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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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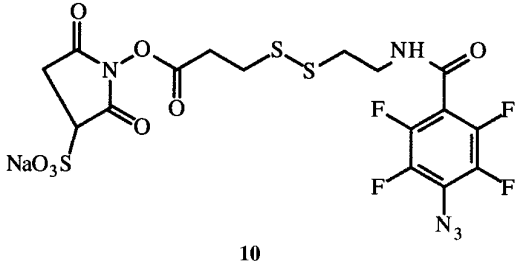
second protein, cleavage of the disulfide (S–S) bridge of the cross-linked protein results in the concomitant transfer of the marker (e.g., radioactivity or chromogenicity or fluorescence) to the specific amino acid(s) at which the cross-link occurs. Hence, a useful photo cross-linker should have a marker 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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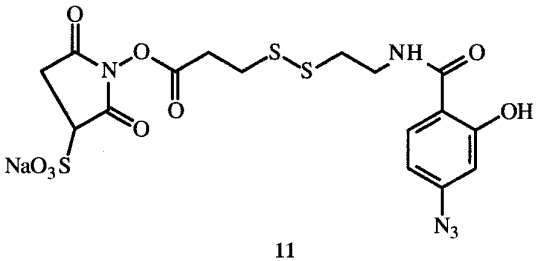

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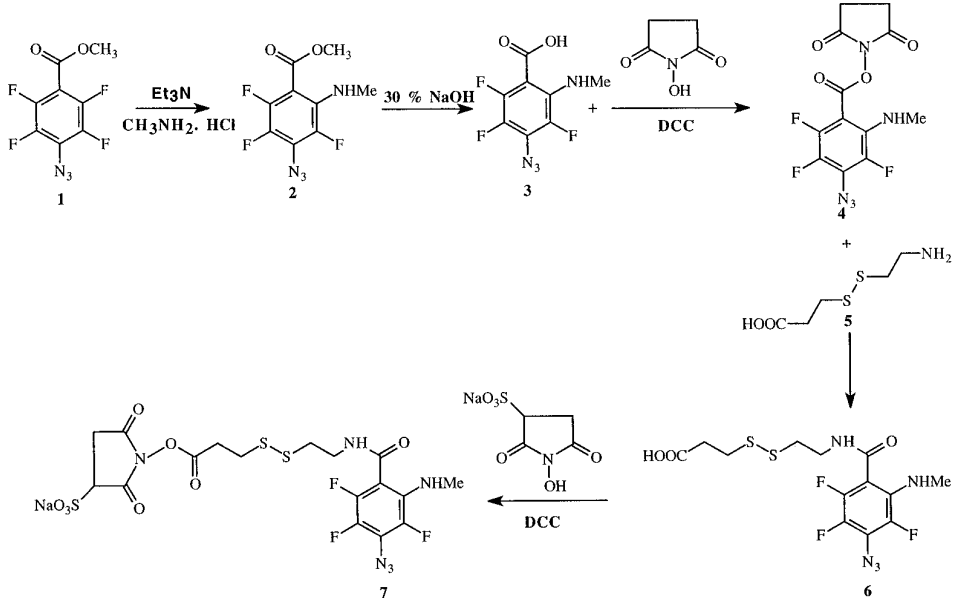
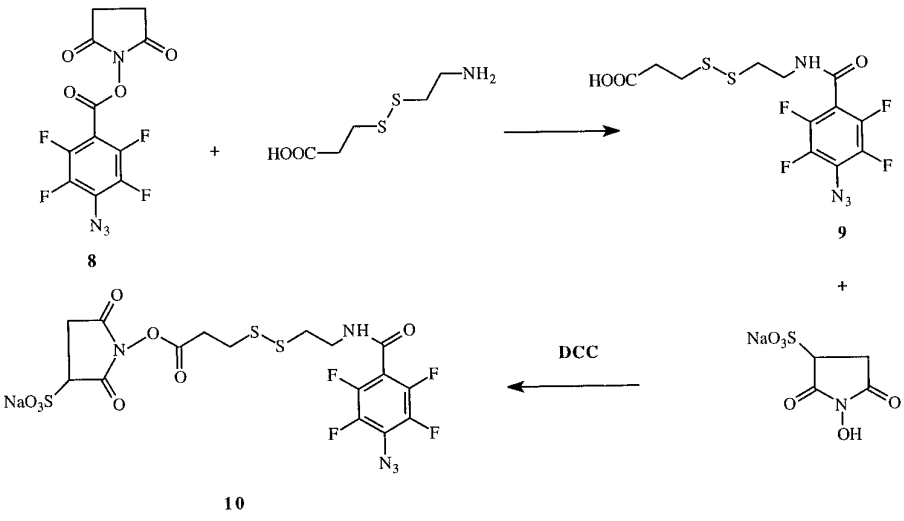
EXPERIMENTAL

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

well and the reaction was monitored for 10 min before stopping with 1 M H_2SO_4 and optical density at 4560 nm was noted using a microwell plate reader.

RESULTS AND DISCUSSION

Chromogenic nitrene precursors which are red shifted from the protein absorption maximum have been shown to be versatile photolabeling agents for human serum albumin (HSA) since these precursors have an additional advantage of absorption at long wavelength (*15*). Nucleophilic substitution of 4-azido perfluoro methyl benzoate **1** by methyl amine hydrochloride leads to the N-methyl trifluoro derivative **2** selectively (Scheme **1**). The interesting aspect of this nucleophilic substitution is a formation of chromogenic derivative **2** shifting λ_{max} to 356 nm, far away from the λ_{max} absorption of biomolecules. Hydrolysis of **2** by 30% NaOH gives rise to the corresponding acid **3** which can be activated to the corresponding succinimide **4** using DCC. The introduction of an S–S cleavable bond is achieved by reaction of **4** with 3-[(2-aminoethyl)dithio]propionic acid **5,** prepared by a method found in the literature (*24*). In the last step, activation of the carboxylic group by the sodium salt of sulfosuccinimide in DMF lead to a chromogenic, cleavable, water soluble, and bifunctional cross-linker **7.** The new cross-linker was characterized by multiprobe NMR (^{1}H, ^{13}C, and ^{19}F), IR and elemental analysis. To illustrate the relative effectiveness of the perfluorinated aryl azide photoprobe **10,** a structural analog of SASD **11** was prepared and used in comparative cross-linking studies.

The biological protocol for cross-linking mouse IgG to the enzyme is outlined in Fig. 1. The procedure involved (a) conjugation of the cross-linker with mouse IgG through the interaction between the sulfosuccinimide group of the cross-linker and α- or ε-amino groups of IgG, followed by a separation of the unconjugated cross-linker by extensive dialysis, and (b) irradiation of the conjugated mouse IgG in the presence of HRP using UV light. Analysis of the cross-linked product was carried out using the ELISA assay. The mouse IgG–HRP conjugate was added and serially diluted (1 : 1) in wells coated with goat anti-mouse IgG. The plate was washed with phosphate buffer (PBS) containing 0.05% Tween 20 to remove any HRP that was not conjugated with mouse IgG. The amount of HRP associated with mouse IgG was estimated from the enzyme immunoassay using 3, 3′,5,5′-tetramethylbenzidine (TMB) as a substrate which allows a detection of HRP (*12*). The absorbance of the enzyme-substrate (OD) is proportional to the concentration of HRP bound to mouse IgG (i.e., cross-linked to antibody). Employing the identical conditions for the SASD cross-linking assay permits a comparison of the cross-linking efficiencies of **10** and SASD. PBS buffer with no conjugate or HRP forms the control.

The results on the comparison of cross-linking efficiencies between **10** and SASD **11** is shown in Fig. 2. The nature of the saturation of optical density followed by a slight increase for nonfluorinated analog **11** is surprising. Although, at present we do not know the explanation for this behavior, at higher concentrations (50 μg/ml) the data indicate that the conjugate made with the perfluoroaryl functionalized cross-linker **10** gave 40% higher OD compared to SASD. This can be attributed to the higher incorporation of HRP cross-linking to mouse IgG. The typical

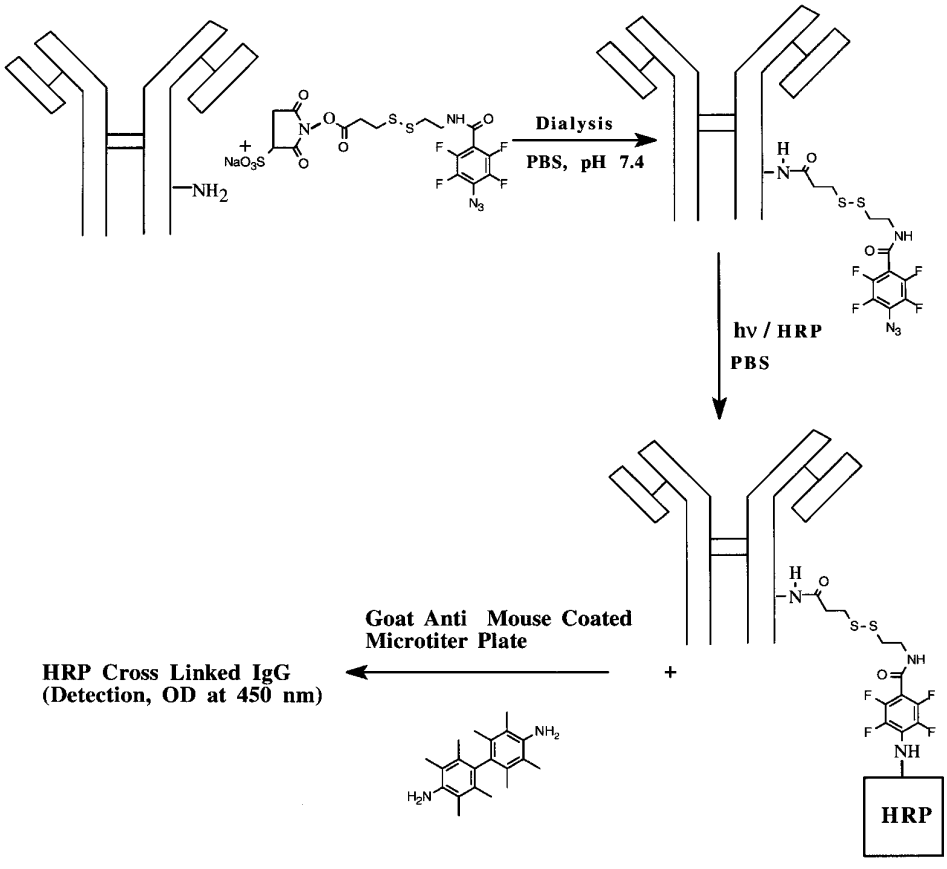


FIG. 1. Cross-linking protocol for compound **10** with IgG followed by irradiation with HRP.

higher photochemical conjugation efficiency of perfluoroaryl azides reported in model solvents (*21*) and proteins (*12*), for example, could, in principle, explain the superiority of perfluoroaryl based cross-linkers compared to the nonfluorinated aryl azide analog which are known to undergo ring expansion rather than the formal insertion of nitrene. Conventional photolabile cross-linking agents involving aryl azides with ^{125}I markers suffer from low insertion efficiency since they either rearrange to didehydroazipines or convert to triplet nitrene intermediates leading to noninsertion polar product (*25*). In addition, the release of iodine radiolabel into the surrounding macromolecules in a nonspecific manner during photolysis results in the labeling of macromolecular domains other than those involved in ligand–affinity interactions (*26*). Moreover, it has been established that the presence of a heavy atom such as iodine in the same ring as the azide functionality reduces the reactivity of the nitrene leading to triplet derived products through intersystem crossing (ISC) rather than the desired singlet derived insertion products (*27*, *28*). Extensive studies

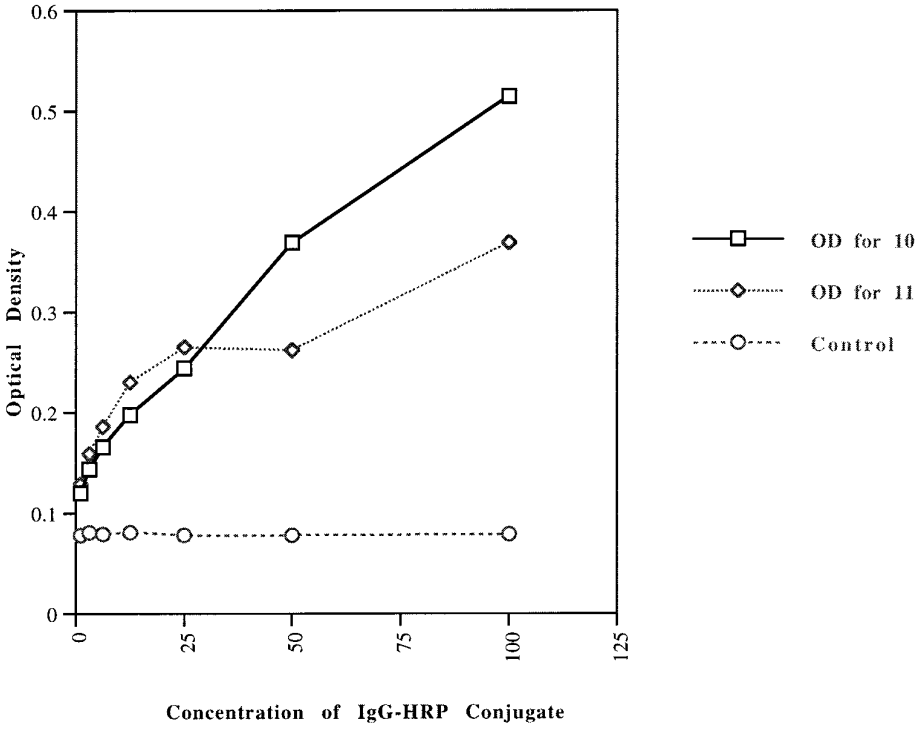


FIG. 2. Relative cross-linking efficiencies of compounds **10** and **11** with IgG–HRP conjugate.

on the photochemistry of phenyl azides by Schuster *et al.* (*19*, *20*) and Platz *et al.* (*21–23*) reveals the limited success of aryl azides as cross-linkers. Although didehydroazipines (photolytic product from aryl azides) are capable of adding to nucleophiles, they might be of limited use in cross-linking studies since they are less reactive than nitrenes and are not stable under the proteolytic conditions typically used for peptide mapping and amino acid identification, thus leading to a low yield of the cross-linked product. On the other hand, moderately successful labeling reactions with azides (e.g., azido nucleotides (*29*)) may be attributed to the preassociation of the probe to a particular region of the macromolecule to be labeled where the generated reactive nitrene is restricted and not allowed to diffuse through the medium and, hence, reacts within the immediate vicinity leading to insertion. Goeldner *et al.* (*30*) have established a hierarchy between nonflourinated, diflourinated, and tetrafluorinated aryl azido probes which supports the potential application of perfluoroaryl based reagents for efficient labeling, for example of GABA receptors. These results also agree with the reports on a similar nonfluorinated cross-linker, SAED, which shows only 15% cross-linking efficiency (*31*).

In the area of cross-linking of proteins, it is important to demonstrate the retention of the native characteristics of biomolecules such as binding affinity in the post-

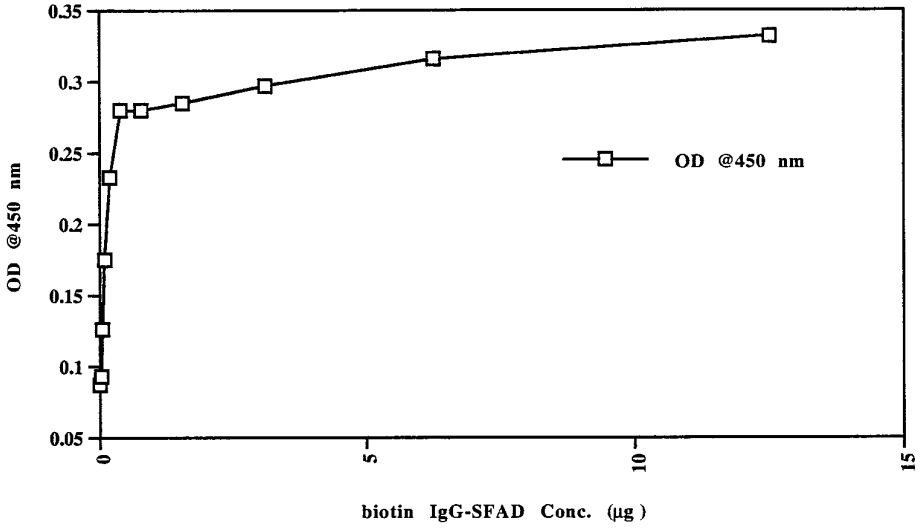


FIG. 3. Binding data on IgG–SFDA biotin conjugate.

cross-linking stage. In this connection, we have photoconjugated IgG–SFAD to biotin which is known to bind streptavidin strongly (*32*). The choice of biotin is based on the establishment of the binding protocol of biotin with streptavidin–HRP, which can be detected and quantified easily. The protocol involves conjugation of IgG with SFAD followed by irradiation with biotin. Binding of biotinylated IgG–SFDP with goat anti-rabbit IgG coated plate followed by repeated wash with buffer gives rise to pure IgG-SFDP cross-linked to biotin. The binding assays involved incubation of the cross-linked product with streptavidin–HRP and monitored through OD at 450 nm after treating the assay with TMB, followed by the addition of 1 M H_2SO_4. Figure 3 shows the binding data for biotinylated IgG–SFAD cross-linker indicating a high binding affinity even at 0.75 mg concentration and showing saturation binding at higher concentrations. These data suggest that protocol adopted for cross-linking biomolecules using SFAD does not interfere with the binding regions and hence, retains the native characteristics of biomolecules. The retention of the binding affinity of biotin can be rationalized in terms of CH insertion of singlet perfluoroaryl nitrene on the hydrophobic part of biotin. In an alternate experiment involving the photo cross-linking in the presence of a triplet sensitizer (e.g., acetophenone), no binding of IgG–SFAD to biotin was observed, indicating that the triplet perfluoroaryl nitrene produced an aniline type of product which cannot form covalent linkage with biotin, unlike CH insertion by singlet nitrene.

CONCLUSIONS

The main consideration for the successful application of covalent attachment of photolabels to biomolecules is the evaluation of their bimolecular insertion efficiencies in buffer conditions. We have introduced chromogenicity (bathochromic shift)

and cleavability into the new design of perfluoroaryl functionalized cross-linker based on our earlier studies on the high efficiency of nitrene insertion into HSA, IgG, and B72.3 antibody by functionalized perfluoroaryl azides (*12–15*). The new photo cross-linker is superior to the nonfluorinated analog, demonstrating 40% higher incorporation into HRP. The technical superiority is correlated well with the differential nature of the reactive intermediates involved and singlet lifetimes between perfluoroaryl azides and conventional aryl azides. The higher efficiency of cross-linking, shown by perfluoroaryl derivatives with a cleavable arm, permits the tranfer of perfluoraryl group to the target protein which can be monitored by ^{19}F NMR and, hence, may find use in better characterization of the binding sites involved in protein–protein cross-linking by macroscopic techniques. Binding studies with biotin by preconjugated IgG–SFAD demonstrates the retention of the binding affinity in the post-cross-linking stage enhancing their utility. Further studies on the peptide mapping of the cross-linked product are in progress and will be published elsewhere.

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