Post-biotinylation of Photocrosslinking by Staudinger–Bertozzi Ligation of Preinstalled Alkylazide Tag

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Post-biotinylation of the alkyl azide derivative of trifluoromethyl phenyldiazirine (TPD) was elucidated to apply a photoaffinity biotinylation technique. A photo-modified polyvinilidene difluoride (PVDF) membrane was used as a photolabeled component and we introduced biotin by Staudinger–Bertozzi ligation. The 15 pmol amount of biotinylated reagent was still effective for the visualization of cross-linked product on the matrix. The results show the potential utility of alkyl azide carrying TPD derivatives in the application of photoaffinity biotinylation, which could be useful for the ligands with tight structural requirements.

Key words diazirine; photoaffinity label; chemical biology; Staudinger reaction; avidin-biotin

Photoaffinity labeling is a powerful method in the chemical biology of protein functions.[1,2] This method is especially useful for protein binding site identification where the application of crystallography or NMR is difficult. Taking advantage of the chemically stable cross-link from (3-trifluoromethyl) phenyldiazirine (TPD) based photolabeling,[3] we have developed a series of biotinyl TPD derivatives as a fish-out approach to improve laborious experimental routines used in binding site identification.[4—10] The application of a *N*-acetylglucosamine photoprobe carrying biotinylated diazirine gave the first information on acceptor site peptides on β1,4-galactosyltransferase.[6,7] We also developed a novel method for the one-step introduction of a biotinylated diazirine photophore into unprotected carbohydrate ligands.[11] Alternatively, biotinylated reagents with a scissile function have been considered for separation of the labeled components from a biotin–avidin complex because the complex is essentially irreversible ($K_d = 10^{-15}$ M).[12] For displacing the labile disulfide group, photoreactive,[13,14] fluoride sensitive,[15] alkali sensitive,[16] oxidative[17,18] and acylsulfonamide[19] linkages have been developed.

Large alternations within the ligand structure, such as through the introduction of a biotinyl group, were reported to decrease the affinity of some ligands.[20] In such cases, the introduction of biotin after photolabeling is one of the rational approaches to overcome the problem, because the process includes minimal chemical modifications of the ligand skeleton to avoid the reduction of ligand activity due to the pre-installed biotin moiety.[21,22] Alkyl azide derivatives were employed as useful post modifiable tags by application of the Staudinger–Bertozzi ligation or azide-alkyne [3+2] cycloaddition (click chemistry).[23,24] The application of the Staudinger–Bertozzi ligation and click chemistry in photoaffinity labeling was examined with aryl azide and diazirine photophores.[23] The results clearly showed that aryl azide and diazirine photophores are selectively photoactivated to leave the alkyl azide as intact for the possible application of the Staudinger–Bertozzi ligation. Although post fluorescent labeling with the Staudinger–Bertozzi ligation was shown for the aryl azide photophore, a TPD derivative carrying an alkyl azide tag was not evaluated for post labeling.[23] The TPD photophore can be photolyzed much faster than aryl azide at a longer wavelength (350 nm), where alkyl azide decomposition is negligible.[3] We report here on the photolysis of alkyl azide carrying TPD following the incorporation of a biotin tag for the possible application of diazirine based photoaffinity biotinylation.

Benzyl bromide derivative **1**[25] having a diazirinyl group was easily converted to alkyl azide analogue **2** with sodium azide (Fig. 1). The half-life of the compound with black light irradiation in methanol was calculated as 3.5 min (Fig. 2). The IR spectrum of the photoirradiated sample showed a strong peak corresponding to the azide moiety at 2100 cm^{-1},

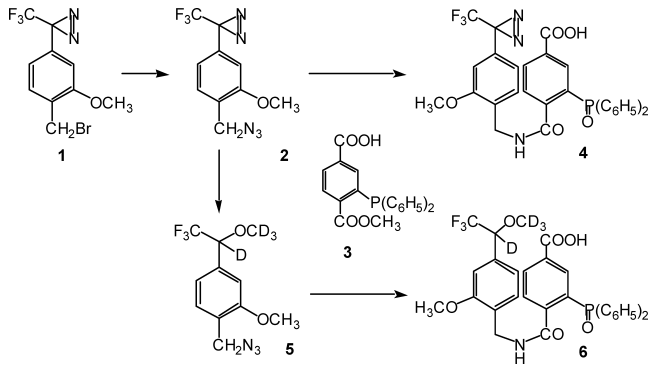

Fig. 1. Synthesis of the Alkylazide Derivative of the (3-Trifluoromethyl) Phenyldiazirine Derivative and Its Application to the Staudinger–Bertozzi Ligation

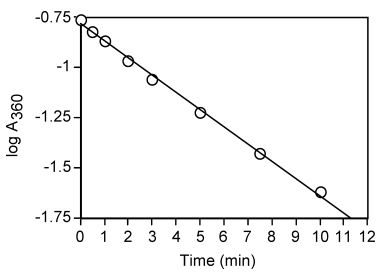

Fig. 2. The Decay in Absorbance at 360 nm of Compound **2** with Black Light Photolysis as a Function of the Time of Photolysis in a Semi-Log Representation

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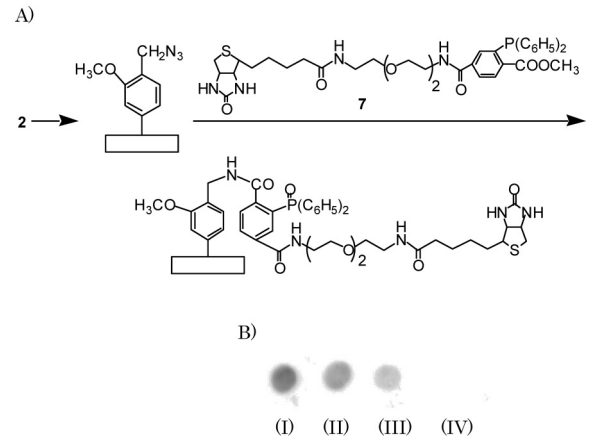

Fig. 3. Chemiluminescence Detection of the Post-Functionalized PVDF Membrane with Compound **7**

(A) Scheme of the post-functional modified PVDF membrane. (B) Chemiluminescence detection of blotted compound **7**. Amounts blotted were, 35, 25, 15 and 7.5 pmol for (I) to (IV), respectively.

and ^{1}H-NMR confirmed the presence of a methylene vicinal to the azide at 4.10 ppm. The results indicate that the azide moiety remained after photolysis, as reported previously.[23,24] The Staudinger reaction with triphenylphosphine derivative[27] **3** with compound **2** afforded the desired ligated product **4** in a moderate yield. Compound **2** in methanol-d_3 was photoirradiated with black light to afford **5**. The photolyzed mixture was subjected to the Staudinger–Bertozzi ligation with compound **3** to afford ligated compound **6**. The method was then examined at the pmol scale, in which many photoaffinity labeling experiments are usually performed. Compound **2** was coated on the surface of a polyvinilidene difluoride (PVDF) membrane followed by photoirradiation[6] with black light to introduce a crosslink (Fig. 3A). The membrane was then subjected to the post biotinylation reaction with biotinylated triphenylphosphine derivative **7** and the ligated biotin was detected with a Streptavidin–horseradish peroxidase (HRP) conjugate.[5] The 15 pmol amount of reagent **7** was still effective for visualization of cross-linked product on the matrix (Fig. 3B).

The results show the potential utility of alkyl azide carrying TPD derivatives in the application of photoaffinity biotinylation, which could be useful for the ligands with tight structural requirements.

Experimental

All ^{1}H-NMR spectra were measured using JEOL JNM-FX270 and ECA-500 spectrometers. MS spectra were obtained using JEOL JNM-LA400 spectrometers. IR spectra were obtained using JASCO FT-IR 420. All solvents were of reagent grade and distilled using the appropriate methods.

3-(4-Azidomethyl-3-methoxy)-3-trifluoromethyl-3*H*-diazirine (2) Compound **1**[25] (0.1304 g, 0.422 mmol) and NaN_3 (0.1478 g, 2.273 mmol) were suspended in DMF (3 ml). The reaction mixture was stirred at room temperature for 6 h and concentrated. The residue was partitioned between ethyl acetate and water. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, filtrated and concentrated. The residue was subjected to silica column chromatography (ethyl acetate : hexane = 1 : 14) to afford **2** as a pale yellow oil (0.0752 g, 66%). ^{1}H-NMR (CD_3OD) δ: 7.377 (1H, d, J=7.4 Hz), 6.886 (1H, d, J=8.0 Hz), 6.762 (1H, s), 4.370 (2H, s), 3.875 (3H, s). IR (neat) 2100 cm^{-1}. FAB-MS m/z 272 ($M+H^+$) HR-FAB-MS Calcd for $C_{10}H_9F_3N_5O$ 272.0738, Found 272.0759.

3-*N*-[2-Methoxy-4-(3-trifluoromethyl-3*H*-diazirin-3-yl)benzyl]-aminocarbonyl-4-(diphenylphosphinyl)-benzoic Acid (4) Compound **2** (15.3 mg, 56.4 μmol) and 1-methyl-2-diphenylphosphinoterephthalate **3**[27] (25.1 mg, 68.9 μmol) were dissolved in THF–H_2O (4 : 1, 0.5 ml). The reaction mixture was stirred for 24 h, and then concentrated. The residue was purified with silica column chromatography ($CHCl_3$: CH_3OH = 8 : 1) to afford **4** as a yellow compound (18.0 mg, 54%). ^{1}H-NMR ($CDCl_3$) 8.629 (1H, t, J=5.6 Hz, NH), 8.177 (1H, d, J=7.9 Hz), 7.922 (1H, dd, J=7.9, 3.6 Hz), 7.814 (1H, dd, J=14.5, 1.6 Hz), 7.65—7.43 (m, 10H), 7.115 (1H, d, J=7.9 Hz), 6.662 (1H, dd, J=7.9 Hz), 6.523 (1H, s), 4.075 (2H, d, J=5.6 Hz), 3.793 (3H, s). FAB-MS m/z 594 ($M+H^+$) HR-FAB-MS Calcd for $C_{30}H_{24}F_3N_3O_5P$ 594.1406, Found 594.1420.

Half Life of Compound 4 Assessed by UV Spectrophotometry A methanolic solution of compound **4** (0.5 mM) was irradiated with black light at 1 cm at 0 °C. The spectrum was measured at 0.5, 1, 2, 3, 5, 7.5 and 10 min. The broad peak at 360 nm decreased over time.

Irradiation of Compound 4 in Methanol-d_3 (5) and 3-*N*-[2-Methoxy-4-(3-trifluoromethyl-2-d_3-methoxy-2-*d*-3-yl)benzyl]aminocarbonyl-4-(diphenylphosphinyl)-benzoic Acid (6) Compound **2** (3.4 mg, 12.5 μmol) was dissolved in CD_3OD (2.5 ml). The solution was irradiated with black light for 20 min. ^{1}H-NMR (CD_3OD) 7.322 (1H, d, J=7.9 Hz), 7.106 (1H, s), 7.052 (1H, d, J=8.0 Hz), 4.357 (2H, s), 3.881 (3H, s), IR (neat) 2100 cm^{-1}.

The irradiated mixture was subjected to the Staudinger reaction with triphenylphosphine derivative **3** (16.5 mg, 45.3 μmol) in THF–H_2O (4 : 1, 0.5 ml). The product was purified with PLC ($CHCl_3$: CH_3OH = 8 : 1×3 times developments) and eluted with $CHCl_3$: CH_3OH = 4 : 1 to afford a colorless amorphous mass (2.9 mg, 39%), ^{1}H-NMR ($CDCl_3$) δ: 8.652 (1H, t, J=5.7 Hz), 8.237 (1H, d, J=8.0 Hz), 7.977 (1H, q, J=7.9, 3.6 Hz), 7.900 (1H, d, J=13.2 Hz), 7.716—7.490 (10H, m), 7.144 (1H, d, J=8.0 Hz), 6.895 (1H, s), 6.883 (1H, d, J=8.0 Hz), 4.101 (2H, d, J=5.7 Hz), 3.856 (3H, s). IR (neat) 2100 cm^{-1}. FAB-MS m/z 602 ($M+H^+$), HR-FAB-MS Calcd for $C_{31}H_{24}D_4F_3NO_6P$ 602.1853, Found 602.1837.

Photoimmobilization of Compound 2 on PVDF Membrane PVDF membrane (10 cm square) was wetted with 0.5 mM of compound **2** in MeOH, dried and irradiated by black light for 20 min on one side. After washing with MeOH, the membrane was dried completely. The biotinylated triphenylphosphine derivatives[27] in PBS were blotted on the dried modified membrane and heated to 50 °C for 8 h. The membrane was well washed with MeOH, Tween-phosphate buffered saline (T-PBS) and treated with 10%-skimmed milk/T-PBS for 1 h. After washing with T-PBS three times, the membrane was immersed in 2000 times diluted Streptavidin–HRP with T-PBS for 1 h, and then washed with T-PBS five times. The treated membrane was subjected to chemiluminescence detection.

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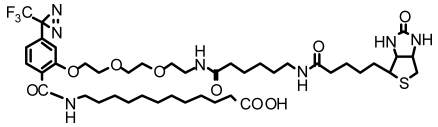

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