Synthesis and Photochemistry of a New Class of Photocleavable Protein Cross-linking Reagents

Lilia Milanesi,[a] Gavin D. Reid,[b] Godfrey S. Beddard,[b] Christopher A. Hunter,*[a] and Jonathan P. Waltho[c]

Abstract: A new series of photocleavable protein cross-linking reagents based on bis(maleimide) derivatives of diaryl disulfides have been synthesised. They have been functionalised with cysteine and transient absorption spectra for the photolysis reaction have been recorded by using the pump-probe technique with a time resolution of 100 femtoseconds. Photolysis of the disulfide bond yields the corresponding thiyl radicals in less than a picosecond. There is a significant amount of geminate recombination, but some of the radicals escape the solvent cage and the quantum yield for photocleavage is 30% in water.

Keywords: disulfide · optical trigger · photolysis · protein folding · thiyl radical

Introduction

Experimental methods to study protein folding are based on perturbing the equilibrium between the folded and unfolded states under denaturing conditions.[1,2] Typically, the unfolding or refolding reaction is initiated by mixing the protein solution with an appropriate buffer. The time resolution of these methods is in the order of milliseconds down to a few microseconds.[3–6] However, key elements of secondary structure and intermediates are formed on a submillisecond timescale.[7–13] To study these processes, new methods have been developed which use laser pulses to initiate folding by triggering a photochemical reaction or by a rapid change in the temperature of the protein solution.[14,15] To date, these methods have been applied almost exclusively to study the folding of specific families of proteins that either bear amenable prosthetic groups, such as porphyrins, or undergo cold denaturation.

Methods for the introduction of photolabile moieties into proteins would allow more general applications of laser technology to study conformational changes that take place in the femtosecond to microsecond time domain. Nitrobenzyl derivatives have been used to prepare photocaged proteins ,[16–18] but the rate of photocleavage leads to a dead time of microseconds.[19,20] A more promising approach is based on photolabile aromatic disulfides which have successfully been used to trigger the folding of small α-helical peptides.[21–23] This looks like an ideal system for the study of fast folding reactions, but the unnatural amino acids must be introduced by solid-phase synthesis; this limits applications to short peptides. In this paper, we report the synthesis of new cross-linking reagents that will allow the introduction of aromatic disulfides into any protein of interest.

Results and Discussion

Approach: The choice of substituents on the aromatic disulfide has a dramatic impact on the photochemical properties. For example, thiyl radicals generated after photolysis of diphenyl disulfide recombine significantly faster than thiyl radicals generated after photolysis of bis(*p*-aminophenyl) disulfide.[21,24,25] The quantum yield of photocleavage is also solvent dependent; more *p*-aminophenyl thiyl radical is generated in polar solvents compared with nonpolar solvents.[26] The two competing processes which determine the quantum yield of photocleavage are geminate recombination and the escape of the radical pair from the solvent cage (Figure 1).[26–28] The rate of intersystem crossing is slow, therefore long-lived triplet radicals are not important in this

[a] Dr. L. Milanesi, Prof. C. A. Hunter
Centre for Chemical Biology, Krebs Institute
for Biomolecular Science, Department of Chemistry
University of Sheffield, Sheffield S3 7HF (UK)
Fax: (+44) 114-2229-346
E-mail: c.hunter@sheffield.ac.uk

[b] Dr. G. D. Reid, Prof. G. S. Beddard
School of Chemistry, University of Leeds
Leeds, LS2 9JT (UK)

[c] Prof. J. P. Waltho
Krebs Institute for Biomolecular Science
Department of Molecular Biology and Biotechnology
University of Sheffield, Sheffield S10 2TN (UK)

 DOI: 10.1002/chem.200305405 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

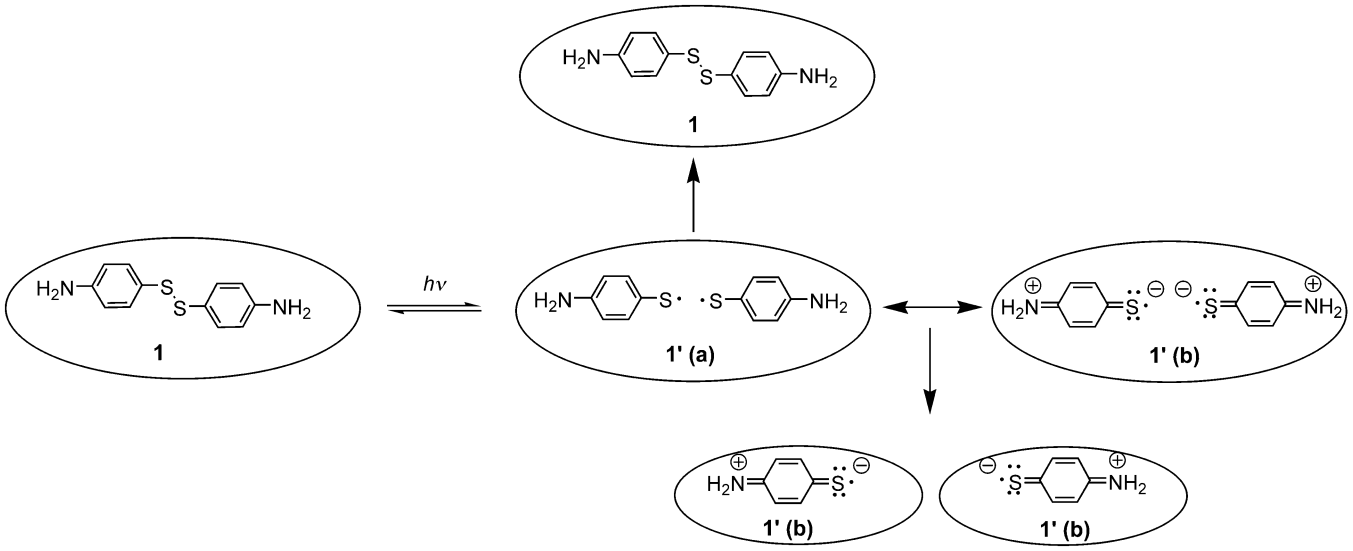


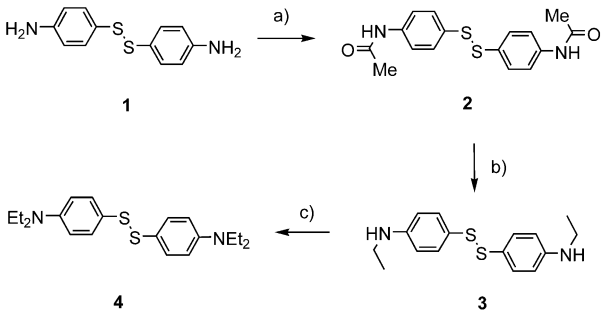
Figure 1. Photocleavage of **1** generates two *p*-aminophenylthiyl radicals. Two structures have been sugggested for the radicals, a neutral form **1′(a)** and a polar form **1′(b)**. The large ellipsoids represent the solvent shell and after photolysis, the radicals can either recombine (top) or escape from the solvent shell to yield stable photodissociated products (bottom).

system.[23] The differences between the photochemical properties of diphenyl disulfide and bis(*p*-aminophenyl) disulfide have been rationalised based on the electronic structure of the radical. The *p*-aminophenyl thiyl radical could be stabilised by the polar form shown in Figure 1. This form would be further stabilised in polar solvents and would decrease the rate of geminate recombination due to repulsion between the negatively charged sulphur atoms.[29]

The first step of this investigation was therefore to prepare a range of bis(*p*-aminophenyl) disulfide derivatives to explore the importance of conjugation between the amino group and the aromatic ring and the significance of interactions of the substituents with polar hydrogen-bonding solvents. Ultimately, we propose to introduce the aromatic disulfide group into proteins by selectively functionalising the thiol side chains of cysteine residues engineered into the protein. The maleimide group is the reagent of choice to achieve this.[30] We therefore designed a series of bis(*p*-aminophenyl) disulfide derivatives and investigated their reaction with cysteine and the photochemical properties of the products in water.

Synthesis: Compound **3** was obtained by the reaction of **1** with acetic anhydride followed by the reduction of intermediate **2** with lithium aluminium hydride. Compound **4** was obtained from **3** by using trifluoromethanesulfonic acid ethyl ester as an alkylating agent (Scheme 1). The yield of **4** was lowered by the formation of an unusual side product **4a**, which was characterised by X-ray crystallography (Figure 2).

Synthesis of **6** (Scheme 2) was carried out by mixing **1** with maleic anhydride in toluene at reflux followed by the cyclisation of acid **5** by using a mixture of acetic anhydride and sodium acetate as dehydrating agents. Initial attempts to synthesise **8** by reaction of **1** with the tosylate derivative of *N*-(2-hydroxyethyl) maleimide failed to give the expected



Scheme 1. Synthesis of compound **4**; a) $(CH_3OC)_2O$, 90%; b) $LiAlH_4$, 46% and c) $EtOSO_2CF_3$, 20%.

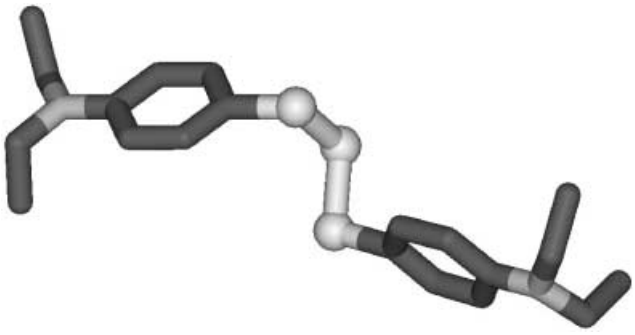
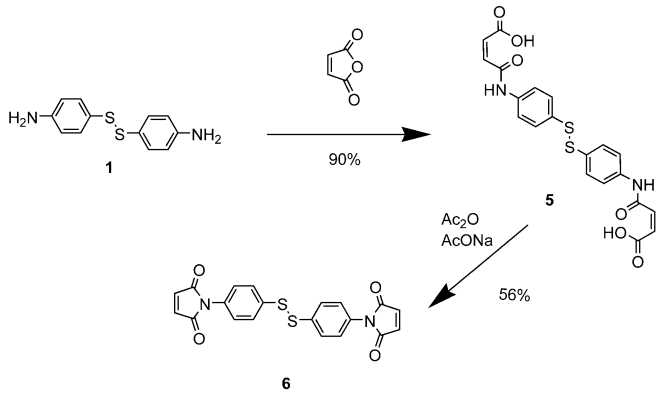


Figure 2. X-ray crystal structure of **4a**. The three sulphur atoms are shown as space-filling spheres.

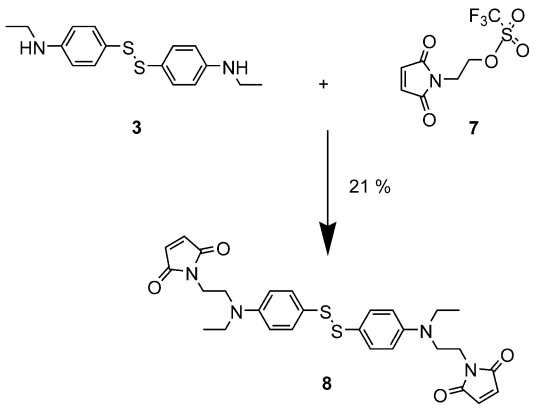
product. Instead, the use of **7**, which was prepared following a literature procedure, afforded the expected product (Scheme 3).[31] Compound **10** was obtained by reaction of **1** with **9**, which was prepared following a literature procedure (Scheme 4).[32]

Functionalisation of L-cysteine methyl ester hydrochloride with the cross-linking reagents was carried out by mixing

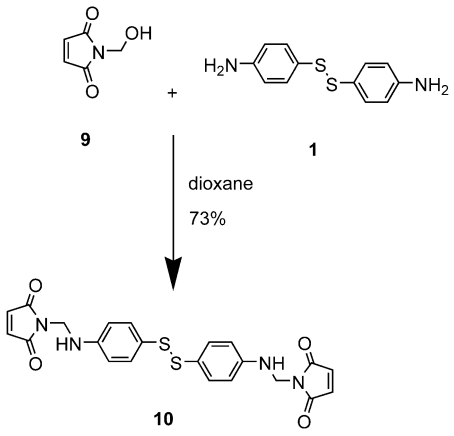
© 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 2.



Scheme 3.



Scheme 4.

the compounds in a solution of acetonitrile or tetrahydrofuran and water (Scheme 5). At a maleimide to thiol stoichiometry of 1:1, side reactions caused by thiol disulfide exchange were appreciable (~10%). However in the presence of excess maleimide, quantitative conversion of the cysteine thiol to the succinimide adduct was achieved with no disulfide exchange.

Photochemistry: The photochemistry of the aryl disulfides was investigated by using a pump-probe experiment with a 100 fs pulse width. Excitation at 260 nm dissociated the disulfide bond and the absorption of the resulting *p*-aminophenylthiyl radicals was monitored at 550 nm.[25,29,33] The time evolution of the radical absorption on photodissociation of **1**, **3** and **4** in acetonitrile is shown in Figure 3. When the cross-linking reagents **6**, **8** and **10** were photolysed, the thiyl radicals that were generated reacted with the maleimide moieties, which complicated the analysis of these systems. The reactions could be reduced by decreasing the concentration, but it is the properties of the cysteine conjugates that we are really interested in. The photolysis of **11**, **12** and **13** could not be carried out in acetonitrile, due to low solubility, therefore these compounds were studied in methanol and water (Figure 4 and 5).

The experimental data could all be fitted to a monoexponential decay, shown in Equation (1),

$$I(t) = Ae^{(-t/\tau)} + B \quad (1)$$

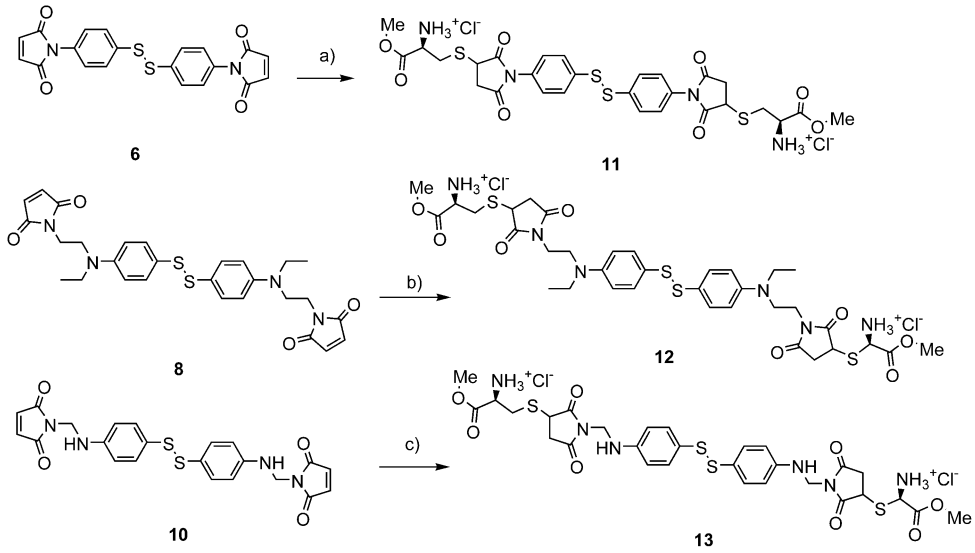
in which $I(t)$ is the absorbance at time t, B is the absorbance at $t=\infty$, $(A+B)$ is the absorption at $t=0$ and τ^{-1} is the rate constant for the decay of radical absorption. The initial process of photodissociation is complete in less than 100 fs for bis(*p*-aminophenyl) disulfide.[26,34] The decay process therefore reflects the rate of geminate recombination. The fraction of radicals that escape the solvent cage and remain after the fast decay process due to geminate recombination is given by Equation (2) below.

$$\phi = \frac{B}{A+B} \quad (2)$$

The values of A, B and τ were determined from the fits to the experimental traces and the results are shown in Table 1. For bis(*p*-aminophenyl) disulfide **1**, essentially no recombination is observed and the yield of radical is almost quantitative. In the literature, this has been attributed to stabilisation of the polar radical form by polar solvents (Figure 6).[25,33b,35] There is slightly more recombination for **3** and **4** and the differences could be attributed to the stabilisation of the polar radical form by hydrogen-bonding interactions with the solvent. The more hydrogen bonds the radical is able to form, the more stable it is (Figure 6). However, the quantum yields for all three compounds are high in acetonitrile, so we conclude that it is possible to functionalise the aniline nitrogen without dramatically altering the photochemical properties.

For **11**, **12** and **13** in methanol, more recombination takes place and the quantum yields fall to about 50%. Compound **13**, which has an NH group available to hydrogen bond with the solvent, again gives a slightly more stable radical. In water, the cysteine derivatives show a further decrease in the quantum yield of radicals (~30%). The results correlate with the viscosity of the solvent. More viscous solvents prevent escape from the solvent shell and increase the rate of geminate recombination.[24,26,27] A 30% yield of the thiyl radicals on photolysis of the aromatic disulfides in water is sufficient to encourage testing of these systems on proteins. However, it is probable that the conformational constraints

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 5. Functionalisation of L-cysteine methyl ester hydrochloride with the cross-linking reagents in a) CH_3CN/H_2O; b) CH_3CN/H_2O and c) THF/H_2O.

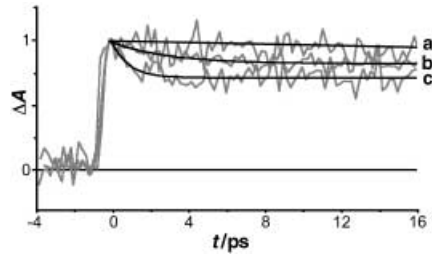


Figure 3. Evolution of the radical absorption signal at 550 nm after photolysis of **1** (a), **3** (b) and **4** (c) in acetonitrile. The curves are fits to a monoexponential decay [Eq. (1)]. The vertical axis is the change in absorbance at 550 nm relative to the starting disulfide (ΔA) in arbitrary units.

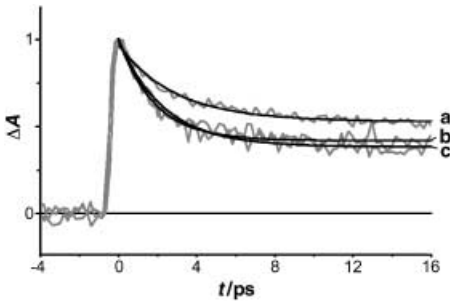


Figure 4. Evolution of the radical absorption signal at 550 nm after photolysis of **13** (a), **12** (b) and **11** (c) in methanol. The curves are fits to a monoexponential decay [Eq. (1)]. The vertical axis is the change in absorbance at 550 nm relative to the starting disulfide (ΔA) in arbitrary units.

of a large protein will further increase the proportion of geminate recombination, as was observed on photolysis of aryl disulfides incorporated into short peptides.[21,22]

Conclusion

A new series of photocleavable protein cross-linking reagents have been synthesised, functionalised with cysteine and transient absorption spectra for the photolysis reaction have been recorded by using the pump-probe technique with a time resolution of 100 femtoseconds. Photolysis of the aromatic disulfides yields the corresponding thiyl radicals in less than a picosecond. There is a significant amount of geminate recombination, which results in a quantum yield for photocleavage of about 30%. This system is currently being investigated

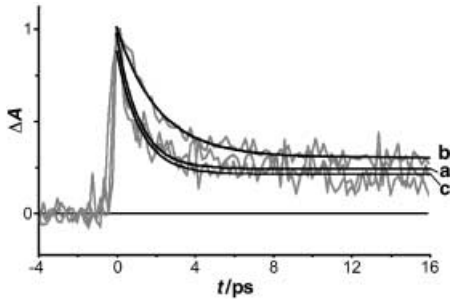


Figure 5. Evolution of the radical absorption signal at 550 nm after photolysis of **13** (a), **12** (b) and **11** (c) in water. The curves are fits to a monoexponential decay [Eq. (1)]. The vertical axis is the change in absorbance at 550 nm relative to the starting disulfide (ΔA) in arbitrary units.

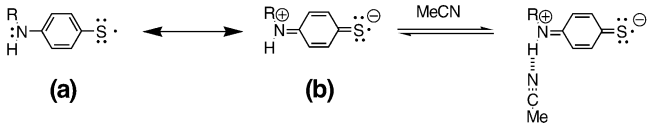


Figure 6. Model for the solvent stabilisation of the polar form for the thiyl radical **1′(b)** through hydrogen-bonding interactions with the solvent.

Table 1.

Compound	Solvent Viscosity[a]	τ [ps]	A [x10^5]	B [x10^5]	ϕ[b]
1	CH_3CN (0.38)	n.d.	n.d.	n.d.	1.0[c]
3	CH_3CN (0.38)	2.9	5.6	26.3	0.8±0.3
4	CH_3CN (0.38)	0.9	5.6	11.7	0.7±0.3
11	MeOH (0.59)	2.3	17.8	9.9	0.4±0.1
12	MeOH (0.59)	1.8	19.7	14.2	0.4±0.1
13	MeOH (0.59)	2.9	8.5	10.6	0.6±0.1
11	H_2O (1.00)	1.2	5.9	2.0	0.3±0.1
12	H_2O (1.00)	2.1	14.1	6.1	0.3±0.1
13	H_2O (1.00)	1.1	9.2	3.1	0.3±0.1

[a] Viscosities are given in cP at 293 K and are taken from ref. [36] [b] The error in the parameters from the fitting of the curves is taken as twice the standard deviation of the experimental data points calculated on the A value. [c] n.d. not determined.

© 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

as a new optical trigger for initiating protein-folding reactions on a picosecond timescale.

Experimental Section

Photolysis: The disulfides were dissolved in HPLC grade solvents (Merck) at concentrations of 1–2 mM, the cuvette path length was 1 mm for all of the measurements.

A detailed description of the femtosecond laser and transient absorption spectrophotometer is reported elsewhere.[37] For the experiments described here, the laser set-up was modified as follows.

The pump beam was 520 nm and it was frequency doubled to 260 nm by using a 100 micro-metre thick BBO crystal. Samples were excited with 100 fs of the 260 nm light (maximum energy of 100 nJ). The zero time for the probe wavelength was determined by excitation of a solution of *trans* stilbene in acetonitrile. For each experiment, the difference in absorbance between the excited and ground-state species measured at different pump/probe delay times was less than 10^{-4}. The signal to noise ratio was improved by averaging 10–20 scans. The data were analysed with the same program used for data collection (ExptPPC1, version 3.0).

Typically, each experiment was an average of 10–20 scans to improve the signal to noise ratio. A sample of solvent was photolysed under the same experimental conditions used for the aromatic disulfides. No signal was found for the photolysis of the solvents; this was probably due to the low intensity (~100 nJ) of the UV pulses.

Synthesis of 2: 4-Aminophenyldisulfide (2.48 g, 10 mmol) dissolved in toluene (20 mL) was added dropwise to a solution of acetic anhydride (2.06 g, 20 mmol) in toluene (150 mL). The solution was then stirred for an hour under reflux, while the product slowly precipitated from the reaction mixture. After cooling, amide **2** was collected by filtration (2.98 g, yield: 90%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 10.10 (s, 2H), 7.59 (d, J = 8.5 Hz, 4H), 7.42 (d, J = 8.5, 4H), 2.05 ppm (s, 6H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 169.0, 140.0, 130.6, 129.8, 120.1, 24.5 ppm; MS (ES$^+$): m/z: 333 [M+H$^+$], 355 [M+Na$^+$], $C_{16}H_{16}N_2O_2S_2$ requires 332.44; m.p.: 218–220 °C.

Synthesis of 3: A solution of **2** (2.37 g, 7.1 mmol) in dry THF (100 mL) was added dropwise to lithium aluminium hydride solution (23.5 mL, 21.4 mmol, 1 M) in THF at 0 °C under nitrogen. The resulting solution was allowed to warm to room temperature and then it was heated under reflux overnight. It was then cooled to 5 °C and water was added until no more gas was produced. The mixture was filtered and the solid was washed with CH_2Cl_2 (100 mL). The organic phase was dried with anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The resulting oil was purified by column chromatography by using a mixture of CH_2Cl_2/hexane 95:5 v/v as an eluent. Disulfide **2** was obtained as a yellow oil that solidified slowly (1 g, yield: 46%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 7.13 (d, J = 8.5, 4H), 6.50 (d, J = 8.8, 4H), 6.01 (t, J = 5.2, 2H), 3.04 (m, 4H), 1.16 ppm (t, J = 7.3, 6H); ^{13} C NMR (75 MHz, [D]CHCl$_3$): δ = 149.0, 134.6, 124.0,112.9, 38.3, 14.8 ppm; MS (ES$^+$): m/z: 152, 305 [M+H$^+$], $C_{16}H_{20}N_2S_2$ requires 304.48; elemental analysis cald (%) for $C_{16}H_{20}N_2S_2$: C, 63.70; H, 7.55; N, 8.74; S, 20.01; found: C, 63.46; H, 7.44; N, 8.61; S, 20.10; UV/Vis (CH_3CN) λ (ε) = 260 nm (16656).

Synthesis of 4: The ethyl ester of trifluoromethanesulfonic acid (0.5 mL, 4 mmol) under nitrogen, was added dropwise to a solution of **3** (0.3 g, 1 mmol) in dry dicholorometane (10 mL) and the resulting solution was stirred overnight. The solvent was then removed under reduced pressure and the residue was suspended in saturated $NaHCO_3$ solution (30 mL) and washed with CH_2Cl_2 (3×30 mL). The organic phase was then dried over Na_2SO_4 and the solvent removed under reduced pressure. The residue was purified by column chromatography by using a mixture of hexane/CH_2Cl_2 70:30 v/v as an eluent. The first fraction eluted was an impurity that corresponded to trisulfide **4a** (78 mg, yield: 20%). Afterwards, compound **4** was obtained as a yellow solid (72 mg, yield: 20%). ^{1}H NMR (250 MHz, [D]CHCl$_3$): δ = 7.33 (d, J = 8.6, 4H), 6.58 (d, J = 8.8, 4H), 3.36 (q, J = 7.0, 4H), 1.17 ppm (t, J = 7.0, 6H); ^{13}C NMR (75 MHz, [D]CHCl$_3$): δ = 141.2, 130.1, 121.9, 113.3, 48.7, 13.3 ppm; HRMS (FAB$^+$): m/z: 361.177924 [M+H$^+$], $C_{20}H_{29}N_2S_2$ requires 361.1777218; UV/Vis (CH_3CN): λ (ε) = 260 nm (11990).

Synthesis of 5: The procedure described for the synthesis of **2** was repeated by using **1** (2.48 g, 10 mmol) and maleic anhydride (1.98 g, 20 mmol) in toluene (200 mL). After one hour at reflux the product slowly precipitated from the reaction mixture. After cooling, amide **5** was collected by filtration (4.00 g, yield: 90%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 13.10 (s, 2H), 10. 50 (s, 2H), 7.65 (d, J = 8.8, 4H), 7.50 (d, J = 6.7, 4H), 6.50 (d, J = 11.9, 2H), 6.34 ppm (d, J = 11.9, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 167.4, 163.8, 139.2, 130.7, 130.4, 132.1, 120.7 ppm; MS (ES$^+$): m/z: 112, 445 [M+H$^+$], $C_{20}H_{16}N_2O_6S_2$ requires 444; m.p. 191.0–193.0 °C.

Synthesis of 6: Acetic anhydride (7 mL) and anhydrous sodium acetate (0.65 g, 8 mmol) were added to **5** (4.14 g, 9.3 mmol) and the mixture was gently heated until there was complete dissolution of **5** (30 min). After cooling, the solution was poured onto ice water (50 mL) and the product was extracted with CH_2Cl_2 (3×50 mL). The organic phase was washed with water (3×50 mL) and dried over Na_2SO_4 anhydrous. After removing the solvent under reduced pressure, the product was recrystallised from acetone/cyclohexane to give a cream coloured solid (2.3 g, yield: 56%). ^{1}H NMR (250 MHz, [D]CHCl$_3$): δ = 7.60 (d, J = 8.8, 4H), 7.33 (d, J = 8.8, 4H), 6.86 ppm (s, 4H); ^{13}C NMR (75 MHz, [D] CHCl$_3$): δ = 169.2, 136.4, 134.3, 130.4, 128.0, 126.5 ppm; MS (FAB $^+$) m/z: 204, 409 [M+H$^+$], $C_{20}H_{12}N_2O_4S_2$ requires 408; UV/Vis (CH_3CN): λ (ε) = 260 nm (19613); elemental analysis (%) cald for $C_{20}H_{12}N_2O_4S_2$: C 58.81, H 2.96, N 6.86, S 15.70; found: C 58.46, H 2.84, N 6.56, S 15.90; m.p. 188–190 °C.

Synthesis of 8: Compound **7** (1.16 g, 4.25 mmol) dissolved in CH_2Cl_2 (10 mL) was added dropwise to a solution of **3** (0.634 g, 2.08 mmol) in dry CH_2Cl_2 (25 mL) under nitrogen. The resulting solution was stirred for 48 h at room temperature. The solvent was then removed under reduced pressure and the residue was purified by flash chromatography by using a gradient mixture of solvent as an eluent, which ranged from CH_2Cl_2/hexane 90:10 v/v; to eliminate the first impurities, pure CH_2Cl_2 and finishing with CH_2Cl_2/ethylacetate 95:5 v/v to obtain the desired product **5** as a yellow solid (0.24 g, 21% yield). ^{1}H NMR (250 MHz, [D]CHCl$_3$): δ = 7.31 (d, J = 7.0, 4H), 6.63 (d, J = 7.0, 4H), 6. 63 (s, 4H), 3.68 (t, J = 7.6, 4H), 3.46 (t, J = 6.7, 4H), 3.36 (q, J = 8.7, 4H), 1.15 ppm (t, J = 7.0, 6H); ^{13}C NMR (75 MHz, [D] CHCl$_3$): δ = 170.6, 147.8, 134.2, 134.1, 123.2, 112.2, 47.5, 44.5, 34.8, 12.2 ppm; HRMS (ES$^+$): m/z: 551.1807 [M+H$^+$], $C_{28}H_{31}N_4O_4S_2$ requires 551.1787; m.p. 120–121 °C; UV/Vis (CH_3CN): λ (ε) = 260 nm (15021).

Synthesis of 10: Compound **1** (0.59 g, 2.34 mmol) and **9** (0.6 g, 4.68 mmol) were dissolved in dry 1,4-dioxane (10 mL). The solution was stirred under reflux for 4 h. After cooling, the solvent was removed by lyophilisation. The residue was washed with acetonitrile, filtered off and dried under vacuum (0.8 g, yield: 73%).

^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 7.17 (d, J = 8.5, 4H), 7.05 (s, 4H), 6.99 (t, J = 6.7, 2H), 6.76 (d, J = 8.55, 4H), 4.82 ppm (d, J = 6.7, 4H). ^{13} C NMR (75 MHz, [D$_6$]DMSO): δ = 171.2, 147.3, 134.8, 133.6, 122.9, 113.0, 46.2 ppm; HRMS (FAB$^+$): m/z: 466.078253 [M^+], $C_{22}H_{18}N_4O_4S_2$ requires 466.076949; UV/Vis (THF): λ (ε) = 260 nm (18762).

Procedure for the preparation of 11, 12 and 13: The reaction of **6**, **8** and **10** with L-cysteine methyl ester hydrochloride was carried out according to the following general procedure.

Solvents were deoxygenated by using the following method: the sample container, provided with a magnetic stirrer, was subjected to a partial vacuum that was broken with nitrogen. This process was repeated at least five times. A deoxygenated water solution (20 mL) of L-cysteine methyl ester hydrochloride (85.5 mg, 0.5 mmol) was added to a deoxygenated solution of disulfide (0.25 mmol) in acetonitrile (20 mL) (THF for **10**) and was stirred under nitrogen. The yellow solution (pH 4.4) became colourless in the first 15 minutes. After 2 h the solution was concentrated under reduced pressure and the aqueous layer was lyophilised to give a white solid, which was used in the photolysis experiments without further purification.

An approximated 90% conversion of starting material disulfide to expected product was estimated from ^{1}H NMR spectroscopic data of the reaction mixture.

*Reaction mixture of **11***: ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 8.87 (s, broad, $-NH_3^+Cl^-$), 7.74 (d, phenyl), 7.68 (d, phenyl), 7.62 (d, phenyl), 7.35 (m, phenyl), 7.1 (m, phenyl), 4.61 (m), 4.36 (m), 4.30 (m), 3.76 (s), 3.75 (s), 3.74 (s), 3.72 (s), 3.36 (m), 2.68 ppm (m); HRMS (FAB$^+$): m/z:

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

679.100871 [$M+H^+$], $C_{28}H_{30}N_4O_8S_4$ requires 679.1024; UV/Vis (H_2O): λ (ε) = 260 nm (13066).

Reaction mixture of ***12***: ^{1}H NMR (250 MHz, [D_4] CH_3OH): δ = 7.31(phenyl), 6.75 (phenyl), 4.5 (m), 3.98 (m), 3.91(s), 3.90 (s), 3.87 (s), 3.84 (q, J = 4.8), 3.69 (m), 3.66 (m), 3.57 (m), 3.45 (m,), 3.3 (d), 3.25 (d), 3.15 (m), 2.50 (m, 1H), 2.42 (m, 1H), 1.36 (t, J = 7.3), 1.19 (t, J = 7, 6H), 0.98 ppm (t, J = 7); HRMS (FAB$^+$) m/z: 821.249671 [$M+H^+$], $C_{36}H_{48}N_6O_8S_4$ requires 821.249475; UV/Vis (H_2O): λ (ε) = 260 nm (13691).

Reaction mixture of ***13***: ^{1}H NMR (250 MHz, [D_4]CH_3OH): δ = 7.21 (d, 4H, phenyl), 6.8 (d, 4H, phenyl), 4.46 (m, 1H), 4.55 (m, 1H), 3.90 (s), 4.07 (m, 2H), 3.89 (s), 3.86 (s), 3.84 (s), 3.76 (m), 3.26 (m), 2.55 ppm (2H, m); HRMS (FAB$^+$): m/z: 737.1565 [$M+H^+$], $C_{30}H_{36}N_6O_8S_4$ requires 737.1556; UV/Vis (H_2O): λ (ε) = 260 nm (13854).

X-ray crystallography: CCDC-215839 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44)1223–336–033; or e-mail: deposit@ccdc.cam.ac.uk).

Acknowledgement

We thank the Universities of Sheffield and Leeds, White Rose Scheme and the BBSRC (LM) for financial support.

[1] H. Wu, *Am. J. Physiol.* **1929**, *90*, 562–566.
[2] C. B. Anfisen, *Science* **1973**, *181*, 223–230.
[3] C.-K. Chan, Y. Hu, S. Takahashi, D. L. Rousseau, W. A. Eaton, J. Hofrichter, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 1779–1784.
[4] M. C. Ramachandra Shastry, J. M. Sauder, H. Roder, *Acc. Chem. Res.* **1998**, *31*, 717–725.
[5] S. Takahashi, S. R. Yeh, T. K. Das, C. K. Chan, D. S. Gottfried, D. L. Rousseau, *Nat. Struct. Biol.* **1997**, *4*, 44–50.
[6] L. Pollack, M. W. Tate, N. C. Darnton, J. B. Knight. S. Gruner, W. A. Eaton, R. H. Austin, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10115–10117.
[7] H. Roder, G. A. Elove, S. W. Englander, *Nature* **1988**, *335*, 700–704.
[8] A. Matouschek, L. Serrano, E. M. Meiering, M. Bycroft, A. R. Fersht, *J. Mol. Biol.* **1998**, *277*, 973–983.
[9] S. T. Gladwin, P. A. Evans, *Folding Des.* **1996**, *1*, 407–417.
[10] K. Kuwajima, H. Yamaya, S. Miwa, S. Sugai, T. Nagamura, *Febs Lett.* **1987**, *221*, 115–118.
[11] K. Kuwajima, S. Sugai, T. Sugawara, *Biochemistry* **1991**, *30*, 2698–2706.
[12] G. V. Semisotnov, N. A. Rodionova, O. I. Razgulyaev, V. N. Uversky. , A. F. Gripas, R. I. Gillmanshin, *Biopolymers* **1991**, *31*, 119–128.
[13] A. F. Chaffotte, C. Cadieux, Y. Guillou, M. E. Goldberg, *Biochemistry* **1992**, *31*, 4303–4308.
[14] a) C. M. Phillips, Y. Mizutani, R. M. Hoachstrasser, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7292–7296; b) R. M. Ballew, J. Sabelko, M. Gruebele, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5759–5764; c) P. A. Thompson, W. A. Eaton, J. Hofrichter, *Biochemistry* **1997**, *36*, 9200–9210; d) W. A. Eaton, W. Munoz, P. A. Thompson, E. R. Henry, J. Hofrichter, *Acc. Chem. Res.* **1998**, *31*, 745–753; e) R. B. Dyer, F. Gai, W. H. Woodruff, R. Gilmanshin, R. H. Callender, *Acc. Chem. Res.* **1998**, *31*, 709–716; f) R. H. Callender, R. B. Dyer, R. Gilmanshin, W. H. Woodruff, *Annu. Rev. Phys. Chem.* **1998**, *49*, 173–202; g) W. A. Eaton, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 5897–5899.
[15] a) C. M. Jones, W. A. Eaton, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11860–11864; b) T. Pascher, J. P. Chesick, J. R. Winkler, H. B. Gray, *Science* **1996**, *271*, 1558–1560; c) J. R. Telford, P. Wittung-Stafshede, H. B. Graay, J. R. Winkler, *Acc. Chem. Res.* **1998**, *31*, 755–763; d) E. Chen, P. Wittung-Stafshede, D. S. Kliger, *J. Am. Chem. Soc.* **1999**, *121*, 3811–3817.
[16] D. Mendel, J. A. Ellman, P. G. Schultz, *J. Am. Chem. Soc.* **1991**, *113*, 2758–2760.
[17] C. Chang, T. Fernandez, R. Panchal, H. Bayley, *J. Am. Chem. Soc.* **1998**, *120*, 7661–7662.
[18] T. Okuno, S. Hirota, O. Yamauchi, *Biochemistry* **2000**, *39*, 7538–7545.
[19] J. W. Walker, G. P. Reid, J. A. McCray, D. R. Trentham, *J. Am. Chem. Soc.* **1988**, *110*, 7170–7177.
[20] A. Barth, K. Hauser, W. Mantele, J. E. T. Corrie, D. R. Trentham, *J. Am. Chem. Soc.* **1995**, *117*, 10311–10316.
[21] H. S. M. Lu, M. Volk, Y. Kholodenko, E. A. Gooding, R. M. Hochstrasser, W. F. DeGrado, *J. Am. Chem. Soc.* **1997**, *119*, 7173–7180.
[22] H. S. M. Lu, M. Volk, Y. Kholodenko, E. A. Gooding, R. M. Hochstrasser, W. F. DeGrado, *J. Phys. Chem. B* **1997**, *101*, 8607–8616.
[23] M. Volk, *Eur. J. Org. Chem.* **2001**, 2605–2621.
[24] T. W. Scott, S. N. Liu, *J. Phys. Chem.* **1989**, *93*, 1393–1396.
[25] N. A. Borisevich, S. V. Mel'nichuk, S. A. Tikhomirov, G. B. Tolstorozev, in *Ultrafast Phenomena in Spectroscopy, Vol. 49*, (Eds.: E. Klose, B. Wilhelmi), Springer, Berlin, **1990**, *49*, pp. 276–281.
[26] N. P. Ernsting, T. Bultmann, *J. Phys. Chem.* **1996**, *100*, 19417–19424.
[27] T. Koenig, H. Fischer, in *Free Radicals, Vol. 1*, (Ed. J. K. Kochi), Wiley, USA, **1973**, pp. 157–158.
[28] N. P. Ernsting, *Chem. Phys. Lett.* **1990**, *166*, 221–226.
[29] a) O. Ito, M. Matsuda, *J. Am. Chem. Soc.* **1982**, *104*, 568–572; b) O. Ito, M. Matsuda, *J. Phys. Chem.* **1984**, *88*, 1002–1005.
[30] a) G. E. Means, R. E. Feeney, *Chemical Modifications of Proteins* **1971**, Chapter 6, Holden-Day, San Francisco, California; b) Y. Kanaoka, *Angew. Chem.* **1977**, *89*, 142; *Angew. Chem. Int. Ed. Engl.* **1977**, *16*, 137–147; c) H. J. Tae, *Methods Enzymol.* **1983**, *91*, 599–600; d) R. Aggeler, K. Chicas-Cruz, S. X. Cai, J. F. W. Keana, R. A. Capaldi, *Biochemistry* **1992**, *31*, 2956–2961; e) S. K. Wright, R. E. Viola, *Anal. Biochem.* **1998**, *265*, 8–14; f) P. Schelte', C. Boeckler, B. Frisch, F. Schuber, *Bioconjugate Chem.* **2000**, *11*, 118–123.
[31] P. T. Litak, J. M. Kauffman, *J. Heterocycl. Chem.* **1994**, *31*, 457–479.
[32] P. O. Tawney, R. H. Snyde, R. P. Conger, K. A. Leibbrand, C. H. Stiteler, A. R. Williams, *J. Org. Chem.* **1961**, *26*, 15–21.
[33] a) N. A. Borisevich, Ya. N. Malkin, Sh. Ruziev, S. V. Melnichuk, S. A. Tikhomirov, G. B. Tolstorozev, V. A. Kuz'min, *Bull. Acad. Sci. USSR Div. Chem. Sci. (Engl. Transl.)* **1990**, *39*, 468–471; b) Y. Hirata, T. Niga, T. Okada, *Chem. Phys. Lett.* **1994**, *221*, 283–288.
[34] The timescale of the rise in the thiyl radical absorption was not determined due to insufficient time resolution. We assume it is similar to the reported value for bis(*p*-aminophenyl) disulfide.
[35] N. A. Borisevich, S. V. Melnichuk, S. A. Tikhomirov, G. B. Tolstorozev, *Bull. Acad. Sci. USSR Phys. Ser* **1992**, 2, 184–189.
[36] J. A. Riddick, W. B. Bunger, T. K. Sakano, in *Techniques of Organic Chemistry, Vol. 2*, 4th ed., (Ed.: A. Weissberger), Wiley-Interscience, New York, **1986**, pp. 75, 190, 582.
[37] G. D. Reid, D. J. Whittaker, M. A. Day, D. A. Turton, V. Kayser, J. M. Kelly, G. S. Beddard, *J. Am. Chem. Soc.* **2001**, *123*, 5518–5527.

Received: July 30, 2003
Revised: November 17, 2003 [F5405]

© 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim