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Enzyme cleavable and biotinylated photoaffinity ligand with diazirine

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Abstract—The efficient synthesis of an enzyme cleavable biotinylated diazirinyl photoaffinity ligand is described to allow the effective manipulation of the photolabeled biocomponents. The compound contains a glutamic acid γ-methyl ester, which is a precursor of the substrate for V8 protease, between the diazirinyl photophor and biotin moiety. After alkaline hydrolysis of the ester, the compound can be proteolyzed at the Glu moiety by V8 protease. The photophore was introduced to L-Phe *p*-nitroanilide and the ligand was applied to photolabel of chymotrypsin to manipulate the application of the concept.

Photoaffinity labeling is a powerful method in the study of biological structures and functions.[1–5] It will be suitable for the analysis of biological interactions in vivo because it is based on the affinity of the ligand moiety. Various photophores, such as phenyldiazirine, arylazide, and benzophenone, were used. Comparative irradiation studies of these three photophors in living cells suggested that a carbene precursor (3-trifluoromethyl) phenyldiazirine is the most promising.[6] Recently, photoreactive amino acid derivatives containing benzophenone units were utilized, as they are commercially available. However, comparative incorporation studies of a photoreactive plasminogen activator peptide having benzophenone or (3-trifluoromethyl) phenyldiazirine at the same position revealed that the latter peptide gave higher labeling efficiency.[7] Low photolabeling yield hampers purification and isolation of the labeled components.[3,8] We have attempted to resolve these difficulties by a combination of avidin–biotin systems (photoaffinity biotinylation)[9–15] or immune-interaction for photolabeling reagents.[16] We developed a series of biotinyl probes for the photoaffinity labeling of biofunctional proteins. The covalent introduction of a biotinyl tag to the target molecule by photoaffinity labeling followed by the use of avidin–biotin interaction, facilitated the structural analyses of photolabeled components. The combination enabled us to detect and isolate photolabeled proteins and peptides without the use of radioisotopes. However, it is difficult to retrieve the biotinylated labeled components from the avidin–biotin complex quantitatively.[10] This is due to the higher affinity between the avidin–biotin complex ($K_d = 10^{-15}$ M).[17] Two techniques were considered to solve this problem. One method was the utilization of monomeric avidin to retrieve the biotinylated components under mild conditions.[11,12] Monomeric avidin has less affinity to biotin ($K_d = 5 \times 10^{-8}$ M) than native tetrameric avidin[18], however, a very high concentration and large amount of this protein is sometimes required. The other method is a chemical cleavage between the photophore and biotin. It includes photocleaving,[19] periodate-cleaving,[14] and utilizing thiol-cleavable[15] biotinylated diazirinyl derivatives. However, these chemical cleavages sometimes damage biomolecules due to their reaction with functional groups in biomolecules under cleavage conditions. In this paper, we describe a new method by which the enzyme cleavable component is inserted between the diazirinyl photophore and biotin moiety and subsequent use of it to remove biotin from labeled biomolecules. Figure 1 shows the new enzyme cleavable biotinylated photoaffinity ligand for chymotrypsin (Fig. 2).

Keywords: Photoaffinity labeling; Diazirine; Avidin–biotin; V8 protease; Chymotrypsin.

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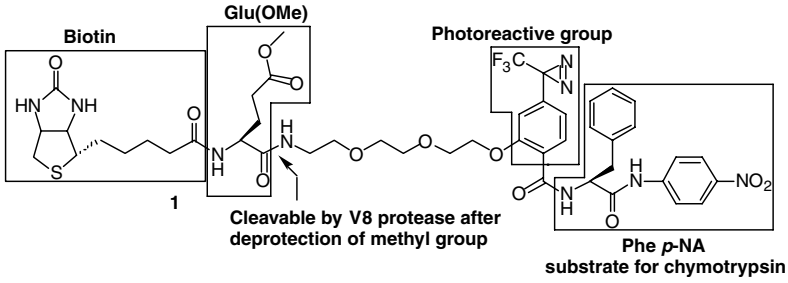

Figure 1. Structure of enzyme cleavable biotinylated photolabel reagent for chymotrypsin.

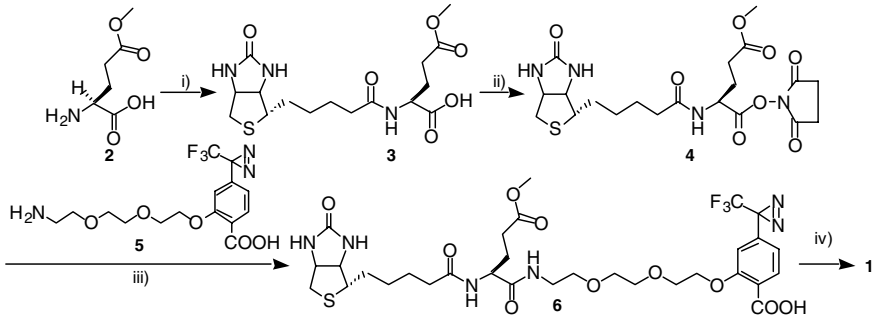

Figure 2. Synthesis of compound **1**. (i) Biotin *N*-hydroxysuccinimide ester, DMF, rt, 5 h, 81%, (ii) *N*-hydroxysuccinimide, DCC, DMF, rt, 2 h, 90%, (iii) TEA, DMF, rt, 5 h, 91%, (iv) *N*-hydroxysuccinimide, DCC, DMF, rt, then L-Phe *p*-NA, TEA, DMF, rt, 4 h, 60%.

The compound consisted of (1) photoreactive diazirinyl photophor, (2) L-glutamic acid γ-methyl ester (L-Glu γ-Me) spacer, (3) biotin moiety, and (4) L-phenylalanine *p*-nitroanilide (L-Phe *p*-NA) attached to diazirine, a substrate for chymotrypsin. Enzymatic cleavage was controlled with alkaline hydrolysis of the L-Glu γ-Me. After hydrolysis, the labeled component became the substrate for V8 protease and released the biotin moiety from the photolabeled site during enzymatic cleavage.

The compound **1** was synthesized from the Glu γ-methyl ester **2**. Biotin was introduced to the N-terminal **3**, and the C-terminal was converted to succinimide ester **4**. The diazirine derivative **5**[15] was condensed to afford compound **6**. L-Phe *p*-NA was introduced to the carboxylic acid of the diazirinyl moiety to obtain compound **1**.[20] Each step proceeded with a moderate yield.

The biological activity of compound **1** as a chymotrypsin substrate was tested by spectrometric assay.[21] We performed the assay in a 96-well titer plate. There were no differences between this and a normal assay in a tube. Compound **1** and L-Phe *p*-NA were applied to the assay. The obtained K_m value of compound **1** (175 μM) indicated that **1** is better substrate of chymotrypsin than L-Phe *p*-NA (750 μM) (Fig. 3). Cleavage of the photophore and biotin by V8 protease[22] was then inspected. Compound **1** was subjected to several condition as follows: (i) alkaline hydrolysis with aqueous NaOH at room temperature for 2 h, (ii) enzymatic digestion with V8 protease in phosphate buffer (pH 7.8) at 37 °C for 24 h and (iii) alkaline hydrolysis and V8 digestion in an identical manner for (i) and (ii). After each treatment, the mixtures were blotted on a PVDF membrane, then

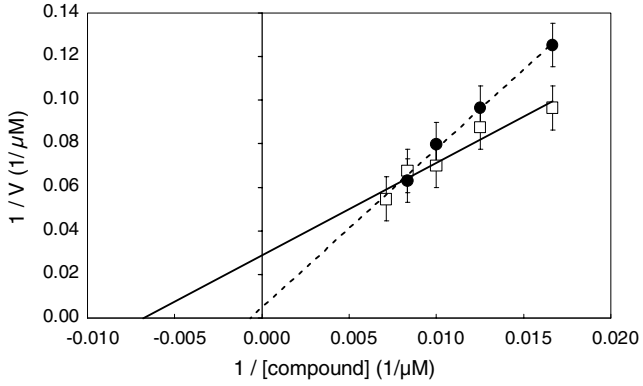

Figure 3. Lineweaver–Burk plots for photoprobe **1** (open square) and L-Phe *p*-NA (closed circle) in a 96-well titer plate. The assay mixture (0.1 mL), consisting of various concentrations of the photoprobe or L-Phe *p*-NA, 8.4 mM chymotrypsin and 0.1 M sodium phosphate buffer (pH 7.0), was incubated at 37 °C for 24 h. Released *p*-NA was measured at 405 nm.

irradiated with black light to attach the samples to the membrane covalently.[11] The irradiated membrane was subjected to chemiluminescence detection with a streptavidin–HRP conjugate.[10] The results indicated that a chemiluminescence signal was observed in the sample without treatment, alkaline hydrolysis, and V8 digestion without alkaline hydrolysis (Fig. 3, A–C). In contrast, no signal was observed in the sample of V8 digestion after alkaline hydrolysis (Fig. 3, D). These results indicated that effective cleavage of the synthetic compounds with V8 protease was regulated by alkaline hydrolysis of methyl ester (Fig. 4).

Figure 4. Dot–blot analysis of compound **1** under various conditions. Compound **1** (1 pmol) was subjected to the conditions indicated: (A) no treatment, (B) alkaline hydrolysis with 10 mM NaOH at room temperature for 2 h, (C) digestion with V8 protease in phosphate buffer (pH 7.8) at 37 °C for 24 h, and (D) alkaline hydrolysis then digestion with V8 protease. Each sample was blotted on a PVDF membrane, then dried. The membrane was irradiated with black light for 40 min for immobilization, then chemiluminescence detection was applied with streptavidin–horseradish peroxidase conjugate in the same manner as described previously.[10,11]

Photoaffinity labeling of chymotrypsin with compound **1** was performed. After incubation of a stoichiometric amount of protein and **1** at 30 °C for 10 min, the sample was irradiated with black light (15 W) at 0 °C for 40 min. Competitive inhibition with L-Phe *p*-NA was also performed. The irradiated samples were subjected to SDS–PAGE, and the gel was electro-transferred to a PVDF membrane. The membrane was subjected to chemiluminescence detection in an identical manner as for dot–blot analysis. A chemiluminescence signal was detected on 26 Kda, which corresponds to chymotrypsin (Fig. 5, lane A). The signal was reduced to 63% or less than 5% in the presence of 7.5 or 15 mM of L-Phe *p*-NA, respectively, (lanes B and C) with densitometer analysis. The results of inhibition clearly showed that compound **1** competed with Phe *p*-NA at the substrate-binding site of chymotrypsin.

The photolabeled samples were subjected to alkaline hydrolysis, then digested with V8 protease without denaturation. The V8 digested sample without alkaline hydrolysis (Fig. 6C) afforded identical chemiluminescence signals on 26 KDa to the samples without treatment (A) and with hydrolysis alone (B) due to the resistance of chymotrypsin to digestion under denaturation conditions. However, no signal was observed when the sample was treated with both alkaline hydrolysis and V8 digestion (D). These results indicated that photobiotinylated chymotrypsin released the biotinyl moiety selectively and quantitatively with V8 treatment after alkaline hydrolysis.

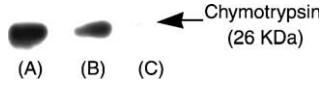

Figure 5. Chemiluminescence detection of photoaffinity labeled chymotrypsin with compound **1**. A mixture of chymotrypsin and compound **1** (3 nmol each) in phosphate buffer (pH 7.8) was incubated at 37 °C for 30 min, and irradiated with black light at 0 °C for 40 min. L-Phe *p*-NA was used as a competitive inhibitor during incubation. The samples contained 0, 30, and 60 nmol inhibitor for (A), (B), and (C), respectively. Each mixture (1 μg protein) underwent SDS–PAGE (12.5%) and chemiluminescence detection in the manner described previously.

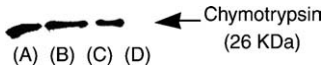

Figure 6. Chemiluminescence detection of photoaffinity labeled chymotrypsin under various conditions. Labeled mixtures of chymotrypsin were subjected to the following conditions: (A) no treatment, (B) alkaline hydrolysis with 10 mM NaOH at room temperature for 2 h, (C) digestion with V8 protease in phosphate buffer (pH 7.8) at 37 °C for 24 h, and (D) alkaline hydrolysis followed by digestion with V8 protease. Each mixture (2.5 μg protein) underwent SDS–PAGE (12.5%) and chemiluminescence detection in the manner described previously.

In conclusion, new diazirinyl photoprobe containing an amino acid linker is useful in overcoming the problem to remove photobiotinylated components from an avidin–biotin complex. The results indicated that the reagent may be useful for the functional analysis of the proteins that are stable V8 protease digestion.

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References and notes

1. Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483–514.
2. Kotzyba-Hilbert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296–1312.
3. Hatanaka, Y.; Nakayama, H.; Kanaoka, Y. *Rev. Heteroat. Chem.* **1996**, *14*, 213.
4. Dorm, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64–77.
5. Hatanaka, Y.; Sadakane, Y. *Curr. Top. Med. Chem.* **2002**, 271.
6. Gillingham, A. K.; Koumanov, F.; Hashimoto, M.; Holman, G. D. Detection and analysis of glucose transporters using photolabelling techniques. In *Membrane Transport: A Practical Approach*; Baldwin, S. A., Ed.; Oxford University Press: Oxford, 2000; p 193.
7. Ploug, M.; Ostergaard, S.; Hansen, L. B. L.; Holm, A.; Dano, K. *Biochemistry* **1998**, *37*, 3612.
8. Hatanaka, Y.; Kanaoka, Y. *Heterocycles* **1998**, *47*, 625.
9. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *Bioorg. Med. Chem.* **1994**, *2*, 1367.
10. Hatanaka, Y.; Hashimoto, M.; Nishihara, S.; Narimatsu, H.; Kanaoka, Y. *Carbohydr. Res.* **1996**, *294*, 95.
11. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *J. Am. Chem. Soc.* **1998**, *120*, 453.
12. Hashimoto, M.; Hatanaka, Y. *Chem. Pharm. Bull.* **1999**, *47*, 667.
13. Hashimoto, M.; Hatanaka, Y.; Nabeta, K. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2481.
14. Hashimoto, M.; Yang, J.; Holman, G. D. *ChemBioChem* **2001**, *2*, 52.
15. Hashimoto, M.; Hatanaka, Y.; Yang, J.; Dhesi, J.; Holman, G. D. *Carbohydr. Res.* **2001**, *331*, 119.
16. Hashimoto, M.; Hatanaka, Y.; Nabeta, K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 89.
17. Green, N. M. *Biochem. J.* **1963**, *89*, 585.
18. Green, N. M.; Toms, E. J. *Biochem. J.* **1973**, *133*, 687.
19. Fang, K.; Hashimoto, M.; Jockusch, S.; Turro, N. J.; Nakanishi, K. *J. Am. Chem. Soc.* **1998**, *120*, 8543.
20. Compound **3**: ^{1}H NMR (CD_3OD) 4.54 (dd, 1H, $J = 5.0$, 7.6 Hz), 4.47 (dd, 1H, $J = 5.0$, 9.2 Hz), 4.36 (dd, 1H,

$J = 4.6$, 7.6 Hz), 3.71 (s, 3H), 3.34 (m, 1H), 2.97 (dd, 1H, $J = 5.0$, -12.9 Hz), 2.97 (d, 1H, $J = -12.9$ Hz), 2.48 (t, 2H, $J = 7.6$ Hz), 2.31 (m, 2H), 2.10 (m, 2H), 1.74 (m, 4H), 1.51 (m, 2H), FAB-MS m/z 388 (MH^+).

Compound **6**: ^{1}H NMR ($CDCl_3$) 7.98 (d, 1H, $J = 8.3$ Hz), 6.85 (d, 1H, $J = 8.3$ Hz), 6.70 (s, 1H), 4.54 (m, 1H), 4.34 (m, 1H), 4.25 (m, 2H), 3.90 (m, 2H), 3.72 (m, 2H), 3.68 (m, 1H), 3.64 (s, 3H), 3.54 (m, 2H), 3.42 (m, 2H), 3.13 (m, 1H), 2.90 (m, 1H), 2.74 (m, 1H), 2.41 (m, 2H), 2.20 (m, 2H), 2.10 (m, 2H), 1.74 (m, 4H), 1.51 (m, 2H), FAB-MS m/z 747 (MH^+).

Compound **1**: ^{1}H NMR ($CDCl_3$) 8.15 (d, 2H, $J = 8.9$ Hz), 8.07 (d, 1H, $J = 8.3$ Hz), 7.70 (d, 2H, $J = 8.9$ Hz), 7.20 (m, 5H), 6.89 (d, 1H, $J = 8.3$ Hz), 6.70 (s, 1H), 4.53 (m, 1H), 4.35 (m, 1H), 4.28 (m, 2H), 3.83 (m, 2H), 3.70–3.20 (m, 13H), 3.18 (m, 1H), 2.96–2.62 (m, 4H), 2.32 (m, 2H), 2.20 (m, 2H), 2.0 (m, 2H), 1.80 (m, 4H), 1.43 (m, 2H), FAB-MS m/z 1015 (MH^+).

21. Lottenberg, R.; Christensen, U.; Jackson, C. M.; Coleman, P. L. *Methods Enzymol.* **1981**, *80*, 341.
22. Drapeau, D. R. *Methods Enzymol.* **1977**, *47*, 469.