. Third, the molar extinction coefficients are high ($>45,000 \text{ M}^{-1}\text{cm}^{-1}$) making these bright photoswitches.

As with previously studied azobenzene based cross-linkers, the new bis-azo species can be used to photocontrol peptide conformation and the effects on helical content of designed helical peptides can be approximately predicted by comparing the end-to-end distance distributions of the cross-linkers with Cys spacings in the target peptides. Because these bis-azo species are substantially longer than BSBCA, the extent of conformational change upon isomerization is necessarily larger. Compared to the mono-azo BSBCA cross-linker however, the interplay between cross-linker isomerization and peptide conformation is more complicated. First, we observed a strong dependence of the yield of *cis* isomers in the photostationary state on the nature of the peptide attachment. Whereas the SS11-BPDBS cross-linked peptide gave ~80 % *cis*, the SS19-BPDBS gave only ~50%.

A further complication of the bis-azo cross-linkers is that one cannot tell from the UV spectra what fraction of *cis* containing species are *cis-cis* versus *cis-trans/trans-cis*. Thus, for a case like the SS19-BPDBS peptide, it is unclear whether the decrease in helix content in the irradiated state reflects a mixture of very helical *trans-trans* species and very disordered *cis-cis* species, or a large fraction of *cis-trans/trans-cis* species of intermediate helicity. For a peptide or protein with a particular biological activity, these two scenarios would be expected to have very different functional consequences. The conformational response of an attached peptide or protein is thus likely to be case specific. In favorable cases however, such as SS11-BPDBS, substantial conformational change is possible. Peptides with Cys residues spaced $i, i+11$ in the sequence have been cross-linked with monoazo BSBCA cross-linker and studied in detail.^[11, 32] Such peptides are predominantly helical in the dark and undergo a substantial loss of helical structure upon UV irradiation. The SS11-BPDBS peptide shows precisely the opposite behavior as diagrammed in Figure 9. In the dark, the long bis-azo BPDBS cross-linker strongly inhibits helical formation. UV irradiation to produce *trans-cis*, *cis-trans*, and *cis-cis* peptides leads to increased helical content.

Experimental Section

Synthesis of the cross-linkers

Compounds **2a** (benzidine), **2b** (4,4'-diaminobiphenyl-2,2'-disulfonic acid) and aniline were purchased from Sigma-Aldrich, and were used. All ^1H and ^{13}C NMR spectra were recorded using a Varian Unity 400 or Bruker 400 spectrometer. High resolution mass spectra were obtained by electron spray ionization (ESI), and mass spectra of peptides were obtained by MALDI TOF mass spectrometry.

1) Synthesis of sodium α -anilinophenylmethanesulfonate (**3**)

Following a literature procedure^[24] formaldehyde (0.65 g, 21.5 mmol) was added to a solution of sodium bisulfite (2.2 g, 21.5 mmol) in water (12 mL). After stirring the reaction for 1 h at room temperature, aniline (2.0 g, 21.5 mmol) was introduced into the reaction and it was stirred for 6 h at 75°C. The reaction was first cooled to room temperature and then in an ice bath. The precipitate obtained was filtered, washed with cold water and dried *in vacuo*. The product, α -anilinophenylmethanesulfonate (2.4 g, 53%) was pure enough to use for further reaction. ^1H NMR (400 MHz, D_2O): δ = 4.45 (s, 2H), 6.87 (t, J = 7.4 Hz, 1H), 6.93 (d, J = 8.7 Hz, 2H), 7.30 (t, J = 8.7 Hz, 1H).

2) Synthesis of 4,4'-bis(4-aminophenyl)diazenylbiphenyl (1a)

To a round-bottomed flask containing benzidine, **2a** (0.8 g, 3.1 mmol) was added a mixture of ice (10 g) and concentrated hydrochloric acid (1.3 mL). A solution of sodium nitrite (0.46 g, 6.6 mmol) in water (2 mL) was added slowly with stirring at 0-5 °C. After 1 h of stirring at this temperature, the excess nitrous acid was consumed by adding urea. In parallel, a coupler solution was prepared by dissolving α -anilinophenylmethanesulfonate (1.6 g, 7.8 mmol) in 20 mL of water and stirred at 0-5 °C. The diazo solution was kept at 0-5 °C and was added dropwise with stirring. The pH of the reaction mixture was maintained in the range of 4 to 7 with an aqueous NaOAc solution. The resulting reaction mixture was then allowed to stir at 0-5 °C for 2 h and at room temperature for a further 12 h. After the reaction, the precipitate was removed by filtration and washed with water. The cake obtained was boiled together with a 20% aqueous NaOH solution for 2 h. The resulting precipitate was filtered, washed thoroughly with water until the washing water become neutral and dried *in vacuo* to yield **1a** (0.23 g, 20%) as an orange solid. ^1H NMR (400 MHz, DMSO- d_6): δ = 6.14 (br s, NH₂), 6.68 (d, J = 9.0 Hz, 4H), 7.69 (d, J = 8.7 Hz, 4H), 7.84 (d, J = 9.0 Hz, 4H), 7.90 (d, J = 8.7 Hz, 4H); ^{13}C NMR (100 MHz, DMSO- d_6): δ = 114.1, 123.1, 126.0, 128.1, 140.6, 143.7, 152.5, 153.7; ESI-HRMS: m/z calc'd for C₂₄H₂₁N₆: 393.1822 [M+H]⁺, found: 393.1807.

3) Synthesis of 4,4'-bis(4-aminophenyl)diazenylbiphenyl-2,2'-disulfonate (1b)

4,4'-Diaminobiphenyl-2,2'-disulfonic acid, **2b** (1.6 g, 4.6 mmol) was dissolved in water (20 mL) with sodium carbonate (0.49 g, 4.6 mmol), and a solution of sodium nitrite (0.76 g, 11.0 mmol) in water (1 mL) was added. This solution was added dropwise to a mixture of ice (30.0 g) and concentrated hydrochloric acid (2.0 mL), and the reaction was stirred at 0-5 °C for 1 h. Excess nitrous acid in the reaction mixture was decomposed with urea. To this tetrazonium solution was added dropwise an ice cold solution of α -anilinophenylmethanesulfonate (2.4 g, 11.5 mmol) in water (10 mL). After stirring the coupling reaction mixture for 2 h at 0-5 °C and at room temperature for 12 h, a 20% aqueous NaOH solution was added. The reaction was then heated at 80 °C for 2 h. After cooling to room temperature, the aqueous solution was saturated with NaCl, which led to the precipitation of the required product. This was filtered, thoroughly washed with aqueous saturated NaCl solution, and dried *in vacuo* to yield **1b** (1.2 g, 43% yield) as a red solid. ^1H NMR (400 MHz, DMSO- d_6): δ = 6.09 (br s, NH₂), 6.68 (d, J = 8.7 Hz, 4H), 7.42 (d, J = 8.4 Hz, 2H), 7.61 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 2H), 7.69 (d, J = 8.7 Hz, 4H), 8.24 (d, J = 2.0 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6): δ = 114.1, 120.5, 121.8, 125.8, 133.8, 139.7, 143.7, 146.3, 151.2, 153.5; ESI-HRMS: m/z calc'd for C₂₄H₂₁N₆O₆S₂: 553.0958 [M+H]⁺, found: 553.0942.

4) Synthesis of 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl, BPDB

To an ice cold solution of **1a** (80 mg, 0.20 mmol) in an ether/pyridine mixture (1:1, 5 mL) was added dropwise a solution of chloroacetic anhydride (105 mg, 0.61 mmol) in ether (2 mL). The resulting reaction was warmed to room temperature and allowed to stir for 1 h. The resulting precipitate was filtered and then washed with ether. The crude product was recrystallized from dimethylformamide/water to yield BPDB (78.8 mg, 70% yield) as orange solid. ^1H NMR (400 MHz, DMSO- d_6): δ = 4.32 (s, 4H), 7.84 (d, J = 9.0 Hz, 4H), 7.95 (d, J = 8.7 Hz, 4H), 7.99 (d, J = 9.0 Hz, 4H), 8.02 (d, J = 8.7 Hz, 4H), 10.67 (br s, NH); ^{13}C NMR (100 MHz, DMSO- d_6 at 60 °C): δ = 44.2, 120.5, 123.8, 124.4, 128.5, 142.1, 142.2, 148.9, 152.4, 165.7; ESI-HRMS: m/z calc'd for C₂₈H₂₃N₆O₂Cl₂: 545.1254 [M+H]⁺, found: 545.1269.

5) Synthesis of 4,4'-bis(4-acetamidophenyl)diazenylbiphenyl, BPDB(r)

To a solution of **1a** (33.0 mg, 0.08 mmol) in pyridine (2 mL) was added acetic anhydride (26.0 mg, 0.25 mmol) dropwise in ice cold conditions. The reaction mixture was allowed to warm to room temperature and then allowed to stir for 1 h. The reaction was quenched with water. The resulting precipitate was filtered, washed with water and finally recrystallized from dimethylformamide/water to yield BPDB(r) (27.3 mg, 68% yield) as yellow solid. ^1H NMR (400 MHz, DMSO- d_6): δ = 2.10 (s, 6H), 7.81 (d, J = 8.6 Hz, 4H), 7.91 (d, J = 9.0 Hz, 4H), 7.97 (d, J = 8.6 Hz, 4H), 8.00 (d, J = 8.6 Hz, 4H), 10.31 (br s, NH); ^{13}C NMR (100 MHz, DMSO- d_6 at 60 °C): δ = 24.5, 119.7, 123.4, 124.1, 128.2, 141.7, 143.0, 148.2, 152.2, 169.2; ESI-HRMS: m/z calc'd for $\text{C}_{28}\text{H}_{24}\text{N}_6\text{O}_2$: 476.1947 $[\text{M}+\text{H}]^+$, found: 476.1961.

6) Synthesis of 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl-2,2'-disulfonate, BPDBS

1b (135 mg, 0.23 mmol) was dissolved in water (4 mL) with sodium carbonate (25.0 mg, 0.23 mmol), and chloroacetyl chloride (126 mg, 1.1 mmol) was added dropwise in ice cold conditions. After stirring the reaction for 15 min, another aliquot of chloroacetyl chloride (126 mg, 1.1 mmol) was introduced dropwise without further cooling and the resulting reaction was allowed to stir at room temperature for 30 min. The aqueous reaction mixture was saturated with NaCl, which led to precipitation of the desired product. The precipitate was filtered, washed with saturated aqueous NaCl solution and ether, and dried *in vacuo* to yield BPDBS (141 mg, 80% yield, <5 % *trans-cis* isomer) as an orange red solid. ^1H NMR (400 MHz, DMSO- d_6): δ = 4.33 (s, 4H), 7.54 (d, J = 8.0 Hz, 2H), 7.76 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 2H), 7.83 (d, J = 9.0 Hz, 4H), 7.94 (d, J = 9.0 Hz, 4H), 8.35 (d, 2H, J = 2.0 Hz, 2H), 10.66 (br s, NH); ^{13}C NMR (100 MHz, DMSO- d_6): δ = 44.3, 120.3, 121.2, 122.4, 124.4, 133.9, 141.2, 142.1, 146.6, 148.8, 150.9, 165.8; ESI-HRMS: m/z calc'd for $\text{C}_{28}\text{H}_{23}\text{N}_6\text{O}_8\text{S}_2\text{Cl}_2$: 705.0390 $[\text{M}+\text{H}]^+$, found: 705.0370.

7) Synthesis of 4,4'-bis(4-acetamidophenyl)diazenylbiphenyl-2,2'-disulfonate, BPDBS(r)

To an ice cold aqueous solution of **1b** (36.0 mg, 0.06 mmol) containing sodium carbonate (7.2 mg, 0.06 mmol) was added acetic anhydride (60.0 mg, 0.6 mmol) dropwise with stirring. The resulting reaction was stirred at room temperature for 2 h. The same work up procedure as above led to a solid orange product BPDBS(r) (29.0 mg, 76% yield). The product was further purified by a reverse phase column chromatography using Varian Bond Elut C18. The product was eluted with a 20-30% methanol-water mixture. ^1H NMR (400 MHz, D_2O): δ = 2.20 (s, 6H), 7.34 (d, J = 8.2 Hz, 2H), 7.58 (d, J = 9.0 Hz, 4H), 7.67 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 2H), 7.78 (d, J = 9.0 Hz, 4H), 8.41 (d, J = 2.0 Hz, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 23.4, 120.8, 121.9, 122.9, 124.0, 133.2, 139.5, 140.9, 142.0, 148.4, 151.0, 172.7; ESI-HRMS: m/z calc'd for $\text{C}_{28}\text{H}_{23}\text{N}_6\text{O}_8\text{S}_2$: 635.1024 $[\text{M}+\text{H}]^+$, found: 635.1051.

Peptide synthesis

The peptides FZ21 (Ac-WGACEAAAREAAAREAAAREAAARCAQ-NH₂), SS19 (Ac-WGACEAAAREAAAREAAAREAAACREAAQ-NH₂), SS14 (Ac-WGACEAAAREAAAREAAAREAAACREAAQ-NH₂), FK11 (Ac-WGEACAREAAAREAAACRQ-NH₂) and SS7 (Ac-WGEAAARECAAREAAACRQ-NH₂) were prepared by using standard protocols for Fmoc-based solid-phase peptide synthesis. All peptides were HPLC-purified on a Zorbax SB-18 column with linear gradient of 5-70% acetonitrile/water containing (+0.1% trifluoroacetic acid) over a course of 35 min. The molecular compositions of peptides were confirmed by MALDI-MS $[\text{M}^+]$: FZ21 ($\text{C}_{112}\text{H}_{182}\text{N}_{42}\text{O}_{37}\text{S}_2$) calc'd: 2773.1 Da, found: 2773.6 Da; SS19 ($\text{C}_{114}\text{H}_{185}\text{N}_{42}\text{O}_{39}\text{S}_2$) calc'd: 2832.1 Da, found: 2832.2 Da; SS14 ($\text{C}_{114}\text{H}_{185}\text{N}_{42}\text{O}_{39}\text{S}_2$) calc'd: 2832.1 Da, found: 2832.2 Da; SS11 ($\text{C}_{112}\text{H}_{182}\text{N}_{42}\text{O}_{37}\text{S}_2$) calc'd: 2773.1 Da, found: 2773.5 Da.

Peptide cross-linking

Intramolecular cross-linking of Cys residues in FZ21 peptide with BPDBS cross-linker was performed as follows: to a final concentration of 0.5 mM peptide in sodium phosphate buffer (50 mM, pH 8.5) was added tris(carboxyethyl)phosphine (10 mM), and the solution was stirred under nitrogen for 45 min to ensure that cysteine residues were in their reduced state. A solution of the cross-linker BPDB in DMSO was added to the reaction to a final concentration of 2 mM, and the solvent system for the reaction was adjusted to 15-20 % DMSO/water. The reaction mixture was allowed to stir at 45°C protected from light for 24 h. A similar protocol was used for intramolecular cross-linking of Cys residues in SS19, SS14 and SS11 with BPDBS. The cross-linked peptides were HPLC-purified using the same conditions as described above and were characterized by ESI-MS: m/z calc'd for FZ21-BPDBS ($C_{140}H_{202}N_{48}O_{45}S_4$): 3406.70 [M+H]⁺, found: 3405.69; SS19-BPDBS ($C_{142}H_{204}N_{48}O_{47}S_4$): 3464.74 [M+H]⁺, found: 3463.40; m/z calc'd for SS14-BPDBS ($C_{142}H_{204}N_{48}O_{47}S_4$): 3464.74 [M+H]⁺, found: 3463.60; m/z calc'd for SS11-BPDBS ($C_{140}H_{202}N_{48}O_{45}S_4$): 3406.70 [M+H]⁺, found: 3405.47.

UV-Vis spectra and photoisomerization

UV-Vis absorption spectra were obtained using either a Perkin-Elmer Lambda 35 spectrophotometer or using a diode array UV-Vis spectrophotometer (Ocean Optics Inc., USB4000) coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc. Spokane, WA, USA). The latter arrangement was used to measure thermal relaxation rates, and steady-state spectra under UV-Vis illumination. For steady state spectra, typically, a 8-10 μ M solution of cross-linked peptide in 10 mM sodium phosphate buffer (pH 7.0), contained in a 1cm path length quartz cuvette in the temperature controlled cuvette holder, was irradiated (at 90° to the measuring beam) with LEDs emitting at 365 nm or 385 nm at 4°C, 20°C and 37°C for 5 min until no further decrease in absorbance was observed, and under this steady-state irradiation condition the spectra were recorded.

Rates of thermal reversion of the irradiated samples were measured for a series of temperatures by monitoring absorbance at 365 nm or 385 nm with time. All curves were fitted to mono-exponential and/or biexponential decay kinetics. The light used for the absorbance measurement was of sufficiently low intensity to cause negligible isomerization.

The concentrations of all cross-linked peptides were measured by using the molar extinction coefficient of cross-linkers, which were determined as follows: ¹H NMR spectra of solutions of the cross-linkers BPDB and BPDBS in DMSO-*d*₆ containing a known concentration of 1,2-dichloroethane (DCE) were recorded. By comparing the intensity of methylene signal of DCE with the cross-linker's α -methylene signal, the concentration of the cross-linker in the NMR sample was determined. These NMR solutions were later diluted in DMSO for BPDB and in phosphate buffer (10 mM, pH 7.0) for BPDBS, and the UV-Vis spectra were recorded to calculate the molar extinction coefficient.

Circular dichroism measurements

Circular dichroism (CD) measurements were performed on an Olis RSM 1000 circular dichroism spectrophotometer with a Quantum Northwest Peltier accessory. 80-100 μ M solutions of cross-linked peptides in 10 mM phosphate buffer (pH 7.0) contained in 1 mm path length quartz cuvette were used to record the CD spectra at 4°C, 20°C and 37°C. Tris(carboxyethyl)phosphine (1 mM) was used in the uncross-linked peptide solutions to ensure that cysteine residues were in their reduced form. Each spectrum was scanned from 260 to 190 nm with an integration time of 2 s at each wavelength. Spectra for non-irradiated samples were the average of two individual experiments of two scans each, with appropriate background spectrum subtracted. At different temperatures, the cross-linked peptides were

irradiated for 5 min, and the CD spectra immediately recorded. In this case, the spectra were the average of two individual experiments of one scan each, with the appropriate background spectrum subtracted. Mean residual ellipticities were calculated using this standard equation: $[\theta]_r = \theta / (10 nc)$; where θ is the measured ellipticity in mdeg, n is the number of backbone amide bonds, c is the concentration in M, and l is the pathlength in cm. The percentage of helix content was calculated by using this standard equation: % helicity = $-100 n [\theta]_{r, 222} / 40\,000 (n-4)$; where ' n ' is the number of amide bonds in the peptide. ^[33] $[\theta]_{r, 222}$ is the measured ellipticity in mdeg at 222 nm. Peptide concentrations were determined using a value of $\epsilon = 50,000 \text{ M}^{-1}\text{cm}^{-1}$ at the absorption maximum of the cross-linked dark-adapted peptides. This value was used since the absorption spectrum of the cross-linker attached to a peptide was more similar to the free cross-linker in DMSO ($\lambda_{\text{max}}=380 \text{ nm}$, $\epsilon = 52,000 \text{ M}^{-1}\text{cm}^{-1}$) than that of the free cross-linker in phosphate buffer ($\lambda_{\text{max}}=360 \text{ nm}$, $\epsilon = 45,000 \text{ M}^{-1}\text{cm}^{-1}$).

Molecular modeling

Models of *trans-trans*, *trans-cis* and *cis-cis* cross-linkers were built using HyperChem (v.8, Hypercube Inc.) with the linker terminated with methyl groups representing the β carbon of Cys in the cross-linked peptide, and minimized using the Amber99 forcefield. Restraints were added to the azo bond for the *trans-cis* and *cis-cis* conformations (force constant 16). Molecular dynamics runs were performed *in vacuo* essentially as described previously ^[34] with a distant dependent dielectric, and 1–4 scale factors of 0.833 for electrostatic and 0.5 for van der Waals interactions, a step size of 1 fs and 300 K as the simulation temperature. Trajectories were analyzed to verify that numerous torsion angle changes occurred for all single bonds during the course of the simulation to ensure that conformational space was adequately sampled. All points histograms were then produced for the S–S distance during the full set of simulations for each isomer.

Acknowledgments

We wish to thank the Natural Sciences and Engineering Research Council of Canada, the Canadian Institutes of Health Research and the National Institutes of Health (U.S.A.)(R01 MH086379) for financial support.

References

- Garner J, Harding MM. Org Biomol Chem. 2007; 5:3577–3585. [PubMed: 17971985]
- Bhattacharya S, Zhang H, Debnath AK, Cowburn D. J Biol Chem. 2008; 283:16274–16278. [PubMed: 18417468] Henchey LK, Jochim AL, Arora PS. Curr Opin Chem Biol. 2008; 12:692–697. [PubMed: 18793750] Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, Kung AL, Gilliland DG, Verdine GL, Bradner JE. Nature. 2009; 462:182–188. [PubMed: 19907488] Walensky LD, Pitter K, Morash J, Oh KJ, Barbuto S, Fisher J, Smith E, Verdine GL, Korsmeyer SJ. Mol Cell. 2006; 24:199–210. [PubMed: 17052454]
- Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ, Datta SR, Pitter KL, Bird GH, Wikstrom JD, Deeney JT, Robertson K, Morash J, Kulkarni A, Neschen S, Kim S, Greenberg ME, Corkey BE, Shirihaï OS, Shulman GI, Lowell BB, Korsmeyer SJ. Nat Med. 2008; 14:144–153. [PubMed: 18223655]
- Renner C, Moroder L. ChemBioChem. 2006; 7:868–878. [PubMed: 16642526]
- Woolley GA. Acc Chem Res. 2005; 38:486–493. [PubMed: 15966715]
- Woolley GA, Jaikaran AS, Berezovski M, Calarco JP, Krylov SN, Smart OS, Kumita JR. Biochemistry. 2006; 45:6075–6084. [PubMed: 16681380] Guerrero L, Smart OS, Weston CJ, Burns DC, Woolley GA, Allemann RK. Angew Chem Int Ed Engl. 2005; 44:7778–7782. [PubMed: 16278913] Guerrero L, Smart OS, Woolley GA, Allemann RK. J Am Chem Soc. 2005; 127:15624–15629. [PubMed: 16262429] Kneissl S, Loveridge EJ, Williams C, Crump MP, Allemann RK. ChemBioChem. 2008; 9:3046–3054. [PubMed: 19012295] Zhang F, Zarrine-Afsar A, Al-Abdul-Wahid MS, Prosser RS, Davidson AR, Woolley GA. J Am Chem Soc. 2009; 131:2283–2289.

- [PubMed: 19170498] Schierling B, Noel AJ, Wende W, Hien le T, Volkov E, Kubareva E, Oretskaya T, Kokkinidis M, Rompp A, Spengler B, Pingoud A. *Proc Natl Acad Sci U S A*. 2010; 107:1361–1366. [PubMed: 20080559]
7. Zhang F, Timm KA, Arndt KM, Woolley GA. *Angew Chem Int Ed Engl*. 2010; 49:3943–3946. [PubMed: 20419720]
8. Dias AR, Minas da Piedade ME, Martinho Simoes JA, Simoni JA, Teixeira C, Diogo HP, Meng-Yan Y, Pilcher G. *J Chem Thermodynamics*. 1992; 24:439–447. Dokic J, Gothe M, Wirth J, Peters MV, Schwarz J, Hecht S, Saalfrank P. *J Phys Chem A*. 2009; 113:6763–6773. [PubMed: 19453149] Rau H. *Angew Chem Int Ed Engl*. 1973; 12:224–235.
9. Burns DC, Zhang F, Woolley GA. *Nat Protoc*. 2007; 2:251–258. [PubMed: 17406582]
10. Burns DC, Flint DG, Kumita JR, Feldman HJ, Serrano L, Zhang Z, Smart OS, Woolley GA. *Biochemistry*. 2004; 43:15329–15338. [PubMed: 15581345]
11. Flint DG, Kumita JR, Smart OS, Woolley GA. *Chem Biol*. 2002; 9:391–397. [PubMed: 11927265]
12. Kumita JR, Smart OS, Woolley GA. *Proc Natl Acad Sci USA*. 2000; 97:3803–3808. [PubMed: 10760254]
13. Zhang F, Sadvovskii O, Woolley GA. *ChemBioChem*. 2008; 9:2147–2154. [PubMed: 18729291]
14. Khan A, Hecht S. *Chemistry*. 2006; 12:4764–4774. [PubMed: 16528779] Yu Z, Hecht S. *Angew Chem Int Ed Engl*. 2011
15. Kusebauch U, Cadamuro SA, Musiol HJ, Lenz MO, Wachtveitl J, Moroder L, Renner C. *Angew Chem Int Ed Engl*. 2006; 45:7015–7018. [PubMed: 17009283]
16. Kuil J, van Wandelen LT, de Mol NJ, Liskamp RM. *J Pept Sci*. 2009; 15:685–691. [PubMed: 19714714]
17. Standaert RF, Park SB. *J Org Chem*. 2006; 71:7952–7966. [PubMed: 17025282]
18. Shinkai S, Manabe O. *Top Curr Chem*. 1984; 121:67–104. Tamaoki N, Ogata K, Koseki K, Yamaoka T. *Tetrahedron*. 1990; 46:5931–5942. Norikane Y, Katoh R, Tamaoki N. *Chem Comm*. 2008:1898–1900. [PubMed: 18401511] Vogtle F, Muller WM, Muller U, Bauer M, Rissanen K. *Angew Chem Int Ed Engl*. 1993; 32:1295–1297. Rau H, Rottger D. *Mol Cryst Liq Cryst Sci Tech A: Mol Cryst Liq Cryst*. 1994; 246:143–146. Bencini A, Bernardo MA, Bianchi A, Ciampolini M, Fusi V, Nardi N, Parola AJ, Pina F, Valtancoli B. *J Chem Soc Perkin Trans II*. 1998:413–418.
19. May, EM.; Fono, A. U. S. P. Office. CAplus. Otto B. May Inc.; U.S.A.: 1966. p. 4. Claussen, U. CAplus. Offen, G., editor. Bayer, A.G.; Germany: 1982. p. 26
20. Matejka L, Dusek K. *Makromol Chem Macromol Chem Phys*. 1981; 182:3223–3236.
21. Cisnetti F, Ballardini R, Credi A, Gandolfi MT, Masiero S, Negri F, Pieraccini S, Spada GP. *Chem Eur J*. 2004; 10:2011–2021. [PubMed: 15079841]
22. Ojala WH, Ojala CR, Gleason WB. *Antiviral Chem Chemother*. 1995; 6:25–33.
23. Mal P, Breiner B, Rissanen K, Nitschke JR. *Science*. 2009; 324:1697–1699. [PubMed: 19556504]
24. Neelakantan L, Hartung WH. *J Org Chem*. 1959; 24:1943–1948.
25. Knoll, H. *CRC Handbook of organic photochemistry and photobiology*. 2. Horspool, W.; Lenci, F., editors. CRC Press; Boca Raton: 2004. p. 1-16.
26. Zhang Z, Burns DC, Kumita JR, Smart OS, Woolley GA. *Bioconjug Chem*. 2003; 14:824–829. [PubMed: 12862437]
27. Merutka G, Shalongo W, Stellwagen E. *Biochemistry*. 1991; 30:4245–4248. [PubMed: 2021618]
28. Borisenko V, Woolley GA. *J Photochem Photobiol A Chem*. 2005; 173:21–28.
29. Riener CK, Kada G, Gruber HJ. *Anal Bioanal Chem*. 2002; 373:266–276. [PubMed: 12110978]
30. Barshop BA, Wrenn RF, Frieden C. *Anal Biochem*. 1983; 130:134–145. [PubMed: 6688159]
31. Lopez-Mirabal HR, Winther JR. *Biochim Biophys Acta*. 2008; 1783:629–640. [PubMed: 18039473]
32. Ihalainen JA, Paoli B, Muff S, Backus EH, Bredenbeck J, Woolley GA, Caflisch A, Hamm P. *Proc Natl Acad Sci U S A*. 2008; 105:9588–9593. [PubMed: 18621686]
33. Jackson D, King D, Chmielewski J, Singh S, Schultz P. *J Am Chem Soc*. 1991; 113:9391–9392.
34. Chi L, Sadvovskii O, Woolley GA. *Bioconjug Chem*. 2006; 17:670–676. [PubMed: 16704204]

Figure 1.

A) Conformational isomers of a BPDB cross-linked peptide where the β -positions of peptide cysteine side-chains are replaced by methyl groups. B) Sulfur to sulfur distances observed during molecular dynamics simulations. Rotation around the bonds indicated in the cis-cis isomer can produce two families of conformers (cis-cis (s) and cis-cis (l)) with very different end-to-end distances.

Figure 2.

A) UV-Vis spectra of dark-adapted (black lines) and irradiated (grey lines) BPDB(r) in DMSO. B) UV-Vis spectra of dark-adapted (black lines) and irradiated (grey lines) BPDBS(r) in DMSO (—) and in 10 mM sodium phosphate buffer, pH 7.0 (.....).

Figure 3. Expanded ^1H NMR spectra showing methyl proton signals (highlighted in (A)) of BPDB (r) in $\text{DMSO}-d_6$ (B), and BPDBS (r) in $\text{DMSO}-d_6$ (C) and in D_2O (D) recorded before and after irradiation (at 380 nm or 365 nm respectively) with different irradiation times as indicated.

Figure 4.

A) Primary sequences of cross-linked peptides: FZ21-BPDBS, SS19-BPDBS, SS14-BPDBS, SS11-BPDBS. B) Histograms of end-to-end distances for *trans-trans*, *trans-cis* and *cis-cis* isomers of BPDB calculated from molecular dynamics simulations with S-S spacing in ideal helical peptides with Cys residues spaced at *i*, *i*+21 (FZ21); at *i*, *i*+19 (SS19); *i*, *i*+14 (SS14); *i*, *i*+11 (SS11), *i*, *i*+7 and *i*, *i*+4 shown.

Figure 5.

A) UV-Vis absorption spectra of all BPDBS cross-linked peptides without and with continuous irradiation at 365 nm at 20°C. Grey lines represent spectra with continuous irradiation at 365 nm, while black lines represent spectra without 365 nm irradiation. (FZ21-BPDBS (—), SS19-BPDBS (....), SS14-BPDBS (–.–), SS11-BPDBS (–.–)) B) UV-Vis spectra of SS19-BPDBS without (black solid line) and with continuous irradiation (grey lines) at different temperatures (4°C (....), 20°C (–.–), 37°C (–.–), 45°C (–)). C) UV-Vis spectra of SS11-BPDBS without (black solid line) and with continuous irradiation (grey lines) at different temperatures (4°C (....), 20°C (–.–), 37°C (–.–)). D) Spectra of SS11-BPDBS (20°C) before irradiation (black solid line), after irradiation with 365 nm light (grey solid line), and at different time intervals after irradiation (5, 10, 15, 30, 45, 60, 75, 90, 109 and 120 min)(grey dotted lines). (10–12 μ M solutions of cross-linked peptides in 10 mM phosphate buffer at pH 7.0 were used for all experiments)

Figure 6.

A) Mono-exponential fit (black line) of the measured absorbance decay (grey line) of irradiated FZ21-BPDBS at 20°C. B) Mono-vs. biexponential fits of the measured absorbance decay of irradiated SS11-BPDBS at 37°C.

Figure 7. CD spectra of A) FZ21-BPDBS, B) SS19-BPDBS, C) SS14-BPDBS, and D) SS11-BPDBS before (black lines) and after irradiation (grey lines). Each spectrum was scanned at 37°C (—), 20°C (....) and 4°C (---). (80-100 μ M peptide solutions in 10 mM phosphate buffer at pH 7.0.)

Figure 8. Time-dependent CD signal for SS19-BPDBS at 222 nm after irradiation at 365 nm at higher temperatures.

Figure 9. Models showing the increase in helical content of the SS11-BPDBS peptide upon UV irradiation.

Scheme 1.

Structures of the new bis-azobenzene structures reported here (BPDB, BPDBS) together with previously studied azobenzene-based cross-linkers BSBKA and DDPBA.

Scheme 2.

Synthetic route to 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl (BPDB) and 4,4'-bis(4-(2-chloroacetamido)phenyl)diazenylbiphenyl-2,2'-disulfonate (BPDBS) and the corresponding reference compounds BPDB(r) and BPDBS(r).

Scheme 3.

Kinetic processes of thermal reversion in cross-linked peptides

Table 1

Thermal relaxation half-lives of all cross-linked peptides and the cross-linker BPDBS(r).

Peptide	$\tau_{1/2}$ (min)	
	20°C	37°C
BPDBS(r)	42.5 $\pm$ 5.0	11.2 $\pm$ 1.3
FZ21-BPDBS	16.0 $\pm$ 0.5	4.9 $\pm$ 0.2
SS19-BPDBS	29.1 $\pm$ 1.2	7.6 $\pm$ 0.2
SS14-BPDBS	10.6 $\pm$ 1.1	3.5 $\pm$ 0.1
SS11-BPDBS	12.6 $\pm$ 0.3	3.5 $\pm$ 0.1

Table 2

Percentage helicity of cross-linked peptides before and after irradiation.

peptide	α -helix content (%)					
	37°C		20°C		4°C	
	dark	irradiated	dark	irradiated	dark	irradiated
FZ21-BPDBS	51	41	55	49	58	56
SS19-BPDBS	63	51	66	60	70	67
SS14-BPDBS	37	40	46	49	54	56
SS11-BPDBS	10	21	11	28	15	31