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Chemistry of Bifunctional Photoprobes[1]

4. Synthesis of the Chromogenic, Cleavable, Water Soluble, and Heterobifunctional Sulfosuccinimidyl (*N*-methylamino Perfluoroaryl Azido Benzamido)-ethyl-1,3′-Dithiopropionate: An Efficient Protein Cross-Linking Agent

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Synthesis of a new photo cross-linking agent incorporating chromogenicity, cleavability, and water solubility is described. The high efficiency of nitrene insertion observed upon photolysis of perfluoroaryl azides into organic solvents and proteins is extended to the design and synthesis of new multifunctional cross-linking agents useful for protein–protein interactions. The new cross-linker sulfosuccinimidyl (perfluorobenzamido)-ethyl-1,3′-dithiopropionate (SFAD) **10** was conjugated to IgG and cross-linked to horseradish peroxidase (HRP). The analysis of the cross-linked product using ELISA assays leads to a higher yield of IgG–HRP cross-linked product than that seen with a similar nonfluorinated analog. The efficiency of photo cross-linking by SFAD is also extended to small molecule biotin via CH insertion and checked for the retention of binding affinity of the cross-linked product.

INTRODUCTION

The potential utility of photo cross-linking reagents in the topological mapping of the binding sites of receptors requires careful consideration of several design factors in the synthetic steps and an understanding of the productive vs nonproductive avenues for photoprecursors (*2*, *3*). The combination of chemical and photochemical activation for cross-linking two different proteins is a powerful procedure for identifying the specific amino acids involved within the protein–protein binding sites. Protein photo cross-linking agents typically contain a chemical functional group (e.g., succinimide, sulfosuccinimide, NCS, etc.) for nucleophilic substitution on the first protein, a disulfide bond, and a photochemically active group which remains chemically inert until photoactivation in the presence of a second protein (*4*). Following irradiation and photochemically induced covalent linking with the

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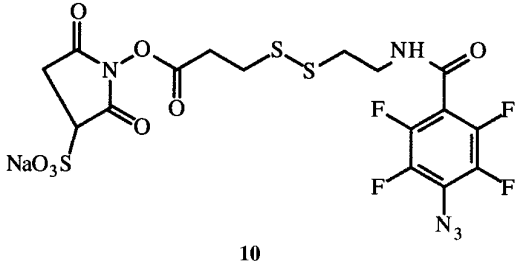
second protein, cleavage of the disulfide (S–S) bridge of the cross-linked protein results in the concomitant transfer of the marker (e.g., radioactivity or chromogenicity or fluorescence) to the specific amino acid(s) at which the cross-link occurs. Hence, a useful photo cross-linker should have a marker and be water soluble and cleavable under mild conditions (*5–8*). Introduction of additional features like fluorescence, chromogenicity, or provision for attachment of a radioactive marker may enhance the utility of cross-linking agents. However, the efficiency of photoconjugation and the stability of the covalent bond formed by the photoprecursors under the conditions used for the analysis of the cross-linked products essentially determine the final yield of the cross-linked conjugate (*9*). A high efficiency of photoconjugation is necessary for the recovery of the cross-linked product in sufficient yield for characterization by macroscopic techniques.

Study of the fundamental photochemistry of perfluoroaryl azides by Keana *et al.* (*10*, *11*) and the development of high efficiency heterobifunctional perfluoroaryl azide based chelating agents for covalent attachment to proteins and antibodies in our laboratory (*12–16*) and by others (*17*, *18*) suggest that perfluoroaryl azides are potentially good candidates as cross-linking agents. The promising nature of perfluoroaryl azides as cross-linkers stems from the fact that singlet perfluoroaryl nitrene rearranges more slowly to didehydroazipines, allowing the reactive intermediate to react with even unactivated CH bonds by formal insertion (*19–23*). Recently, we have shown from flash photolysis studies that the bifunctional nitrenes carrying electron withdrawing groups (amide and ester groups) exhibited a longer lifetime for singlet nitrenes (>250 ns), leading to the highest CH insertion in model solvents (*1*) and in proteins (*12*, *15*, *16*). Such observations prompted us to design and report, here, a first example of the synthesis of a chromogenic, cleavable, water soluble, and heterobifunctional sulfosuccinimidyl (*N*-methylamino perfluoroazido benzamido)-ethyl-1,3′-dithiopropionate **7** (Scheme **1**). We also wish to report the preparation of sulfosuccinimidyl (perfluoroaryl azido benzamido)-ethyl-1,3′-dithiopropionate **10** (Scheme **2**) and comparison of its relative cross-linking efficiency with the commercially available sulfosuccinimidyl 2-(*p*-azidosalicylamido)-ethyl-1,3′-dithiopropionate **11** (SASD) cross-linker. We have extended our studies to cross link small molecules and demonstrate the retention of native characteristics of biotin (e.g., binding affinity toward streptavidin) in the post-cross-linking stage.



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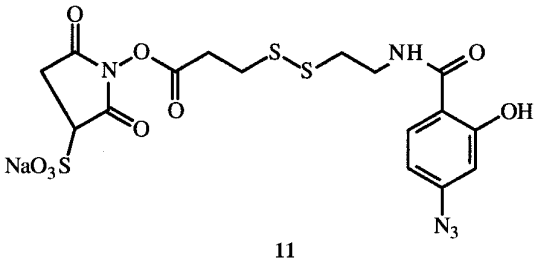
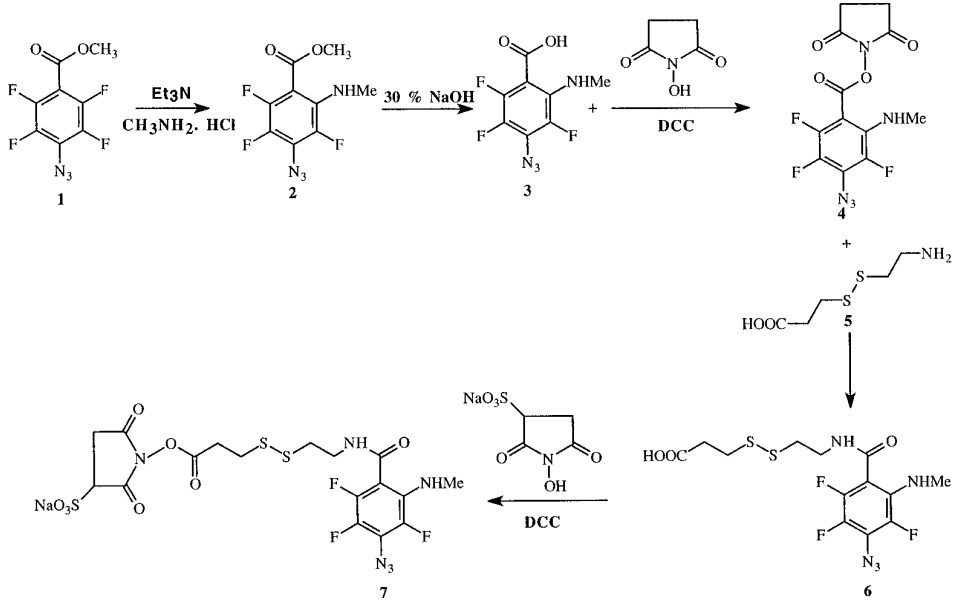


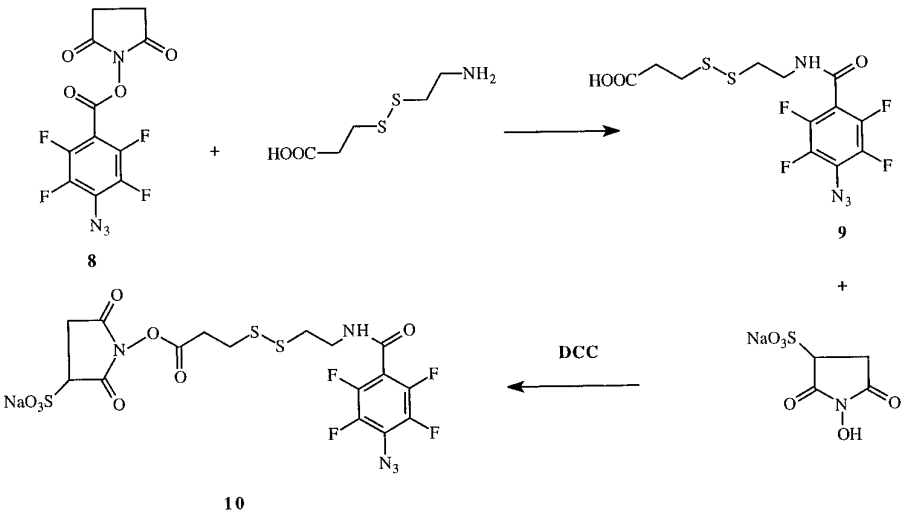
11

EXPERIMENTAL

All synthetic procedures were conducted in a dry nitrogen atmosphere using standard Schlenk tube techniques and prepurified solvents. Reactions involving the synthesis of azides were carried out under subdued light by wrapping the flasks with aluminum foil. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ on a Bruker 250 MHz spectrometer and chemical shifts are reported in ppm downfield from $SiMe_4$ for ^1H NMR. ^{19}F NMR chemical shifts are reported with respect to $CFCl_3$ as an external standard. Infrared spectra were recorded as KBr pellets on a Nicolet 20DXB FT-IR spectrophotometer. Elemental analysis for the new compounds were performed by Oneida Research Services, Inc. (New York). UV/visible spectra were recorded either in water or in buffer (0.1 M sodium phosphate,



SCHEME 1



SCHEME 2

150 mM NaCl, pH 7.4) on a Hewlett Packard (8452A) diode array spectrophotometer. Photolyses were carried out with a 200-W high-pressure Hg lamp with water as a filter. Compounds **5** (*24*) and **8** (*10*) were prepared by literature methods. The nonfluorinated cross-linker **11** is available commercially from Pierce Co.

Synthesis of ***2***

A solution of methyl-4-azidotetrafluorobenzoate **1** (1.97 g, 7.91 mmol), prepared as reported earlier (*10*), $MeNH_2 \cdot HCl$ (1.33 g, 19.78 mmol), and 8 ml Et_3N in 10 ml acetonitrile was refluxed for 18 h. The solution was cooled in ice and $Et_3N \cdot HCl$ was filtered and the solvent was evaporated in vacuum. The crude product was washed with $NaHCO_3$ solution and extracted into CH_2Cl_2 and dried using $MgSO_4$. The filtrate was evaporated to leave methyl 4-azido-*N*-methyl-2-amino trifluorobenzoate **2** (1.696 g, 51.6%). ^1H NMR ($CDCl_3$), δ, 7.00, (br, s, 1H), 3.91, (s, 3H), 3.06, (m, 3H). ^{19}F NMR ($CDCl_3$), δ, -135.47 (1F), -147.25 (1F), -163.95 (1F).

Synthesis of ***3***

A solution of methyl 4-azido-*N*-methyl-2-amino trifluorobenzoate **2** (583 mg, 2.24 mmol) in methanol was stirred overnight with 2 ml 30% aqueous NaOH at RT. The solution was acidified by 2 N HCl in an ice bath to pH <1 and extracted by $CHCl_3$. The extract was dried using $MgSO_4$ and the solvent was evaporated to leave 4-azido-*N*-methyl-2-aminotrifluorobenzoic acid **3** (523.79 mg, 95%). ^1H NMR (DMSO-d_6), δ, 3.75, (br, s, 1H), 3.89, (d, 3H). ^{19}F NMR (DMSO-d_6), δ, -134.48 (1F), -146.95 (1F), -163.71 (1F).

*Synthesis of **4***

The carboxylic group of 4-azido-*N*-methyl-2-aminotrifluorobenzoic acid **3** in dry CH_2Cl_2 (246.14 mg, 1 mmol), was activated by *N*-hydroxysuccinimide (NHS, 115 mg, 1 mmol), in the presence of dicyclohexylcarbodiimide (DCC, 220 mg, 1.1 mmol), also taken in CH_2Cl_2 (10 ml) and stirred at RT overnight. The mixture was filtered. The filtrate was evaporated to obtain of *N*-succimidyl-4-azido-2,3,5,6-tetrafluorobenzoate **4** (340 mg, 99%). ^{1}H NMR ($CDCl_3$), δ, 3.16, (m, 3H), 2.98, (s, 4H). ^{19}F NMR ($CDCl_3$), δ, −130.97 (1F), −146.84 (1F), −163.39 (1F).

*Synthesis of **6***

A mixture of 3-[(2-aminoethyl)dithio]propionic acid in 1ml of water (78 mg, 0.43 mmol), sodium bicarbonate (72 mg, 0.86 mmol) was added to a solution of *N*-succimidyl-4-azido-*N*-methyl-2-amino-3,5,6-trifluorobenzoate **4** (147.6 mg, 0.43 mmol) taken in 14 ml dioxane. After stirring the mixture for 18 h, the solvent was evaporated and reduced to 2 ml volume, cooled in ice, and adjusted to pH 2 with concentrated HCl to yield the solid product (79.2 mg, 45%) which was directly used for conjugating sodium salt of sulfosuccinimide. ^{1}H NMR ($CDCl_3$), δ, 7.75, (b, s, 1H), 6.89, (br, m, 1H), 3.12 (m, 3H), 2.93–1.9 (m, 8H). ^{19}F NMR ($CDCl_3$), δ, −141.94 (1F), −146.04 (1F), −163.87 (1F).

*Synthesis of **7***

A mixture of **6** (135 mg, 0.33 mmol), sodium salt of *N*-hydroxysuccinic acid (71 mg, 0.33 mmol), and DCC (72 mg, 0.35 mmol) was dissolved in 2 ml DMF and stirred at room temperature overnight. The reaction mixture was cooled and the precipitated dicyclohexylurea was removed by filtration. The filtrate was washed with a small quantity of dry DMF. The required product **7** was then precipitated from the solution by addition of ~20 vol of ethyl acetate, filtered, and dried in high vacuum. ^{1}H NMR (DMSO-d_6), δ, 8.81 (b, 1H), 5.61, (b, 1H), 3.95 (m, 1H), 3.21 (m, 3H), 3.01–2.6 (m, 10H). ^{19}F NMR (DMSO-d_6), δ, −143.95 (m, 1F), −146.97 (m, 1F), −164.51 (m, 1F). UV (water), λ_{max} 356 nm. *Anal.* Calcd for $C_{17}H_{16}N_6F_3O_8S_3Na$: C, 33.56; H, 2.65; N, 13.81. Found: C, 33.12, H, 2.55; N, 13.45.

*Synthesis of **9***

A mixture of 3-[(2-aminoethyl)dithio]propionic acid in 1 ml of water (78 mg 0.43 mmol), sodium bicarbonate (72 mg, 0.86 mmol) was added to a solution of *N*-succimidyl-4-azido-2,3,5,6-tetrafluorobenzoate prepared as reported earlier (*10*) (149.5 mg, 0.45 mmol) taken in 5 ml dioxane. After stirring the mixture for 18 h, the solvent was evaporated partially to 2 ml volume, cooled in ice, and adjusted to pH 2 with concentrated HCl to yield the solid product (68.1 mg, 38% yield) which was directly used for conjugating sulfosuccinimide. ^{1}H NMR ($CDCl_3$/DMSO-d_6), δ, 8.85, (b, s, 1H), 2.9–1.8 (m, 8H) ^{19}F NMR ($CDCl_3$/DMSO-d_6), δ, −141.57 (2F), −151.44 (2F).

*Synthesis of **10***

A mixture of **9** (50 mg, 0.126 mmol), the sodium salt of *N*-hydroxysuccinic acid (27 mg, 0.13 mmol), and DCC (30 mg, 0.17 mmol) was dissolved in 2 ml DMF and stirred at room temperature overnight. The reaction mixture was cooled and the precipitated dicyclohexylurea was removed by filtration. The product **10** was then reprecipitated from the solution by addition of ~20 vol of ethyl acetate, filtered, and dried in high vacuum. ^{1}H NMR (DMSO-d_6), 8.31 (b, s, 1H), 3.85 (m, 1H), 2.9–1.8 (10H). ^{19}F NMR (DMSO-d_6), δ, −135.54 (2F), −151.30 (2F). UV (Water), λ_{max} 275 nm, 310 nm(sh). *Anal.* Calcd for $C_{16}H_{12}N_5F_4O_8S_3Na$: C, 32.17; H, 2.02; N, 11.72. Found: C, 32.27; H, 2.12; N, 11.82.

Biological Assay

All the following steps were carried out under diffused light. The protocol for conjugation of cross-linkers SASD (10 μl) and **10** [10 μl, made up at 1 mg/ml] in PBS, (0.1 M sodium phosphate, 150 mM NaCl, pH 7.4)] involved incubation of each one of them separately with 2 mg mouse IgG (at 11 mg/ml) in the dark for 2 h. Samples were injected into 0.1–0.5 ml Slide-A-Lyzers dialysis cassettes (Pierce) and dialyzed overnight versus 5 L of PBS. The samples were removed from the dialysis cassettes and placed in 16 × 100-mm glass tubes. Horseradish peroxidase (HRP, Pierce, 10 mg) was dissolved in 1 ml PBS and 200 μl of the HRP was added to the activated mouse IgG. A hand-held long wavelength UV light was placed above the samples and the samples were irradiated with UV light for 30 min before storing them at 4°C.

The analysis of the conjugates was done using the ELISA assay. The two mouse IgG–HRP conjugates and HRP (10 mg/ml, diluted with 190 μl of PBS) were further diluted (1 : 100) with PBS. 100 μl of each dilution was added to separate wells (the assay was performed in duplicate) of a goat anti-mouse IgG coated microtiter plate. The last row was a PBS blank with no conjugate or HRP. Prior to the addition of the sample, the plate had been washed with 3 × 200 μl PBS. The samples were incubated in the plate wells for 1 h at room temperature. The plate was washed again with 3 × 200 μl PBS. Turbo TMB (tetramethyl benzidine, 100 μl) was added to each well. The plate was incubated for 5 min at room temperature before adding 1 M H_2SO_4 to each well to stop the reaction. Subsequently, the plates were read at 450 nm for optical densities.

Biotinylation of Rabbit IgG

About 1 mg of rabbit IgG in 90 μl of PBS was added to the solution of SFAD at 12 mg/ml and the reaction was monitored for 2 h at room temperature. The sample was desalted using 5 ml cross-linked Dextran column (Pierce) before adding 2 ml of 2 mM biotin. The sample was exposed to UV light at 312 nm for 30 min. A goat anti-rabbit coated plate was washed (3 × 20 ml) with PBS/0.05% Tween 20 (Pierce). Biotinylated rabbit IgG was serially diluted (1 : 1) and incubated for 1 h. The plates were washed again before adding streptavidine–HRP (1 mg/ml, Pierce). Quantification of IgG cross-linked product was determined by adding TMB substrate to each incubation