Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 12, pp. 1367–1373, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00129-4

A Novel Biotinylated Heterobifunctional Cross-linking Reagent Bearing an Aromatic Diazirine

Yasumaru Hatanaka,[1]* Makoto Hashimoto[2] and Yuichi Kanaoka[3]

[1]*Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama, 930-01 Japan* [2]*Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan* [3]*Toyama Women's College, 444 Gankaiji, Toyama, 930-01 Japan*

Abstract—The synthesis of a *p*-[(3-trifluoromethyl)diazirine-3-yl]benzoic acid derivative is described as a new carbene generating heterobifunctional cross-linking reagent. The cross-linker carries a biotin moiety in order to make use of avidin–biotin technology for specific manipulation of cross-linked components. To evaluate the ability of this reagent, the inter-subunit cross-linking of egg-white avidin tetramer was investigated. As a typical application of avidin–biotin technology for cross-linking experiments, a chemiluminescent detection method was examined to identify photobiotinylated components. A cross-linked dimeric product with an apparent molecular mass of 38 kDa was clearly visualized by the combined use of a horseradish peroxidase–streptavidin conjugate and a luminol-based chemiluminescent system.

Introduction

One of the major difficulties encountered during the processes of photolabeling is a step to selectively retrieve labeled components from complex mixtures. For example, the cleavage of labeled proteins into small fragments followed by isolation of the labeled peptides by high performance liquid chromatography (HPLC) is a typical step prerequisite to sequence analysis. In our case, however, low yield of photoaffinity labeling of the electroplax sodium channel hampered us to isolate labeled fragments by HPLC, and the sequence analysis of cross-linked regions by a conventional Edman degradation method have so far not been possible.[1] The recent development of several biotinylated analogs of the photoreactive reagent would be particularly noteworthy in this regard.[2] An example of affinity isolation of labeled fragments by means of avidin–biotin technology was recently reported for identification of a ligand binding site within insulin receptor.[3] Herein we describe a synthesis of the first example of carbene-generating heterobifunctional cross-linking reagent carrying a biotin moiety within its molecular structure.

Chemical cross-linking reagents have been used for the determination of ligand–receptor complex, three-dimensional protein structures, as well as the topology of oligomeric proteins.[4] The efficiency and the specificity of the cross-linking has been increased by the development of the category of heterobifunctional cross-linkers bearing one photosensitive and one chemically reactive functional groups.[5] By taking advantage of the differential reactivities of the two functional groups, cross-linking can be controlled both selectively and sequentially. To date, arylazide derivatives are predominantly used as photoactivatable cross-linking reagents because of their ease of synthesis.[6] Among many different photoreactive groups, however, the aryl(trifluoromethyl) diazirine functionality is expected that the formation of cross-links would be more efficient than their aryl azide counterparts and covalent adducts would be stable enough to the subsequent digestion, HPLC purification, and sequencing steps. Indeed, we have successfully determined the binding region for tetrodotoxin within the sodium channel polypeptide by using a photoaffinity ligand carrying a diazirine.[7,8] A carbene-generating dihydropyridine with a diazirine moiety is also useful for the identification of the ligand-binding region of calcium channels.[8,9] Neither of the corresponding azide derivatives gave positive results. Although the photoproducts of a diazirine can undergo an elimination reaction resulting loss of cross-links was reported,[10] the diazirine-based cross-linker seems more promising than the use of aryl azides. In favorable cases a cross-linked component may be degraded by proteolysis or chemical cleavage to identify the region where the cross-linking occurred. While several heterobifunctional cross-linkers bearing aryl diazirines are available,[11] their biotinylated versions which may develop the convenient use of avidin–biotin technologies in the field of photolabeling is not reported yet.

Results

Synthesis

We have recently developed a novel family of phenyldiazirine including a salicylic acid derivative which contains an easily functionalizable phenol hydroxyl by alkylation reactions.[12] By using this compound, a spacer arm to link diazirines with ligands as well as radioactive diazirines labeled with carbon-14 or tritium were readily prepared. Owing to the improved interaction between avidin and biotinylated macromolecules which bear extended spacer arms, long-chain derivatives of biotin are usually required to obtain optimal biotin-binding capabilities.[13] Following the same strategy described above, biotin could be connected to the diazirinylphenol through a polyether spacer to give a

cross-linker **1** (Fig. 1). As well as to gain better binding between avidin and photobiotinylated products, the hydrophilic spacer introduced between biotin and a phenyl diazirine would improve solubility of a hydrophobic diazirine moiety into aqueous media.

A polyether bromide **4** was prepared from 2-[2-(2-chloroethoxy)ethoxy]ethanol as shown in Scheme I. Conversion of the starting chloride to the azide **2** was followed by reduction to give an amine which was subsequently protected with the *tert*-butoxycarbonyl (Boc) group to provide **3**. The alcohol **3** was then converted to the bromide **4**.

The alkylation of the phenol **5** with **4** was successfully performed at 60 °C to give **6** in a good yield. Deprotec-

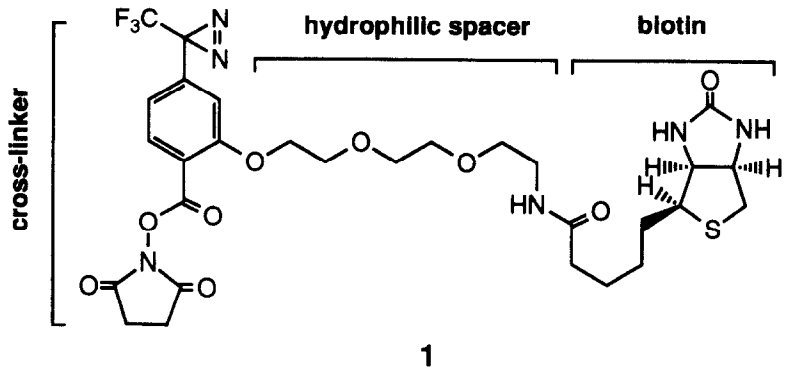
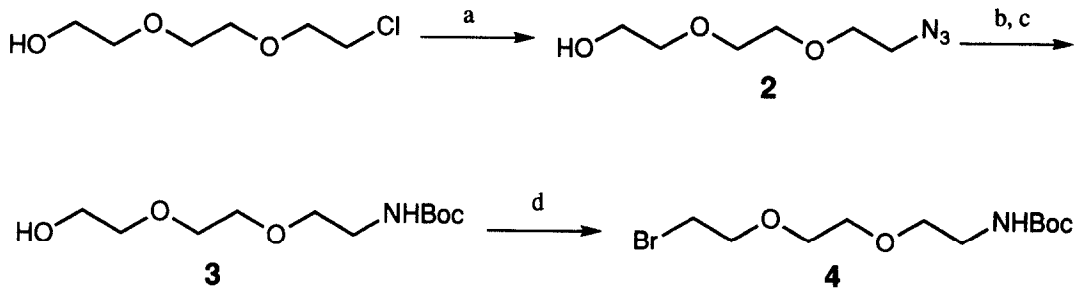
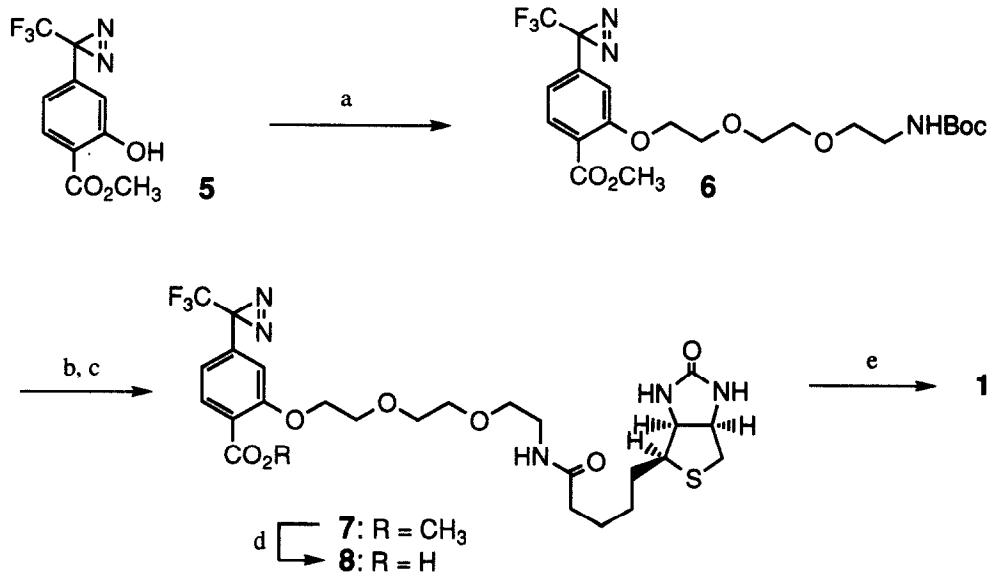


Figure 1. Structural feature of biotinylated heterobifunctional cross-linker **1**.



Scheme I. (a) NaN_3, NaI, H_2O, 50 °C; (b) Ph_3P, H_2O, THF, r.t.; (c) $(Boc)_2O$, 1N NaOH, CH_3CN, r.t.; (d) Ph_3P, CBr_4, K_2CO_3, CH_2Cl_2, r.t.



Scheme II. (a) **4**, K_2CO_3, $Bu_4N{\cdot}I$, DMF, 60 °C; (b) TFA/CH_2Cl_2 (1:1), 0 °C; (c) biotin-OSu, Et_3N, DMF, r.t.; (d) 1N NaOH, MeOH, r.t.; (e) HOSu, EDC, THF/CH_3CN (1:1), r.t.

tion of **6** followed by acylation with biotin *N*-hydroxysuccinimide ester to yield a biotinylated methyl ester **7** which was hydrolyzed to an acid **8**. The benzoic acid derivative **8** was converted to its *N*-hydroxysuccinimide ester to result the desired heterobifunctional cross-linker **1** (Scheme II).

Photolysis

All the phenyldiazirine derivatives show the characteristic absorption bands of diazirine ring at *ca* 350 nm (see Experimental Section). Irradiation of a methanolic solution of **7** was performed with a black light lamp which emits lights around 350 nm. Figure 2 shows the changes of the diazirine bands upon photolysis and a half-life of photodecomposition was determined as 1.8 min under our photolytic conditions (inset to Fig. 2).

Cross-linking

Modification of egg-white avidin was first performed in the dark to achieve selective modification of primary amino functional groups. Cross-linking was then initiated by irradiation at the diazirine bands around 350 nm. The results of cross-linking were analyzed by SDS-PAGE and its silver-staining pattern is shown in Figure 3 A. For the detection of biotinylated components, proteins were electrophoretically transferred onto a membrane surface and their blotting patterns were visualized with a chemiluminescence detection method (Fig. 3B).[14]

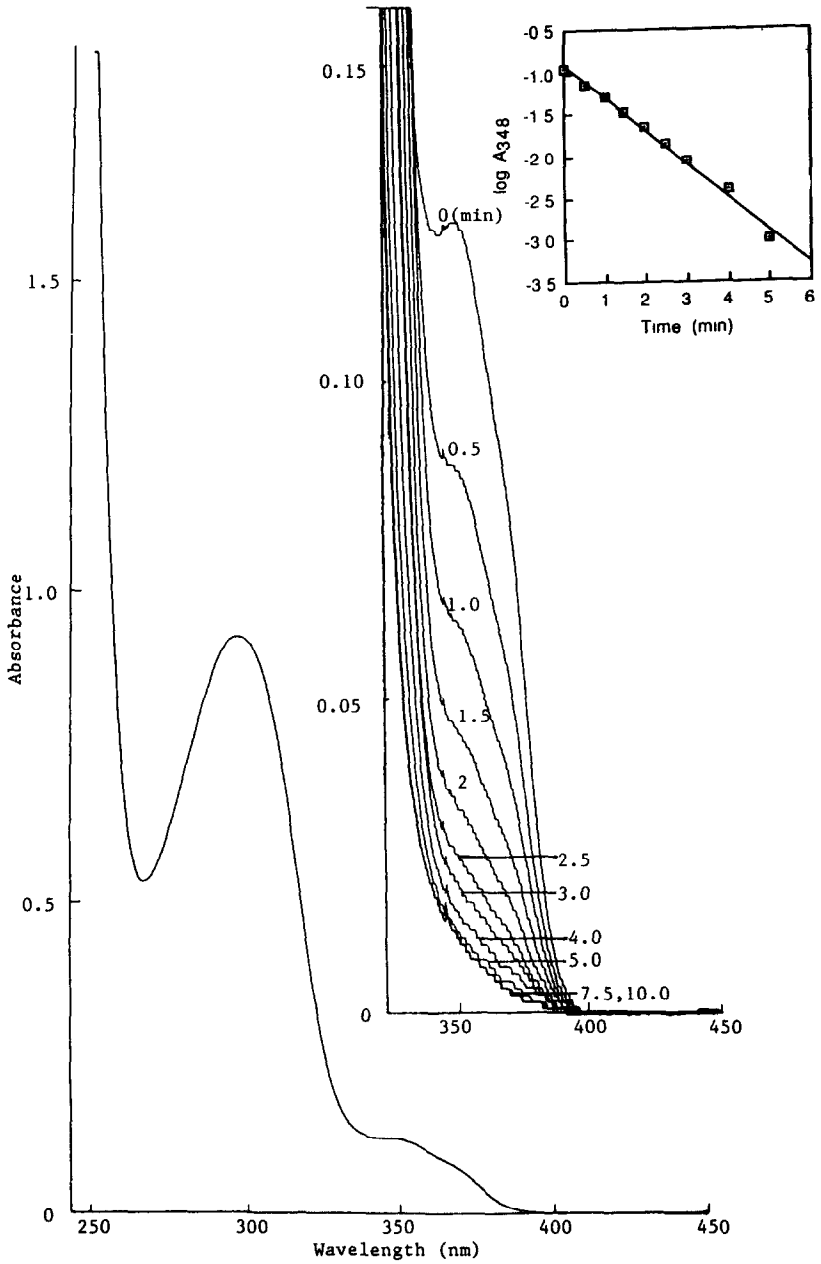


Figure 2. Photolysis of the methyl ester of biotinylated diazirine. UV/vis spectra of compound **6** (left; t = 0 min), and of the photolysis reaction mixture (right) at the times (in min) indicated with numbers. The inset shows the decay of the absorbance at 348 nm as a function of time of photolysis in a semilogarithmic representation.

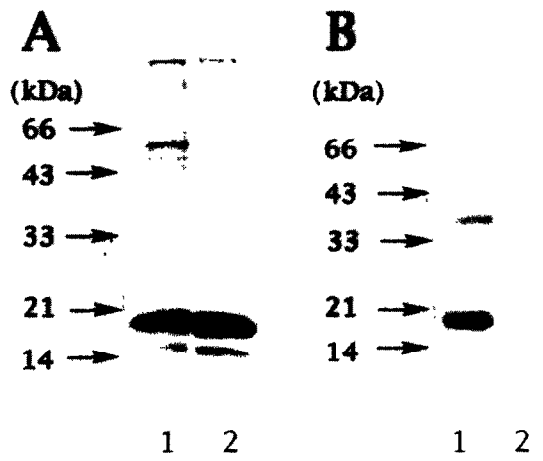


Figure 3. Photochemical cross-linking of avidin subunits with the reagent **1**. Egg white avidin was incubated with ca 0.8 equivalent of **1** per subunit and photolyzed with a black light lamp at 4 °C. The silver-staining pattern (A) of a 12.5 % SDS–polyacrylamide gel and the chemiluminescent detection pattern of a electroblotted membrane (B) are shown of the following samples: μg of proteins in each lane (A); 0.4 μg of proteins in each lane (B).

Upon photolysis of modified avidin, a weak band migrating with an apparent molecular mass of 38 kDa was detected by silver-staining (Fig. 3 A, lane 1). The molecular weight of observed band is in good agreement with the value of 36 kDa expected for the dimer of glycosylated forms.[15] The chemiluminescent detection of this band clearly indicates a presence of biotinylated components corresponding to dimeric products (Fig. 3 B, lane 1). Besides the major formation of biotinylated monomers which failed to cross-link intermolecularly, trace amounts of oligomeric products with higher molecular mass was also detected. The lane of starting avidin (Fig. 3 A, lane 2) shows no presence of a SDS resistant dimeric form which was reported for Sigma avidin.[15] Thus, the possibility of the formation of a biotinylated dimer which is not covalently linked together can be eliminated. As no detectable band was observed in a control lane (Fig. 3 B, lane 2), the non-specific absorption of the chemiluminescent detection reagent onto blotted proteins should be negligible.

Discussion

Diazirines are known to be unstable at elevated temperatures,[16] and the alkylation of diazirinylphenols have been performed with reactive alkyl halides at room temperature or below.[12,17] Although the alkylation with an aliphatic bromide **4** required a rather harsh alkylation condition, the phenol **5** was found to be sufficiently stable during the reaction. All diazirines described in Scheme II are possessing similar alkoxyphenyl structures and may be considerably stable for further derivatization. These diazirine building blocks could be potentially useful for the preparation of other photoreactive cross-linkers with cleavable arms as well as various photoaffinity labeling reagents. The water solubility of cross-linkers may have special attention because they have to be used in aqueous media. The biotinylated acid **8** is bearing a hydrophilic spacer arm and is as much soluble as biotin itself in water despite the introduction of a large hydrophobic aryl diazirine moiety.[18] Thus, the conditions already established for protein biotinylation may similarly be applicable to the cross-linker **1**.[19]

At the present stage, there are no experimental evidence for the sites of cross-link formed between avidin subunits. The following speculations, however, could be possible for the sites of acylation with the reagent **1**. Egg white avidin contains nine lysines and an N-terminal alanine within the polypeptide structure.[20] Among these sites, the presence of three reactive lysines, Lys-45, Lys-94 and Lys-111, were already identified by dinitrophenylation.[21] The recent X-ray crystallography of the avidin–biotin complex revealed that Lys-45 and Lys-111 are located at the same site forming a rim of biotin binding pocket whereas Lys-94 is included at the other end of the avidin barrel.[22] The lysine residues on the rim, therefore, may be preferentially acylated with reagent **1** after the rapid incorporation of biotin moiety into the binding site.[23] It seems likely that the diazirine function on these lysines possibly have a crucial role for cross-linking to adjacent subunits resulting in the observed dimeric product.

Owing to the low efficiency of photolabeling, radioisotopes are commonly used for the detection of trace amount of photolabeled components. The catalyzed reporter deposition to the signal amplification based on chemiluminescence has been developed as a radioisotope-free highly sensitive detection method.[14] The present results demonstrate that the photochemically cross-linked proteins were readily visualized by exposing a blotted membrane on a detection film for 3 min. Combined use of the reagent **1** with avidin–biotin technology could be useful for the high-sensitive detection of photolabeled macromolecules as well as the affinity isolation of biotinylated components from a complex mixture.

Experimental

General

All chemical reagents were commercially available and were used without further purification. Tetrahydrofuran (THF) was freshly distilled from $LiAlH_4$. Acetonitrile and dichloromethane used as reaction solvents were distilled and stored over molecular sieves. IR spectra were measured on a Shimadzu IR-408 spectrophotometer. ^1H NMR spectra were recorded with a JEOL JNM GX-400 spectrometer with tetramethylsilane as an internal standard. Low-resolution mass spectra (MS) or high-resolution mass spectra (HRMS) were obtained with a Jeol JMS-HX110 spectrometer. The fast atom bombardment (FAB) mass spectra were obtained by using nitrobenzyl alcohol as the matrix. Silica gel for column chromatography was Kieselgel 60 (Merck, No. 7734, 70–230 mesh), and alumina refers to aluminum oxide 90 (Merck, No. 1097, activity II–III). Egg white avidin (A9275) were obtained from Sigma and purified

with a gel filtration chromatography on Sephacryl S-300 column (1.8 × 75 cm) eluting with 10 mM sodium bicarbonate, pH 8.3 (10 mL/h). A pooled fraction containing no dimeric form which resists to denaturation[15] was dialyzed against 0.1 M sodium carbonate, pH 8.3, and this was used for cross-linking experiments.

2-[2-(2-Azidoethoxy)ethoxy]ethanol (2)

A suspension of 2-[2-(2-chloroethoxy)ethoxy]ethanol (5.00 g, 30 mmol), NaI (0.90 g, 6.0 mmol) and NaN_3 (19.3 g, 300 mmol) in H_2O (30 ml) was stirred at 50 °C for 12 h. After almost insoluble material was removed, the filtrate was concentrated, the residue was dissolved in ethyl acetate and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel (eluting with ethyl acetate) to give 4.43 g of yellow oil. As the starting chloride can not be separated by this condition, the oil contained about 80 % of the desired azide **2** as judged by NMR and was used without further purification. IR (neat) 3400, 2100 cm^{-1}. ^1H NMR ($CDCl_3$) : δ 3.40 (t, 2H, *J*=5.2 Hz, CH_2N_3)

*2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]ethanol (3)*

The crude azide **2** (0.16 g) was dissolved in THF (4 mL) and solid triphenylphosphine (0.27 g, 1.0 mmol) was added at room temperature. After stirring for 45 min, distilled water (4 mL) was added to the reaction mixture and the turbid mixture was vigorously stirred for 13 h at room temperature. The THF was evaporated *in vacuo* and CH_3CN (6 mL) was added to the residue. Di-*tert*-butyl carbonate (0.23 g, 0.9 mmol) and 1 N NaOH (0.75 mL) were added to this solution and the reaction mixture was stirred for 3 h at room temperature. As the product **3** was considerably soluble in water, the reaction mixture was evaporated to dryness and the residue was taken in ethyl acetate. This solution was dried over $MgSO_4$ and the solvent was removed by evaporation. Chromatography of the residue on aluminum oxide (eluting with 10 % ethanol–ethyl acetate) afforded the protected amine **3** (0.13 g, 47 % from the starting chloride) as a colorless oil. IR (neat) 3350, 1700 cm^{-1}. ^1H NMR ($CDCl_3$) : δ 3.74 (t, 2 Hz, *J*=4.9), 3.67–3.63 (m, 4H), 3.61 (t, 2H, *J*=4.6 Hz), 3.56 (t, 2H, *J*=5.2 Hz), 3.35 (t, 2H, *J*=5.2 Hz), 1.44 (s, 9H). FABMS m/z 250 $[MH]^+$; HRFABMS calcd for $C_{11}H_{24}NO_5$ 250.1655, found 250.1635.

*2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]ethyl bromide (4)*

2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]ethanol **3** (0.50 g, 2 mmol), carbon tetrabromide (0.83 g, 2.5 mmol), K_2CO_3 (0.42 g, 3 mmol) and CH_2Cl_2 (5 mL) were placed in a flask. Triphenylphosphine (0.79 g, 3 mmol) in CH_2Cl_2 (5 mL) was added to above suspension over 15 min at room temperature. After stirring for 13 h at room temperature, the reaction mixture was filtered and the filtrate was evaporated. The residue was treated with 10 mL of hexane–ethyl acetate (1:1) to remove insoluble triphenylphosphine oxide by filtration, and the filtrate was evaporated. Chromatography of the residue on silica gel (eluting with ethyl acetate : hexane, 1:1) afforded the bromide **4** (0.48 g, 77 %) as a colorless oil. IR (neat) 3370, 1700 cm^{-1}. ^1H NMR ($CDCl_3$) : δ 3.81 (t, 2H, *J*=6.4 Hz), 3.66 (t, 2H, *J*=4.2 Hz), 3.63(t, 2H, *J*=4.2 Hz), 3.55 (t, 2H, *J*=5.2 Hz), 3.48 (t, 2H, *J*=6.4 Hz), 3.32 (t, 2H, *J*=5.2 Hz), 1.44 (s, 9H). FABMS m/z 312, 314 $[MH]^+$; HRFABMS calcd for $C_{11}H_{23}BrNO_4$ 312.0810, found 312.0826.

*2-[2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]-ethoxy]- 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid methyl ester (6)*

The diazirine **5**[12b] (0.52 g, 2 mmol) was dissolved in DMF (2 mL) followed by the addition of potassium carbonate (0.28 g, 2 mmol) at 0 °C. 2-[2-(2-*tert*-butoxycarbonylaminoethoxy)ethoxy]ethyl bromide (**4**) (0.69 g, 2.2 mmol) and tetrabutylammonium iodide (80 mg, 0.2 mmol) were added at room temperature, and the reaction flask was wrapped with aluminum foil to protect from light. Then the reaction mixture was heated at 60 °C for 19 h with stirring. Insoluble materials were filtered off and the filtrate was evaporated *in vacuo*. The residue was partitioned between benzene and H_2O, and the organic layer was dried over $MgSO_4$. The solvent was removed under reduced pressure and chromatography of the residue on silica gel (eluting with ether : hexane, 1:1) afforded **6** (0.79 g, 80 %) as a yellow oil. IR (neat) 3350, 1710 cm^{-1}. ^1H NMR ($CDCl_3$) : δ 7.80 (d, 1H, *J*=8.2 Hz), 6.82 (d, 1H, *J*=8.2 Hz), 6.72 (s, 1H), 5.02 (br, 1H), 4.19 (t, 2H, *J*=4.7 Hz), 3.90 (t, 2H, *J*=4.7 Hz), 3.87 (s, 3H), 3.74 (dd, 2H, *J*=4.9, 6.4 Hz), 3.63 (dd, 2H, *J*=4.9, 6.4 Hz), 3.54 (t, 2H, *J*=5.2 Hz), 3.35 (br, 2H), 1.42 (s, 9H). FABMS m/z 492 $[MH]^+$; HRFABMS calcd for $C_{21}H_{29}F_3N_3O_7$ 492.1958, found 492.1927.

2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid methyl ester (7)

Deprotection of **6** (0.48 g, 1.0 mmol) was performed with 2 mL of 50 % TFA–CH_2Cl_2 at 0 °C. After stirring at 0 °C for 3 h, the reaction mixture was concentrated to provide a yellow oil. The resulting TFA salt of the deprotected amine was dissolved in DMF (1 mL) and the solution was cooled at 0 °C. Triethylamine (0.32 mL, 2.5 mmol) was added followed by a solution of *d*-biotin *N*-hydroxysuccinimide ester (0.36 g, 1.0 mmol) in DMF (6 mL). The reaction flask was wrapped with aluminum foil and the mixture was stirred at room temperature overnight. The mixture was concentrated under reduced pressure and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was dried over $MgSO_4$ and the solvent was evaporated *in vacuo*. Chromatography of the residue on silica gel (eluting with ethanol:CH_3Cl, 1:6) gave **7** (0.51 g, 84 %) as a colorless solid. IR (nujol) 1710 cm^{-1}; UV (MeOH) λ_{max} (ε) 299 (3224), 348 (433, shoulder). ^1H NMR ($CDCl_3$) : δ 8.0, 7.6, 6.1 (br, each 1H), 7.80 (d, 1H, *J*=8.2 Hz), 6.83 (d, 1H, *J*=8.2 Hz), 6.72 (s, 1H), 4.50 (m, 1H), 4.30 (m, 1H), 4.19 (t, 2H, *J*=4.4 Hz), 3.90 (t, 2H, *J*=4.4 Hz), 3.87 (s, 3H), 3.75 (br, 2H), 3.64 (br, 2H), 3.57 (br, 2H),

3.44 (br, 2H), 3.15 (m, 1H), 2.90 (m, 1H), 2.72 (m, 1H), 2.20 (m, 2H), 1.62 (m, 2H), 1.3–1.4 (m, 4H). FABMS m/z 618 $[MH]^+$; HRFABMS calcd for $C_{26}H_{35}F_3N_5O_7S$ 618.2210, found 618.2227.

*2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid (**8**)*

The biotinylated ester **7** (0.49 g, 0.8 mmol) was dissolved in methanol (15 mL). Aqueous NaOH (4 mL of 1N solution, 4 mmol) was added at 0 °C and the yellow solution was stirred at room temperature in the dark overnight. After evaporation of methanol, the residue was dissolved in water and the solution was acidified with 1N HCl. This was extracted with CH_2Cl_2, dried over $MgSO_4$ and evaporated. The crude acid was purified by reprecipitation from $CHCl_3$–hexane to give **8** (0.42 g, 88 %) as a colorless solid. IR (nujol) 3250, 1670 cm^{-1}; UV (EtOH) λ_{max} (ϵ) 285(2600), 354 (408, shoulder). ^1H NMR ($CDCl_3$) : δ 8.0, 7.6, 6.1 (br, each 1H), 7.80 (d, 1H, *J*=8.2 Hz), 6.83 (d, 1H, *J*=8.2 Hz), 6.72 (s, 1H), 4.53 (dd, 1H, *J*=7.3, 4.9 Hz), 4.36 (dd, 1H, *J*=7.3, 4.3 Hz), 4.19 (t, 2H, *J*=4.4 Hz), 3.90 (t, 2H, *J*=4.4 Hz), 3.87 (s, 3H), 3.75 (br, 2H), 3.64 (br, 2H), 3.57 (br, 2H), 3.44 (br, 2H), 3.16 (m, 1H), 2.92 (dd, 1H, *J*=4.9, 12.8 Hz), 2.75 (d, 1H, *J*=12.8 Hz), 2.23 (m, 2H), 1.7–1.6 (m, 4H), 1.45 (m, 2H). FABMS(FAB) m/z 604 $[MH]^+$; HRFABMS calcd for $C_{25}H_{33}F_3N_5O_7S$ 604.2053, found 604.2057.

2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid N*-hydroxysuccinimide ester (**1**)*

A solution of the acid **8** (0.14 g, 0.23 mmol) and *N*-hydroxysuccinimide (0.028 g, 0.24 mmol) in THF–CH_3CN (1:1, 5 mL) was mixed with a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC free base; 0.04 g, 0.26 mmol) in 1 mL of THF–CH_3CN (1:1) at room temperature. The reaction mixture was stirred at room temperature for 14 h in the dark. After evaporation of the solvents, the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to afford **1** (0.13 g, 83 %) as a pale yellow solid. IR (nujol) 1740, 1700 cm^{-1}; UV (H_2O) λ_{max} (ϵ) 310 (3840), 355 (420). ^1H NMR ($CDCl_3$) : δ 8.04 (d, 1H, *J*=7.9 Hz), 6.89 (d, 1H, *J*=7.9 Hz), 6.75 (s, 1H), 6.7, 6.6, 5.5 (br, 1H), 4.47 (dd, 1H, *J*=7.3, 4.8 Hz), 4.28 (dd, 1H, *J*=7.3, 4.9 Hz), 4.23 (t, 2H, *J*=4.3 Hz), 3.91 (t, 2H, *J*=4.3 Hz), 3.72 (dd, 2H, *J*=2.4, 3.7 Hz), 3.63 (dd, 2H, *J*=2.4, 3.7 Hz), 3.55 (t, 2H, *J*=4.9 Hz), 3.40 (br, 2H), 3.11 (m, 1H), 2.91 (s, 4H), 2.87 (dd, 1H, *J*=4.8, 12.8 Hz), 2.72 (d, 1H), 2.19 (m, 2H), 1.7–1.6 (m, 4H), 1.40 (m, 2H). FABMS m/z 701 $[MH]^+$; HRFABMS calcd for $C_{29}H_{36}F_3N_6O_9S$ 701.2217, found 701.2231.

Photolysis of the methyl ester 7 in methanol

A 0.29 μM solution of **7** in methanol was placed in a quartz cuvette. After replacing the inner atmosphere with argon, photolysis was carried out with a 30 W black-light lamp (Ultra-Violet Products Inc., San Gabriel, California, U.S.A.) at a distance of 4 cm from the surface of light source.

*Photocross-linking of egg white avidin subunits with **1***

A 12.1 μM solution of egg white avidin tetramer (determined from the UV absorption: ϵ_{280} = 24,000)[23] in 0.1 M sodium bicarbonate pH 8.3 (200 μl, 9.7 nmol as monomer) was diluted with 0.1 M sodium bicarbonate, pH 8.3 (800 μL). A 1 μL of 7.5 mM solution of **1** (7.5 nmol) in DMF was added and the reaction mixture was kept at room temperature for 10 min in the dark. An aliquot (500 μL) of the mixture was placed in a glass tube (φ1 × 2.5 cm), and the irradiation was carried out from the open end of the tube for 40 min at 4 °C with the same setup described above. Samples were subsequently boiled in 2 % SDS (with 5 % β-mercaptoethanol) for 10 min before SDS–PAGE. After being electrophoresed, the gels were fixed in an aqueous solution of 50 % methanol–10 % acetic acid before silver staining. The gels for chemiluminescent detection were not fixed and processed as described below.

Chemiluminescent analysis of photocross-linking products

Following SDS–PAGE, protein bands were electrotransferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore) in 192 mM glycine, 25 mM Tris, 20 % methanol, 0.1 % SDS. Running time was 12 h at 12 mA and a constant temperature of 4 °C was maintained. The transferred membrane was blocked with 2 % skimmed milk in blocking buffer [0.1 M sodium phosphate, pH 7.3, 0.15 M NaCl, 0.1 % Tween 20: (T-PBS)] for 1 h at room temperature and then washed with T-PBS (5 min, two times). After soaking the membrane in 1500 times diluted streptavidin–horseradish conjugate (Amersham) for 1 h at room temperature, the membrane was developed using chemiluminescent detection reagents (Renaissance, DuPont NEN) for 1 min. The membrane was then wrapped in a plastic sheet and exposed to Hyperfilm–ECL (Amersham) for 3 min in the dark.

Acknowledgment

This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas No. 05274209 and Grant-in-Aid No. 06453193 from the Ministry of Education, Science and Culture, Japan.

References and Notes

1. Yoshida, E.; Nakayama, H.; Hatanaka, Y.; Kanaoka, Y. *Chem. Pharm. Bull.* **1990,** *38*, 982.

2. (a) Fabry, M; Brandenburg, D. *Biol. Chem. Hoppe–Seyler* **1992,** *373*, 143; (b) Marie, J.; Seyer, R.; Lombard, C.; Desarnaud, F.; Aumelas, A.; Jard, S.; Bonnafous, J.-C. *Biochemistry* **1990,** *29*, 8943; (c) Watanabe, Y.; Hirata, M.; Ogasawara, T.; Koga, T.; Ozaki, S. *BioMed. Chem. Lett.* **1991,** *1*, 399.

3. Fabry, M.; Schaefer, E.; Ellis, L.; Kojro, E.; Fahrenholz, F.; Brandenburg, D. *J. Biol. Chem.* **1992,** *267*, 8950.

4. Bäumert, H. G.; Fasold, H. *Methods Enzymol.* **1989,** *172,* 584.

5. Huang, C.-K.; Richards, F. M. *J. Biol. Chem.* **1977,** *252,* 5514.

6. Bayley, H. *Laboratory Techniques in Biochemistry and Molecular Biology: Photogenerated Reagents in Biochemistry and Molecular Biology*, pp. 25-44. Work, T. S.; Burdon, R. H., Eds.; Elsevier, Amsterdam. 1983.

7. Nakayama, H.; Hatanaka, Y.; Yoshida, E.; Oka, K.; Takanohashi, M.; Amano, Y.; Kanaoka, Y. *Biochem. Biophys. Res. Commun.* **1992,** *184*, 900.

8. Nakayama, H.; Hatanaka, Y.; Taki, M.; Yoshida, E.; Kanaoka, Y. *Ann. N. Y. Acad. Sci.* **1993,** *707,* 349.

9. Nakayama, H.; Taki, M.; Striessnig, J.; Glossmann, H.; Catterall, W. A.; Kanaoka, Y. *Proc. Natl Acad. Sci. USA* **1991,** *88,* 9203.

10. Platz, M.; Admasu, A. S.; Kwiatkowski, S.; Crocker, P. J.; Imai, N.; Watt, D. S. *Bioconjugate Chem.* **1991,** *2,* 337.

11. (a) Dolder, M.; Michel, H.; Sigrist, H. *J. Prot. Chem.* **1990,** *9,* 407; (b) Kogan, A.; Bochkariov, D. E.; Baskunov, B. P.; Cheprakov, A. V. *Liebigs Ann. Chem.* **1992,** 879; (c) Collioud, A.; Clémence, J-F.; Sänger, M.; Sigrist, H. *Bioconjugate Chem.* **1993,** *4,* 528; (d) Resek, J. F.; Bhattacharya, S.; Khorana, H. G. *J. Org. Chem.* **1993,** *58,* 7598.

12. (a) Hatanaka, Y.; Yoshida E.; Nakayama, H.; Kanaoka, Y. *Bioorganic Chem.* **1989,** *17,* 482; (b) Hatanaka, Y.; Hashimoto, M.; Kurihara, H.; Nakayama, H.; Kanaoka, Y. *J. Org. Chem.* **1994,** *59*, 383; (c) Hatanaka, Y.; Hashimoto, M.; Nakayama, H.; Kanaoka, Y. *Chem. Pharm. Bull.* **1994,** *42*, 826.

(*Received in U.S.A.* 6 *June* 1994; *accepted* 13 *July* 1994)

13. Wilchek, M.; Bayer, E. A. *Methods Enzymol.* **1990,** *184,* 123.

14. Leong, M. M. L.; Fox, G. R. *Methods Enzymol.* **1990,** *184,* 442.

15. Hiller, Y.; Gershoni, J. M.; Bayer, E. A.; Wilchek, M. *Biochem. J.* **1987,** *248,* 167.

16. Liu, M. T. H. *Chem. Soc. Rev.* **1982,** *11,* 127.

17. (a) Baldwin, J. E.; Pratt, A. J.; Moloney, M. G. *Tetrahedron* **1987,** *11,* 2565; (b) Baldwin, J. E.; Coates, J. B.; Halpern, J. B.; Moloney, M. G; Pratt, A. J. *Biochem. J.* **1989,** *261,* 197; (c) Rimoldi, J. M.; Kingston, D. G.; Chaudhary, A. G.; Samaranayake, G. *J. Nat. Prod.* **1993,** *56,* 1313.

18. The acid **8** is soluble in water up to 1.0 mM at room temperature and the solubility of *d*-biotin in water is about 22 mg/100 mL (ca 1 mM); *The Merck Index, 11th edn.;* Budavari, S. Ed.; Merck & Co. Rahway, NY.; 1989.

19. Bayer, E. A.; Wilchek, M. *Methods Enzymol.* **1990,** *184,* 138.

20. DeLange, R. J.; Huang, T. S. *J. Biol. Chem.* **1971,** *246,* 698.

21. Gitlin, G.; Bayer, E. A.; Wilchek, M. *Biochem. J.* **1987,** *242,* 923.

22. (a) Livnah, O.; Bayer, E. D. Wilchek, M.; Sussman, J. L. *Proc. Natl Acad. Sci. USA* **1993,** *90,* 5076; (b) Pugliese, L.; Coda, A.; Malcovati, M.; Bolognesi, M. *J. Molec. Biol.* **1993,** *231,* 698.

23. Green, N. M. *Methods Enzymol.* **1990,** *184,* 51.