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Positively coded photoaffinity label for altering isoelectric points of proteins

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Abstract—Novel diazirinyl photoaffinity ligand, which contains (3-trifluoromethyl) phenyldiazirine and penta(ε-Boc-Lys) as a photoreactive code, allows the introduction of a positive cascade to alter the pI value of labeled components, facilitating the isolation of photolabeled biocomponents with isoelectric focusing techniques.

Photoaffinity labeling is a powerful method in the study of biological structures and functions.[1] It is suitable for the analysis of biological interactions because it is based on the affinity of the ligand moiety. Various photophores, such as phenyldiazirine, arylazide, and benzophenone, were used. Although comparative irradiation studies of these three photophores in living cells suggested that a carbene precursor (3-trifluoromethyl)phenyldiazirine is the most promising photophore,[2–4] low cross-linking yield of photoaffinity labeling experiments still hamper purification and isolation of the labeled components.[5] We have demonstrated to resolve these difficulties by a combination of avidin–biotin systems (photoaffinity biotinylation).[6–8] The disassociation of the labeled components from the avidin–biotin complex is sometimes hampered because the complex is too stable to disassociate.[9] 2-D electrophoresis is one of the major analytical methods for proteomics studies. The combination of isoelectrofocusing and molecular weight analysis promotes the analysis of whole proteins with a high resolution. Isoelectric pH (pI) values are unique for each protein, therefore altering the pI value of photolabeled components will facilitate their isolation from unlabeled components by isoelectrofocusing. Poly-basic or -acidic amino acids groups can be used to alter pI, and these changes are distinguishable on electrofocusing. For example, the introduction of five additional Lys residues to a neutral protein will increase pI by about 0.5 in a simulation.[10] These new concepts can be applied for manipulation of the photoaffinity-labeled components. In this paper, we describe the synthesis and application of a novel diazirinyl photophore containing (ε-Boc-Lys) which is effective in resolution of the labeled protein by altering the pI value. Figure 1 shows the structure of the novel diazirinyl photoaffinity ligand **1**. The compound consisted of a photoreactive diazirinyl photophore at the C-terminal and biotin moiety at the N-terminal of a penta(ε-Boc-Lys) peptide. After acid treatment, the Boc groups were deprotected, leading to an increased pI of the labeled component. Furthermore, the labeled component became a substrate for trypsin and released the biotin moiety with enzymatic cleavage under the native condition.[11]

Compound **1** was synthesized from commercially available Z-(ε-Boc-Lys)$_5$-OH **2**. After removing Z-group of **2**, biotin was introduced into N-terminal of **3** to afford **4**. The C-terminal was converted to succinimide ester, then diazirine derivative **5**[12] was condensed to afford compound **1** (Fig. 2).[13] The carboxylic acid was converted to succinimide derivative **6**. Each step proceeded with a moderate yield. The properties of compound **1** on altering the isoelectric point were tested. Chromatofocusing of protected and deprotected compound **1** was performed with a pH gradient between pH 7 and 4. The protected form was eluted at pH 5.5 (Fig. 3A, i), on the other hand, the deprotected form was eluted at

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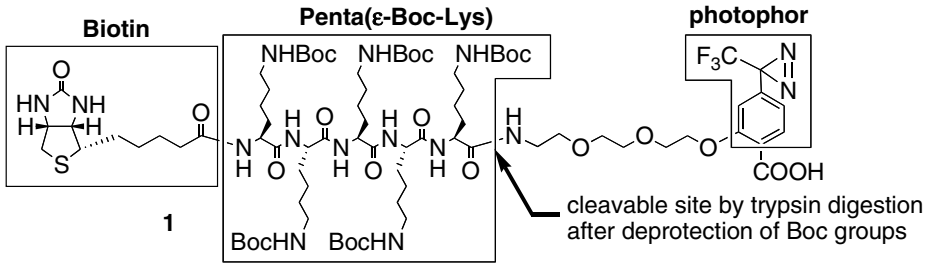

Figure 1. Structure of novel diazirinyl compound **1**.

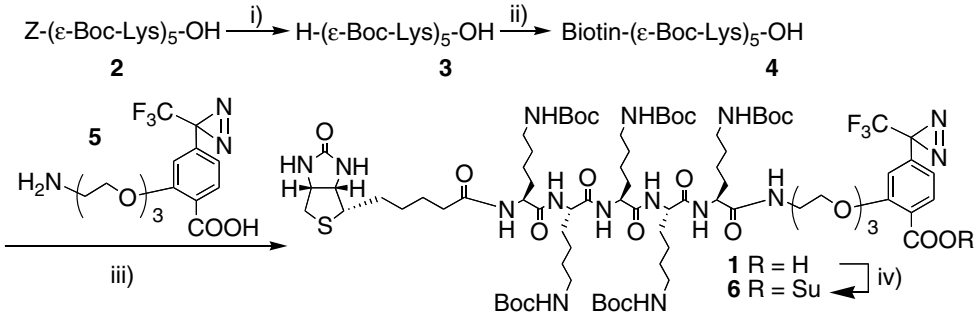

Figure 2. Synthesis of compound **1**. Reagents and conditions: (i) H_2, 10% Pd/C, rt, 5 h, 90%; (ii) biotin *N*-hydroxysuccinimide, triethylamine, DMF, rt, 10 h, 90%; (iii) *N*-hydroxysuccinimide, EDC·HCl, then, **5**, TEA, DMF, rt, 12 h, 78%; (iv) *N*-hydroxysuccinimde, DCC, DMF, rt, 8 h, 71%.

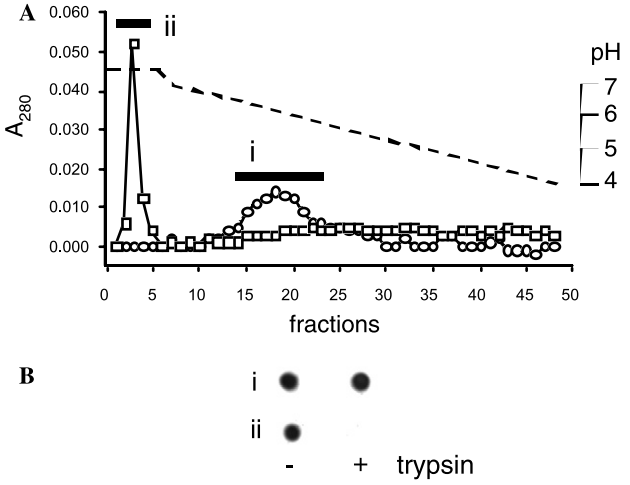

Figure 3. (A) Chromatofocusing profiles of photoprobe **1** without (open circle) and with (open square) deprotection of Boc groups. PBE™ 94 gel (5 ml gel, 1 × 6.4 cm) was equilibrated with 0.025 M imidazole–HCl (pH 7.4). After loading the sample, the column was eluted with Polybuffer 74-HCl and the fraction was collected with each 1.5 mL, then absorbance measured at 280 nm. The pH gradient profile was represented as dashed line. The peaks i and ii were concentrated and subjected to chemiluminescence detection. (B) Chemiluminescence detection of the regions i and ii on (A). The samples with or without trypsin treatment (indicated by + and −) were blotted on PVDF and immobilized with black light irradiation in the similar manner described before.[7]

over pH 7.4 (Fig. 3A, ii). The results indicated that the isoelectric point was altered by deprotection of the Boc group and the reagent utilized to alter the pI value of photolabeled components. Both peaks i and ii were subjected to trypsin digestion. The samples with or without trypsin treatment were immobilized on a poly(vinylidene difluoride) (PVDF) membrane by photolysis of the diazirine part with black light. The membrane was subjected to chemiluminescence detection with streptavidin–horseradish peroxidase conjugate to detect the biotin moiety in a similar manner as described previously.[5] Both peaks i and ii without trypsin treatment and peak i with trypsin digestion afforded chemiluminescence signals. On the other hand, no chemiluminescence signal was detected in peak ii with trypsin treatment (Fig. 3 B). All samples without photolysis did not afford any chemiluminescence signals (data not shown). The results indicated that TFA-treated compound **1** (de-Boc derivative) is a substrate for trypsin and has a potency to cleave between the photophore and biotin efficiently at C-terminal of lysines. Hydroxysuccinimide ester **6** was utilized for the chemical modification of BSA. The modified BSA was subjected to IEF–PAGE, blotting of the PVDF membrane, and then chemiluminescence detection. The samples with acid treatment showed a slightly higher isoelectric point than the samples without acid treatment on IEF–PAGE. The migrated chemiluminescence signals disappeared after the sample was incubated with trypsin (Fig. 4A), but the protein band at 66 kDa was observed with silver staining (Fig. 4B). These results indicated that compound **1** was utilized to alter the pI of labeled components from unlabeled components.

The benzoic acid moiety has an inhibitory activity on D-amino acid oxidase from the porcine kidney.[14] Photoaffinity labeling of the enzyme with compound **1** was performed, and then the labeled component was detected by chemiluminescence with streptavidin–horseradish peroxidase. The chemiluminescence signal for the

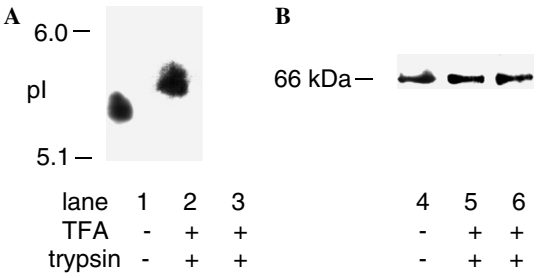

Figure 4. Chemiluminescence detection with IEF–PAGE (pH 3–7) (A) and silver staining detection with SDS–PAGE (10%) (B) of modified BSA with compound **6**. The lyophilized powder of modified BSA was treated with TFA at room temperature for 0.5 h and subjected to IEF– and SDS–PAGE. The gel of IEF–PAGE was subjected to Western blotting on the PVDF membrane. The membrane was treated for chemiluminescence detection with streptavidin–HRP in the same manner as described previously.[5] The treated conditions were indicated as − and + for TFA or trypsin absence and presence, respectively.

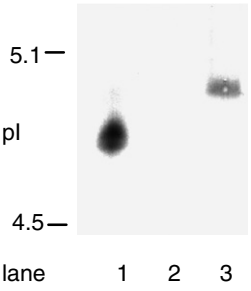

Figure 5. Chemiluminescence detection of photoaffinity-labeled D-amino acid oxidase with compound **1**. The labeled proteins were subjected to IEF–PAGE (pH 3–7), followed by transfer to a PVDF membrane to detect the biotin moiety. The photolabeling was inhibited by excess phenylalanine (lane 2). The labeled protein was treated with TFA in the same manner as described above (lane 3).

enzyme was inhibited in the presence of phenylalanine on IEF–PAGE (Fig. 5, lanes 1 and 2). The specific chemiluminescence was migrated with TFA treatment of the labeled mixture (Fig. 5, lane 3).[15]

In conclusion, the new diazirinyl photoprobe containing penta(ε-Boc-Lys) is useful to detect photolabeled components by virtue of altering pI of labeled components from unlabeled ones. Furthermore, the compounds **1** and **6** were easily introduced ligand moieties by amide bond formation and purified the labeled components by avidin–biotin systems. The results indicated that other peptides consisting of poly-basic or -acidic amino acids would be useful for application using this strategy.

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13. Compound **1** ^{1}H NMR (CD_3OD) 7.889 (d, 1H, J = 8.2 Hz), 7.02 (d, 2H, J = 8.2 Hz), 6.89 (s, 1H), 4.52 (dd, 2H, J = 7.6, 4.9 Hz, biotin H-4), 4.36 (m, 8H, biotin H-3, OCH_2, and Lys-α-CH × 5), 3.93 (m, 2H), 3.77 (m, 2H), 3.66 (m, 2H), 3.59 (m, 2H), 3.36 (m, 2H), 3.24 (m, 1H, biotin H-2), 3.07 (m, 10 H, Lys-ε-CH_2 × 5), 2.97 (dd, 1H, J = 12.9, 4.9 Hz, biotin H-5), 2.74 (d, 1H, J = 12.9 Hz, biotin-5), 2.33 (m, 2H, biotin-α-CH_2), 1.73–1.23 (m, 81H, Lys-β, γ, δ,–CH_2 × 5, biotin-β, γ, δ-CH_2, Boc × 5). FAB-MS m/z 1745 (MH^+).
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15. Typical photoaffinity labeling for D-amino acid oxidase. D-Amino acid oxidase (50 μg protein) and compound **1** (0.25 nmol) in 0.1 M Tris–HCl (pH 7.8) were incubated at 37 °C for 5 min with or without phenylalanine (200 μmol). The labeled mixture was concentrated with N_2 flow, treated with trifluoroacetic acid for 3 h at room temperature, and then concentrated. The residue was reconstituted with 0.1 M $NaHCO_3$ for IEF–PAGE.