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Short-length dimethoxynitrophenyl photo-cleavable crosslinkers, synthesis and photolysis

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ABSTRACT

In order to have short photo-cleavable crosslinkers more suitable for the intramolecular crosslinking; we have synthesized homo-bifunctional photo-cleavable sulfhydryl groups crosslinkers that represent distances of 2.6–5.4 Å between the two reactive sites. These chemical probes were able to react with two equivalents of cysteines, and to photo-release them very efficiently by near-UV irradiation.

1. Introduction

Photolabile protecting groups, or "caging" groups, have become important tools for the study of important biological events such as enzymatic activity, cellular signaling and photoregulation of proteins conformational changes. In the past few years, the use of caged compounds, molecules that have been rendered biologically inert by chemical modification by photolabile protecting groups, has witnessed a considerable increase because of their high degree of spatial and temporal controllability [1]. Among the most popular caging groups are the 2-nitrophenyl derivatives due to their unique properties that include stability under ambient light, clean cleavage upon exposure to UV irradiation and fast fragmentation reactions upon photoexcitation. A photo-cleavable, 2-nitrobenzyl, crosslinker was already used to connect ribosome-inactivating protein and targeting molecules such as monoclonal antibodies or lectins. The photolytic fragmentation of this linker resulted in the release of a nonmodified toxin that is fully active in a cell-free translation system [2].

Chemical crosslinking, a process that forms covalent bonds between different molecules (intermolecular) or parts of a molecule (intramolecular) by using bifunctional reagents containing reactive end groups that react with functional groups, such as primary amines and sulfhydryls, of amino acid residues, has largely contributed to the study of proteins structures and functions [3]. We previously reported the synthesis and the photochemical properties of new photo-cleavable crosslinkers with double 4,5-dimethoxy-2-nitrophenyl core that have a maximum light absorbance at 352 nm [4]. These crosslinkers were able to react and then to photo-release very efficiently two cysteines by means of the photo-controlled cleavage of those crosslinkers. In view to have photo-cleavable crosslinkers more suitable for the intramolecular crosslinking; we have performed the synthesis of new crosslinkers with shorter distances between the two reactive sites by applying different synthetic methods. These new photo-cleavable crosslinkers should allow trapping macromolecules in distinct conformations.

2. Materials and methods

2.1. General

All chemicals were purchased from Sigma–Aldrich, Acros or Alfa Aesar in analytical grade. The NPE-ATP was purchased from Jena Bioscience. ^{1}H NMR and ^{13}C NMR were run at 200, 300 or 400 MHz and 50 or 75 MHz, respectively. Coupling constants (J) are quoted in Hz and chemical shifts (δ) are given in parts per million (ppm) using the residue solvent peaks as reference relative to TMS. ESI mass spectra were recorded on Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS. A Vydac Analytical C18 column (4.6 mm × 250 mm) or an Acclaim C18 column (4.6 mm × 250 mm) were used for HPLC analysis and a XTerra C18 column (10 mm × 250 mm) was used for HPLC purification.

2.2. Synthesis

2.2.1. 1,5-bis(4,5-Dimethoxy-2-nitrophenyl)pentane-1,5-dione **3a**

To a solution of HNO_3 (0.84 ml, 20 mmol) in distilled CH_2Cl_2 (10 ml) was added TFA (3 ml, 40 mmol) at −15 °C under argon atmosphere. After the mixture had been stirred for 30 min at −10 °C,

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a solution of the diketone **2a** (1.86 g, 5 mmol) in distilled CH_2Cl_2 (5 ml) wad added dropwise. The reaction was then stirred at 0 °C for further 2 h. The reaction was quenched by the addition of a saturated $NaHCO_3$ aqueous solution (20 ml) at 0 °C. The two layers were separated and the aqueous layer was extracted with 50 ml of CH_2Cl_2. The combined organic layers were washed with brine (1× 50 ml), dried over anhydrous $CaCl_2$, filtered and concentrated under reduced pressure. The yielded product was purified by flash chromatography (80:20 heptane/EtOAc). Yield: 51%, ^{1}H NMR (300 MHz, CDCl3) δ: 7.60 (s, 2H, H-3arom), 6.83 (s, 2H, H-6arom), 3.95 (s, 6H, OMe), 3.57 (s, 6H, OMe), 2.94 (t, J = 6.4 Hz, 4H, H-2 and H-4), 1.83 (t, J = 6.4 Hz, 2H, H-3). ^{13}C NMR (75 MHz, CDCl3) δ: 202.48, 154.51, 149.63, 138.15, 133.27, 108.89, 107.30, 57.04, 56.78, 41.57, 17.68.

2.2.2. 1,5-bis(4,5-Dimethoxy-2-nitrophenyl)pentane-1,5-diol ***4a***

To a stirred solution of 480 mg (1 mmol) of the diketone **3a** in CH_2Cl_2 (5 ml) were added successfully MeOH (80 ml) and $NaBH_4$ (113 mg, 3 mmol) at room temperature. The reaction was stirred for further 15 min. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in CH_2Cl_2 (50 ml) and washed with water (2× 50 ml), and brine (1× 50 ml). The organic layer was dried over anhydrous $CaCl_2$, filtered and concentrated under reduced pressure to give the pure diol **4a**. Yield: 98%. ^{1}H NMR (400 MHz, CDCl3) δ: 7.54 (s, 2H, H-3arom), 7.27 (s, 2H, H-6arom), 5.62 (m, 2H, H-1 and H-5), 3.97 (s, 6H, OMe), 3.92 (s, 6H, OMe), 1.77 (m, 6H, H-2 H-3 and H-4). ^{13}C NMR (75 MHz, CDCl3) δ: 154.05, 147.97, 139.76, 136.77, 109.37, 108.03, 69.64, 56.78, 56.68, 38.15, 30.06.

2.2.3. 1,3-bis(3,4-Dimethoxy)propane-1,3-diol ***5c***

To a stirred solution of 344 mg (1 mmol) of the enole **2c** in CH_2Cl_2 (5 ml) were added successfully MeOH (40 ml) and $NaBH_4$ (113 mg, 3 mmol) at room temperature. The reaction was stirred for further 15 min. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in CH_2Cl_2 (50 ml) and washed with water (2× 50 ml), and brine (1× 50 ml). The organic layer was dried over anhydrous $CaCl_2$, filtered and concentrated under reduced pressure to give the pure diol **5c**. Yield: 96%. ^{1}H NMR (300 MHz, CDCl3) δ: 6.82 (m, 6H, Harom), 4.85 (m, 2H, H-1 and H-3), 3.83 (s, 6H, OMe), 3.82 (s, 3H, OMe), 3.81 (s, 3H, OMe), 1.98 (m, 2H, H-2). ^{13}C NMR (75 MHz, CDCl3) δ: 149.02, 148.97, 148.40, 148.24, 137.11, 137.09, 117.96, 117.87, 111.04, 111.00, 108.98, 108.89, 74.74, 71.37, 55.97, 55.96, 55.90, 55.88, 47.71, 46.76.

2.2.4. 1,4-bis(4,5-Dimethoxy-2-nitrophenyl)butane-1,4-diol ***4b***

To a solution of HNO_3 (0.84 ml, 20 mmol) in distilled CH_2Cl_2 (10 ml) was added TFA (3 ml, 40 mmol) at −15 °C under argon atmosphere. The mixture was stirred for further 30 min at the same temperature. The reaction mixture was then cooled to −50 °C and another solution of the diol **5b** (2.33 g, 5 mmol) in distilled CH_2Cl_2 (5 ml) was added dropwise. The reaction was then stirred at −50 °C for further 2 h. The reaction was quenched by the addition of a saturated $NaHCO_3$ aqueous solution (20 ml). The two layers were separated and the aqueous layer was extracted with 50 ml of CH_2Cl_2. The combined organic layers were washed with brine (1× 50 ml), dried over anhydrous $CaCl_2$, filtered and concentrated under reduced pressure. The yielded product was purified by flash chromatography (80:20, heptane/EtOAc). Yield: 48%. ^{1}H NMR (200 MHz, CDCl3) δ: 7.56 (s, 1H, H-3arom), 7.54 (s, 1H, H-3′arom), 7.33 (s, 1H, H-6arom), 7.28 (s, 1H, H-6′arom), 5.53 (m, 2H, H-1 and H-4), 3.98 (s, 3H, OMe), 3.97 (s, 3H, OMe), 3.91 (s, 3H, OMe), 3.90 (s, 3H, OMe), 2.00 (m, 4H, H-2 and H-3). ^{13}C NMR (50 MHz, CDCl3) δ: 154.20, 154.19, 148.36, 148.35, 140.23, 140.20, 136.16, 136.13, 109.71, 109.60, 108.50, 108.47, 69.93, 69.73, 57.04, 56.76, 56.74, 56.73, 35.37, 35.29.

2.2.5. 1,3-bis(4,5-Dimethoxy-2-nitrophenyl)propane-1,3-diol ***4c***

This product was synthesized by following the same procedure as for preparing the diol **4b**. Yield: 39%. ^{1}H NMR (200 MHz, CDCl3) δ: 7.51 (s, 1H, H-3arom); δ: 7.44 (s, 1H, H-3′arom), 7.34 (s, 1H, H-6arom), 7.27 (s, 1H, H-6′arom), 5.65 (m, 2H, H-1 and H-3), 3.93 (s, 3H, OMe), 3.92 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.85 (s, 3H, OMe), 2.28 (m, 2H, H-2). ^{13}C NMR (50 MHz, CDCl3) δ: 153.96, 153.90, 148.05, 147.97, 139.74, 139.42, 135.88, 135.38, 109.67, 109.42, 108.22, 107.84, 70.16, 70.11, 56.96, 56.95, 56.74, 56.73, 45.95.

2.2.6. General procedure for the bromination of diols ***4a–c***

1 mmol of the starting material was cooled to 0 °C in a round-bottom flask with an ice bath under argon atmosphere. Acetyl bromide (3 ml) and hydrobromic acid (0.1 ml) were successively added by using a syringe. The reaction mixture was then stirred for 30 min at 0 °C and for further 2 h at room temperature. The reaction was quenched by the addition of a saturated $NaHCO_3$ aqueous solution (50 ml) at 0 °C. The mixture was extracted with CH_2Cl_2 (3× 50 ml). The combined organic layers were washed with brine (1× 150 ml), dried over anhydrous $CaCl_2$, filtered and concentrated under reduced pressure. The obtained products were purified by flash chromatography (95:5, heptane/EtOAc).

2.2.7. 1-[1,5-Dibromo-5-(4,5-dimethoxy-2-nitrophenyl)pentyl]-4,5-dimethoxy-2-nitro-benzene

6a

Yield: 72%. ^{1}H NMR (300 MHz, CDCl3) δ: 7.43 (s, 1H, H-3arom), 7.42 (s, 1H, H-3′arom), 7.21 (s, 1H, H-6arom), 7.20 (s, 1H, H-6′arom), 5.85 (m, 2H, H-1 and H-5), 4.00 (s, 3H, OMe), 3.99 (s, 3H, OMe), 3.93 (s, 3H, OMe), 3.92 (s, 3H, OMe), 2.24 (m, 6H, H-2, H-3 and H-4). ^{13}C NMR (75 MHz, CDCl3) δ: 153.76, 153.75, 148.91, 148.90, 140.27, 140.23, 131.63, 131.57, 112.06, 111.95, 107.75, 107.74, 56.96, 56.95, 56.84, 56.83, 48.93, 48.69, 39.81, 39.55, 26.85. HRMS: calculated: 608.0242, found: 608.0238 $(M+NH_4)^+$.

2.2.8. 1-[1,4-Dibromo-4-(4,5-dimethoxy-2-nitrophenyl)butyl]-4,5-dimethoxy-2-nitro-benzene

6b

Yield: 68%. ^{1}H NMR (300 MHz, CDCl3) δ: 7.49 (s, 1H, H-3arom), 7.44 (s, 1H, H-3′arom), 7.23 (s, 1H, H-6arom), 7.22 (s, 1H, H-6′arom), 5.85 (m, 2H, H-1 and H-4), 4.03 (s, 3H, OMe), 4.02 (s, 3H, OMe), 3.96 (s, 3H, OMe), 3.94 (s, 3H, OMe), 2.30 (m, 4H, H-2, and H-3). ^{13}C NMR (75 MHz, CDCl3) δ: 153.86, 153.77, 149.57, 149.08, 140.30, 140.10, 131.37, 131.29, 111.83, 111.80, 107.90, 107.82, 57.14, 57.01, 56.87, 56.78, 48.25, 47.82, 39.62, 39.50. HRMS: calculated: 594.0088, found: 594.0081 $(M+NH_4)^+$.

2.2.9. 1-[1,3-Dibromo-3-(4,5-dimethoxy-2-nitrophenyl)propyl]-4,5-dimethoxy-2-nitro-benzene

6c

Yield: 68%. ^{1}H NMR (300 MHz, CDCl3) δ: 7.49 (s, 1H, H-3arom), 7.44 (s, 1H, H-3′arom), 7.27 (s, 1H, H-6arom), 7.24 (s, 1H, H-6′arom), 6.21 (m, 2H, H-1 and H-3), 4.01 (s, 3H, OMe), 3.98 (s, 3H, OMe), 3.96 (s, 3H, OMe), 3.95 (s, 3H, OMe), 2.86 (m, 2H, H-2). ^{13}C NMR (75 MHz, CDCl3) δ: 153.86, 153.77, 149.01, 149.00, 140.07, 140.06, 130.18, 130.17, 112.61, 112.60, 107.62, 107.61, 56.79, 56.77, 56.69, 56.68, 49.13, 47.13, 47.02. HRMS: calculated: 579.9926, found: 594.9925 $(M+NH_4)^+$.

2.2.10. General procedure for the synthesis of compounds ***7a–c***

To a stirred solution of one of the bromide derivatives (**6a–c**) (0.5 mmol) in degazed MeOH (3 ml) were added successively the *N*-bezoylcysteine methyl ester **8** (36 mg, 1.5 mmol) and $NaHCO_3$ (25 mg, 3 mmol) under argon atmosphere. When the TLC shows that the reaction is finished, 1 g of silica gel was added and the sol-

vent was removed under reduced pressure. The product was then purified by flash chromatography (75:25, heptane/EtOAc).

2.2.11. 2-(Benzoylamino)-3-[[5-[[(2R)-2-(benzoylamino)-3-methoxy-3-oxopropyl]thio]-1,5-bis(4,5-dimethoxy-2-nitrophenyl)pentyl]thio]-(2R)-propanoic acid methyl ester ***7a***

Yield: 71%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.77 (m, 4H, H-aromatic), 7.4 (m, 8H, H-aromatic), 7.16 (m, 2H, H-aromatic), 6.83 (m, 2H, NH), 4.81 (m, 2H, CHCOO), 4.72 (m, 2H, H-1 and H-5), 3.92–3.76 (m, 18H, OMe and COOMe), 3.04–2.85 (m, 4H, CH_2S), 1.79–1.50 (m, 6H, H-2, H-3, and H-4). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.23, 171.17, 167.08, 166.91, 153.56, 153.48, 147.93, 147.92, 142.06, 141.89, 133.71, 133.67, 132.70, 132.64, 132.33, 132.08, 128.99, 128.78, 127.34, 127.32, 110.85, 110.78, 107.63, 107.54, 56.65, 56.59, 56.57, 56.50, 53.04, 52.97, 52.63, 52.56, 45.03, 44.34, 36.77, 36.55, 33.95, 33.80, 25.49.

2.2.12. 2-(Benzoylamino)-3-[[4-[[(2R)-2-(benzoylamino)-3-methoxy-3-oxopropyl]thio]-1,4-bis(4,5-dimethoxy-2-nitrophenyl)butyl]thio]-(2R)-propanoic acid methyl ester ***7b***

Yield: 65%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.83 (m, 4H, H-aromatic), 7.48 (m, 8H, H-aromatic), 7.14 (m, 2H, H-aromatic), 6.91 (m, 2H, NH), 4.81 (m, 2H, CHCOO), 4.75 (m, 2H, H-1 and H-4), 3.97–3.65 (m, 18H, OMe and COOMe), 3.24–3.02 (m, 4H, CH_2S), 1.79–1.50 (m, 4H, H-2 and H-3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.97, 170.91, 167.36, 167.17, 153.48, 153.38, 147.88, 147.85, 141.81, 141.76, 133.30, 133.23, 132.20, 132.06, 131.96, 131.77, 128.74, 128.67, 127.25, 127.16, 110.85, 110.51, 107.37, 107.59, 56.52, 56.46, 56.36, 56.23, 52.93, 52.85, 52.49, 52.28, 45.12, 44.18, 35.40, 34.97, 33.87, 33.62.

2.2.13. 2-(Benzoylamino)-3-[[3-[[(2R)-2-(benzoylamino)-3-methoxy-3-oxopropyl]thio]-1,3-bis(4,5-dimethoxy-2-nitrophenyl)propyl]thio]-(2R)-propanoic acid methyl ester ***7c***

Yield: 65%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.76 (m, 4H, H-aromatic), 7.44 (m, 8H, H-aromatic), 7.20 (m, 2H, H-aromatic), 6.84 (m, 2H, NH), 4.90 (m, 2H, CHCOO), 4.85 (m, 2H, H-1 and H-3), 4.04–3.59 (m, 18H, OMe and COOMe), 3.24–2.48 (m, 6H, CH_2S and H-2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.30, 170.92, 167.36, 167.31, 153.87, 153.65, 148.46, 147.36, 142.78, 142.31, 133.86, 133.72, 132.52, 132.28, 131.63, 131.25, 129.05, 128.94, 127.68, 127.53, 111.33, 111.98, 108.09, 107.60, 56.97, 56.84, 56.75, 56.70, 53.25, 53.04, 52.82, 52.74, 43.50, 43.20, 41.97, 33.94, 33.68.

2.3. Stability of crosslinkers *6a–c*

Crosslinkers were initially dissolved in CH_2Cl_2 at a concentration of 10 mM. At zero time, the tested compounds were diluted in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) to a concentration of 0.05 mM, incubated at 4 °C, and 500 ml aliquots were injected at various times to HPLC analysing using an Acclaim C18 column (4.6 mm × 250 mm); elution was performed at a flow rate of 1 ml/min with a linear gradient of acetonitrile in an aqueous solution of 0.1% TFA from 0 to 100% (v/v) over a period of 30 min. Absorbance was monitored at 352 nm.

2.4. Reactivity of crosslinkers *6a–c* toward thiol groups

Crosslinkers **6a–c** were initially dissolved in CH_2Cl_2 at a concentration of 10 mM. At zero time, 25 μl of the stock solution of the chemical probes were mixed with 4.75 ml of a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) containing 1 mM of *N*-bezoylcysteine methyl ester **8** at 4 °C. The reaction was followed as in the stability studies.

2.5. Photolysis

A solution (4 ml) of 50 μM of one of the compounds **7a–c** in a mixture 50/50 of EtOH and phosphate buffer (50 mM, pH 8.0) containing 0.5 mM DTT was exposed to a 1000 W Hg lamp from Hanovia focused on the entrance slit of a monochromator at 364.5 nm (±0.2 nm). The reactions were monitored by UV where aliquots of samples (500 μl) were injected into a Waters 600E HPLC carried out on a Acclaim C18 column (4.6 nm × 300 nm); elution was performed at a flow rate of 1 ml/min with a linear gradient of acetonitrile in an aqueous solution of 0.1% TFA from 0 to 100% (v/v) over a period of 30 min. The compounds were detected by a Waters 2996 PDA detector operating between 200 and 600 nm. Compounds **7a–c** have a retention time of 27.3, 25.9 and 25.5 min, respectively. The *N*-bezoylcysteine methyl ester **8**, which has a retention time of 18.5 min, was quantified during the photolysis of **7a–c**. Numerous photolytic by-products were also detected presumably due to the photochemical instability of the 1-(4,5-dimethoxy-2-nitrozophenyl)alkyl-1-one derivatives **9a–c**.

2.6. Quantum yield determination

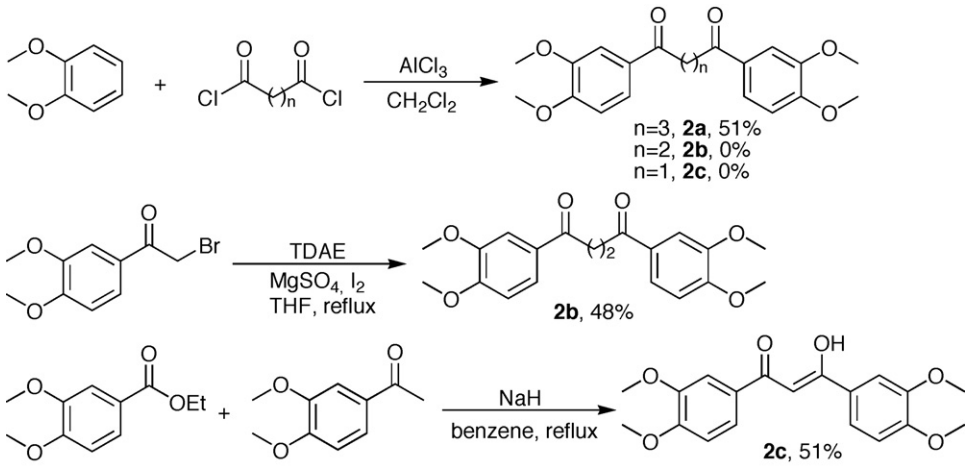
The quantum yield for the photoconversion of compounds **7a–c**, was determined by comparison with the photolysis of 1-(2-nitrophenyl)ethyl-ATP (NPE-ATP) (Φ = 0.63) which was taken as reference in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) at 25 °C. These compounds were tested at identical optical densities at the irradiation wavelengths used. Accordingly, the mixture of one of the compounds **7a–c** and NPE-ATP was photolysed by continuous irradiation at 315 nm, and aliquots were subjected to reversed-phase HPLC to determine the extent of the photolytic conversions. HPLC analysis was carried out on an Acclaim C18 column (4.6 mm × 250 mm); elution was performed at a flow rate of 1 ml/min with a linear gradient of acetonitrile in an aqueous solution of 0.1% TFA from 0 to 100% (v/v) over a period of 30 min. Quantum yields were calculated by considering conversions up to 20%, to limit as much as possible errors due to undesired light absorption during photolysis.

3. Results and discussion

3.1. Synthesis

The synthesis of the photo-cleavable crosslinkers **6a–c** is outlined in Schemes 1 and 2. While the diketone **2a** [5] was obtained from veratrole by Friedel–Crafts reaction using previously described reaction conditions [4]; the later failed to provide the diketones **2b** and **c**. Hence, the diketone **2b** was synthesized by reductive coupling of the commercially available 2-bromo-1-(3,4-dimethoxyphenyl)ethanone by using tetrakis(dimethylamino)ethylene (TDAE) as the reducing agent and iodine as a catalyst [6]. On the other hand, the dibenzoylmethane derivative **2c**, under its enolic form, was synthesized by claisen acylation as already described by Chosho et al. [7] (Scheme 1).

Usual nitration of the diketone **2a** by nitric acid and TFA at 0 °C furnished the *ortho* nitro derivative **3a** with a good yield [8,4]. However, nitration of compounds **2b** and **c** under these conditions failed. This is probably due to the fact that the diketone **2b** undergoes a Paal–Knorr reaction to give the corresponding furan derivative under the used acidic conditions [9]. Meanwhile, the aromatic rings of the compound **2c** became very deactivated toward the electrophilic aromatic substitution due to their conjugation with the α, β unsaturated system. Nevertheless, nitration of the diols **5b** [10]



Scheme 1. Synthesis of the diketones derivatives **2a**–**c**.

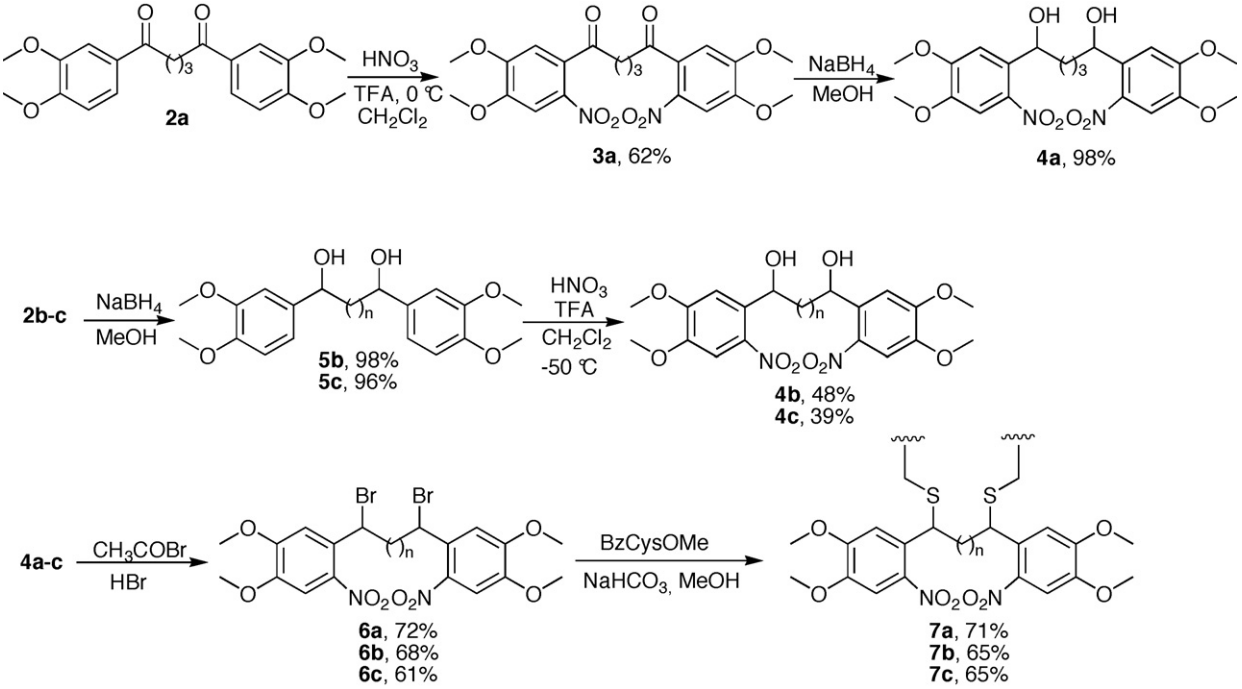
and **5c**, synthesized from the diketones **2b** and **c** by reduction with $NaBH_4$ in methanol, has been achieved in satisfactory yields using nitric acid and TFA at −50 °C (Scheme 2). The regioselectivity of the nitration reaction is in agreement with the previously obtained results by Hess and co-workers [11]. Furthermore, the position of the nitration can be unambiguously assigned by ^{1}H NMR spectroscopy where no coupling constants ^{3}J or ^{4}J between the two aromatic signals could be detected. Final treatment of the diols **5a**–**c** by acetyl bromide and hydrobromic acid [12] afforded the sulfhydryl group crosslinkers **6a**–**c**, with satisfactory yields. Thus we were able to have the attempted crosslinkers with carbon chain spacers ranging from 2.6 to 5.4 Å.

To demonstrate the ability of these molecules to photoregulate the crosslinking of two sulfhydryl groups, the crosslinkers **6a**–**c** were reacted with excess amount of *N*-benzoylcysteine methyl ester **8** [13] in a slightly alkaline medium to give the caged cysteines **7a**–**c**. The *N*-benzoylcysteine methyl ester **8** was chosen to facilitate the UV detection of the released sulfhydryl group by HPLC analysis.

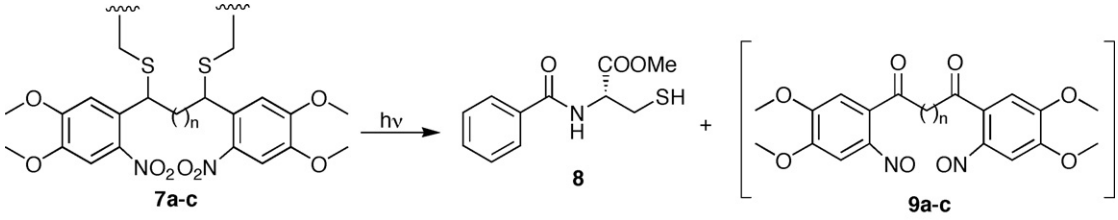
3.2. Stability and reactivity of crosslinkers *6a–c* toward thiol groups

The reactivities of the crosslinkers **6a**–**c** toward thiols and their half-time of reaction ($t_{1/2react}$) were checked by means of reverse phase HPLC (RP-HPLC) analysis of 50 μM solution of **6a**–**c** in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) in the presence of 20-fold excess of *N*-benzoylcysteine methyl ester **8** at 4 °C. RP-HPLC monitoring of the reaction shows the concomitant disappearance of **6a**–**c** and the formation of the mono- and di-substituted crosslinkers by the cysteine derivative **8**. The measurement of $t_{1/2react}$ was adjusted for the disappearance of crosslinkers **6a**–**c**. The crosslinker **6b** revels to be the most reactive with a $t_{1/2react}$ of 83 min, while **6a** and **6c** react with the cysteine **8** with $t_{1/2react}$ of 150 and 179 min, respectively (Table 1).

The hydrolytic stability of our crosslinkers was also checked by RP-HPLC analysis of 50 μM solution of **6a**–**c** in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) at 4 °C in the absence of light. A $t_{1/2}$ of hydrolysis ($t_{1/2hydro}$) of 26 h was measured



Scheme 2. Synthesis of the crosslinkers **6a**–**c**.



Scheme 3. Photo-degradation of the caged cysteines **7a**–**c**.

Table 1
Stability and reactivity of crosslinkers **6a**–**c** ($t_{1/2react}$ half-time of the reaction of the crosslinkers with the thiol derivative **8**, $t_{1/2react}$ half-time of the hydrolysis reaction).

Compound	$t_{1/2react}$ (min)	$t_{1/2hydro}$ (h)
6a	150	25
6b	83	26
6c	179	26

(Fig. 1), indicating that the crosslinkers **6a**–**c** hydrolysis is 8–16 times slower than their sulfhydryl substitution reactions under these conditions. Fig. 1 shows the disappearance of compound **6b** in a mixture EtOH and phosphate buffer, in presence and absence of excess amount of the thiol derivative **8**.

3.3. Photochemical properties

The photolytic reactions of **7a**–**c** (50 μM in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0), containing 0.5 mM DTT at λ = 365 nm) were analysed by UV spectroscopy and HPLC (Scheme 3). All three compounds showed an increase in absorbance at 375 and 280 nm and decrease at 320 nm (Fig. 2A). The isosbestic points at 350 and 300 nm indicate that the photolytic reaction is homogenous. The RP-HPLC analysis (Fig. 2B) of these reactions depicted the concomitant disappearance of the starting compounds and an almost quantitative liberation of 2 equivalents of the unmasked thiol derivative **8** (Table 2). The expected nitroso derivatives **9a**–**c** side-products could not be detected presumably due to their chemical instability.

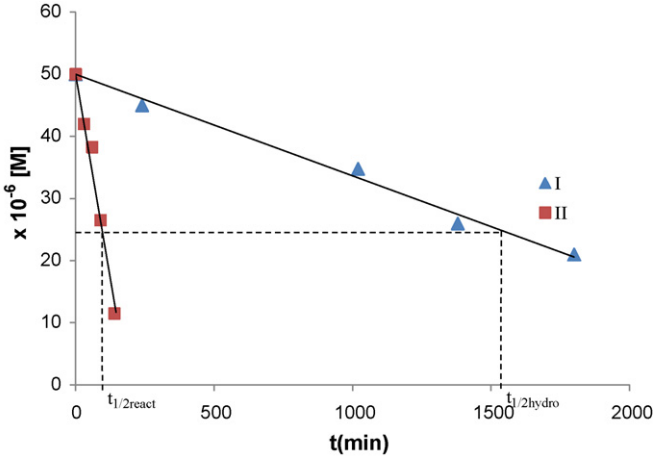


Fig. 1. Decrease of **6b** concentration in function of time. (I) 50 μM solution of **6b** in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) at 4 °C. (II) A mixture of 50 μM of **6b** and 1 mM of the cysteine derivative **8** in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) at 4 °C.

Table 2
Summary of the properties of **7a**–**c** (ε_{352} absorption coefficient at 352 nm, d the distance between the two sulfhydryl groups, ϕ quantum yield).

Compound	d (Å)	ε_{352}	ϕ	Photo-released cysteine (%)
7a	5.4	9,240	0.59	190
7b	3.8	10,050	0.63	200
7c	2.6	9,350	0.63	198

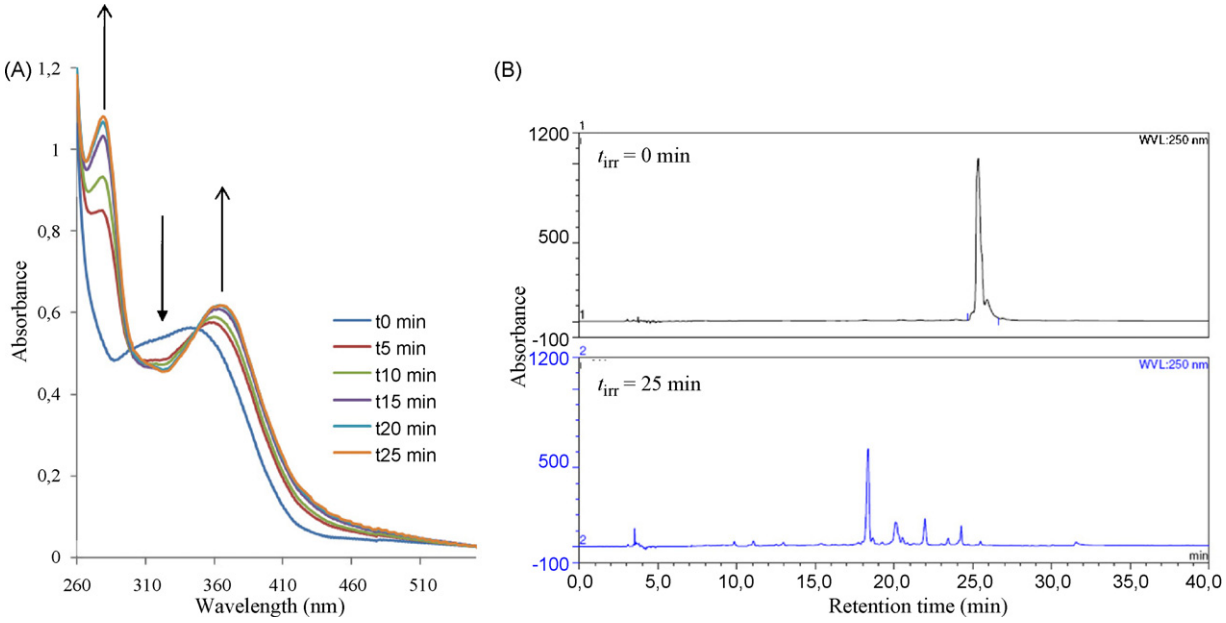


Fig. 2. (A) UV-spectral recording of the photolysis of **7b**. (B) HPLC analysis at 250 nm of the photolytic reaction, after 0 and 25 min of irradiation at 365 nm. **7b** has a retention time of 25.9 min and the appearing peak at 18.5 min corresponds to the *N*-benzoylcysteine methyl ester **8**.

Quantum yields of the photolytic reactions for **7a**–**c** were determined by competition with the 2-(nitrophenyl)ethyl-ATP reference molecule [14]. Quantum yields between 0.59 and 0.63 (Table 2) were determined in a mixture 50:50 of EtOH and phosphate buffer (50 mM, pH 8.0) leading to extremely high photochemical efficiencies (up to 6330 M^{-1} cm^{-1} at 352 nm) for the fragmentation of our photo-cleavable crosslinkers. Therefore, our new photo-cleavable crosslinkers, which can photo-release two cysteines very efficiently, should open new ways for the photoregulation of the proteins activities by intramolecular crosslinking.

In conclusion, we have described here the synthesis and the characterization of short-length photo-cleavable sulfhydryl groups crosslinkers with carbon chain spacers ranging from 2.6 to 5.4 Å. These molecules are able to cage and then to photo-release two cysteines leading to the photoregulation of crosslinkers cleavage. Thus, they should be interesting tools for the photoregulation of biological activities especially by intramolecular crosslinking of proteins.

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