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Convenient Synthesis of Photoaffinity Probes and Evaluation of Their Labeling Abilities

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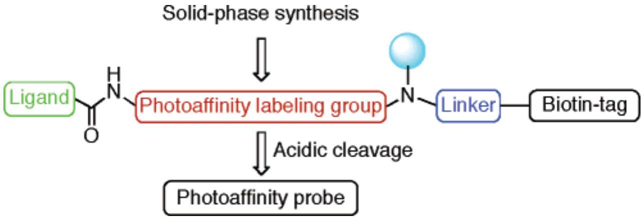
ABSTRACT



Convenient synthesis of a variety of photoaffinity probes was accomplished by utilizing our Ns strategy and novel resin. The synthetic probes were evaluated via the labeling ability with the preseniline 1 C-terminal fragments, which was identified as a therapeutic target for Alzheimer's disease.

Photoaffinity labeling has been demonstrated to be a remarkably efficient method for studying the interaction of biologically significant compounds (ligands) with their target macromolecules.[1] The method allows the identification of the target and also the binding domain within the target protein. An appropriate photoaffinity probe is usually prepared by attachment of a photoreactive labeling group and an indicator such as a biotin tag[2] to the ligand molecule. However, the incorporation of such a bulky group often causes a significant decrease in the affinity with the target molecule. Therefore, the search for a suitable probe for a labeling group and/or a linker continues to be a compelling need in photoaffinity labeling studies. Thus, a convenient synthesis of diverse probes and screening of their labeling abilities would be an attractive prospect, especially considering that the three-dimensional space of the binding site of an enzyme or a receptor protein is generally not known.

Recently, we investigated the screening of a potent inhibitor and the functional analysis of γ-secretase,[3] which was identified as an important therapeutic target for Alzheimer's disease (AD).[4] During the course of our investigation based on potent inhibitor DAPT (**1**),[5] we developed a facile synthetic method for a photoaffinity probe by utilizing the polymer-bound reagent **3**.[6] Furthermore, the utility of **3** was demonstrated by synthesis of the probe **4**,[6] and the photoaffinity labeling experiments of **4** clarified that the major direct target molecules for the DAPT are preseniline 1

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(1) For a review of photoaffinity labeling, see: (a) Kotozyba-Hilbert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296. (b) Fleming, S. A. *Tetrahedron* **1995**, *51*, 12479. (c) Dormán, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64.

(2) For use of a biotin tag for catching the target protein, see: (a) Hofmann, K.; Kiso, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3516. (b) Hofmann, K.; Finn, F. M.; Kiso, Y. *J. Am. Chem. Soc.* **1978**, *100*, 3585. For use in photoaffinity labeling, see: (c) Gilbert, B. A.; Rando, R. R. *J. Am. Chem. Soc.* **1995**, *117*, 8061. (d) Hatanaka, Y.; Hashimoto, H.; Kanaoka, Y. *J. Am. Chem. Soc.* **1998**, *120*, 453.

(3) For a recent review on secretases, see: Wolfe, M. S. *Nat. Rev. Drug Discovery* **2002**, *1*, 859 and references therein.

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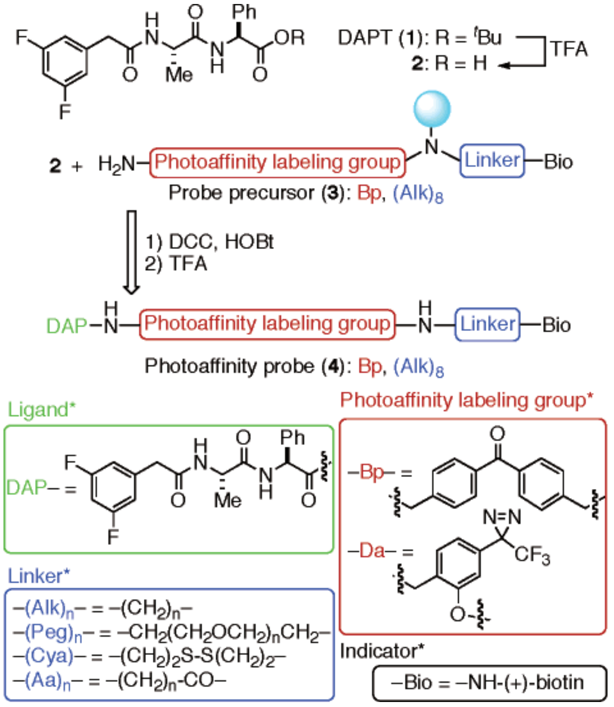
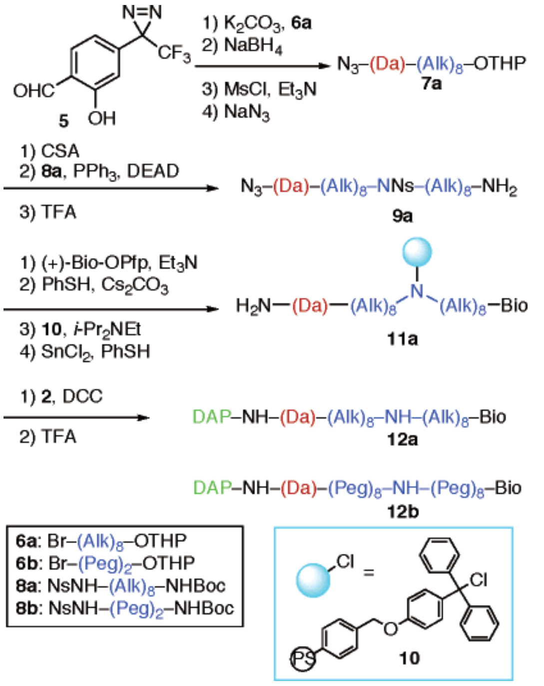
C-terminal fragments (PS1 CTFs),[7] which were discovered from the genetic investigation of familial AD. Additionally, in a competing photolabeling experiment of **4** against various classes of inhibitors, we dissected the inhibitory mechanism of **1**.[7] However, the more effective labeling probe for obtaining a large enough quantity of proteins was strongly required for an exact determination of the DAPT binding site by mass spectrometry at the single amino acid level. Solid-phase synthesis has the advantage of readily providing the affinity probes by a simple operation without any tedious purification steps.[6] We envisioned an application of this methodology to combine other labeling groups and/or linker moieties to afford diverse photoaffinity probes which would be efficient for labeling experiments. Herein, we report a solid-phase synthesis of a variety of photoaffinity probes (Scheme 1) and an evaluation of their labeling ability with the PS1 CTFs.

Scheme 1. Our Synthetic Strategy for Photoaffinity Probes by Solid-Phase Synthesis



First, we planned to replace the diazirine labeling (Da) group in the benzophenone (Bp) of the probe intermediate **3**. Among several reactive diazirines, we found that trifluoromethylaryl diazirine was a reliable photophore, due to its excellent stability. As shown in Scheme 2, synthesis of the probes **12a**,**b** began with the diazirine derivative **5**, which was reported by Hatanaka et al.[8] For the alkyl (Alk) linker probe **12a**, alkylation of **5** with the alkyl bromide **6a**[9] promoted conversion of the aldehyde to the azide. Deprotection of the THP group of **7a** and condensation with the diamine derivative **8a**[9] were accomplished by the Mitsunobu reaction of the Ns amide (Ns strategy).[10,11] After removal of

Scheme 2. Synthesis of Probes **12a** and **12b**



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(4) (a) Takahashi, Y.; Hayashi, I.; Tominari, Y.; Rikimaru, K.; Morohashi, Y.; Kan, T.; Natsugari, H.; Fukuyama, T.; Tomita, T.; Iwatsubo, T. *J. Biol. Chem*. **2003**, *278*, 18664. (b) Fuwa, H.; Okamura, Y.; Morohashi, Y.; Tomita, T.; Iwatsubo, T.; Kan, T.; Fukuyama, T.; Natsugari, H. *Tetrahedron Lett.* **2004**, *45*, 2323. (c) Kan, T.; Tominari, Y.; Rikimaru, K.; Morohashi, Y.; Natsugari, H.; Tomita, T.; Iwatsubo, T.; Fukuyama, T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1983. (d) Takahashi, Y.; Fuwa, H.; Kaneko, A.; Sasaki, M.; Yokoshima, S.; Koizumi, H.; Takebe, T.; Kan, T.; Iwatsubo, T.; Tomita, T.; Natsugari, H.; Fukuyama, T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3813. (e) Fuwa, H.; Hiromoto, K.; akahashi, Y.; Yokoshima, S.; Kan, T.; Fukuyama, T.; Iwatsubo, T.; Tomita, T.; Natsugari, H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4184.

(5) Dovey, H. F.; John, V.; Anderson, J. P.; Chen, L. Z.; de Saint Andrieu, P.; Fang, L. Y.; Freedman, S. B.; Folmer, B.; Goldbach, E.; Holsztynska, E. J.; Hu, K. L.; Johnson-Wood, K. L.; Kennedy, S. L.; Kholodenko, D.; Knops, J. E.; Latimer, L. H.; Lee, M.; Liao, Z.; Lieberburg, I. M.; Motter, R. N.; Mutter, L. C.; Nietz, J.; Quinn, K. P.; Sacchi, K. L.; Seubert, P. A.; Shopp, G. M.; Thorsett, E. D.; Tung, J. S.; Wu, J.; Yang, S.; Yin, C. T.; Schenk, D. B.; May, P. C.; Altstiel, L. D.; Bender, M. H.; Boggs, L. N.; Britton, T. C.; Clemens, J. S.; Czilli, D. L.; Dieckman-MacGinty, D. K.; Droste, J. J.; Fuson, K. S.; Gitter, B. D.; Hyslop, P. A.; Johnstone, E. M.; Li, W. Y.; Little, S. P.; Mabry, T. E.; Miller, F. D.; Audia, J. E. *J. Neurochem*. **2001**, *76*, 173.

(6) Kan, T.; Tominari, Y.; Morohashi, Y.; Natsugari, H.; Tomita, T.; Iwatsubo, T.; Fukuyama, T. *Chem. Commun.* **2003**, 2244.

(7) Morohashi, Y.; Kan, T.; Tominari, Y.; Fuwa, H.; Okamura, Y.; Watanabe, N.; Natsugari, H.; Fukuyama, T.; Iwatsubo, T.; Tomita, T. *J. Biol. Chem.* **2006**, *281*, 14670.

(8) (a) Hashimoto, M.; Kanaoka, Y.; Hatanaka, Y. *Heterocycles* **1997**, *46*, 119. (b) A one-pot incorporation of diazirine and the biotin tag was reported. See: Hatanaka, Y.; Kempin, U.; Jong-Jip, P. *J. Org. Chem*. **2000**, *65*, 5639.

(9) Detailed synthetic procedures are described in the Supporting Information.

(10) For a review of Ns strategy, see: (a) Kan, T.; Fukuyama, T. *J. Syn. Org. Chem. Jpn*. **2001**, *59*, 779. (b) Kan, T.; Fukuyama, T. *Chem. Commun.* **2004**, 353.

(11) (a) Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373. (b) Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831. (c) Fukuyama, T.; Cheung, M.; Kan, T. *Synlett* **1999**, 1301. (d) Kurosawa, W.; Kan, T.; Fukuyama, T. *Org. Synth*. **2002**, *79*, 186.

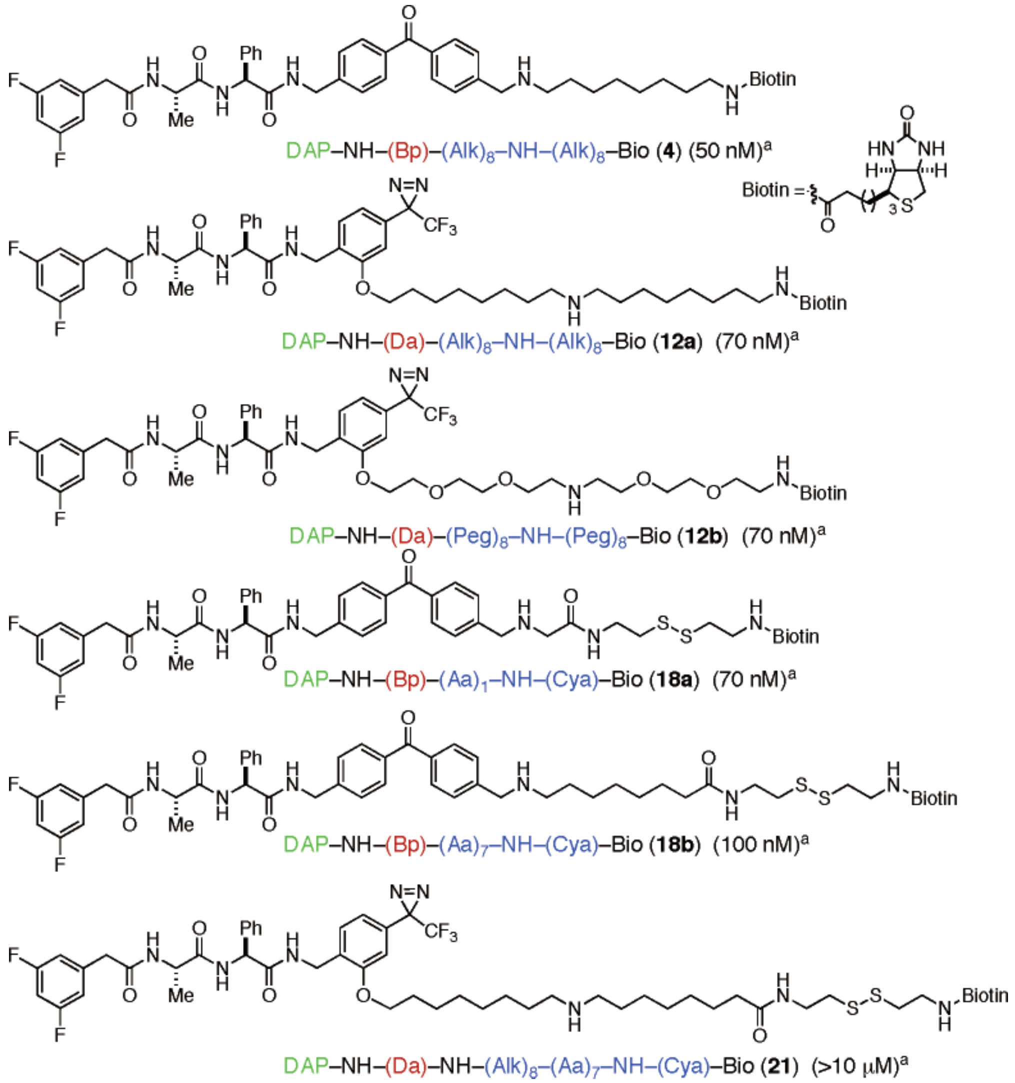

Figure 1. Structure and inhibitory potencies of synthetic probes. ^{a}IC$_{50}$ value for inhibition of Aβ 40 generation. Detailed experimental conditions are described in ref 7.

the Boc group, the biotin unit was incorporated by the active ester method. Removal of the Ns group, loading on the resin **10**,[12] and subsequent reduction of the azide group gave the probe intermediate **11a**. Using a procedure similar to that in the preparation of **4**, we carried out a condensation of the ligand (DAP−OH) **2**[6] and acidic cleavage from the resin to provide the probe **12a** efficiently. The hydrophilic probe **12b**[9] was obtained by a sequence similar to that used for **12a** by using the polyethylene glycol (Peg) linkers **6b**[9] and **8b**,[9] as shown in Scheme 2 (**12a**, 47% from **5**; **12b**, 22% from **5**).

Next we turned our attention to the preparation of a disulfide-bridged probe. Although avidin−biotin affinity for finding a labeled protein is a fine technique, the release of the target protein from the streptavidin complex often requires harsh conditions. Thus, a disulfide linker (Cya) would be an attractive and useful contribution since it would cleave under mild conditions and readily enable isolation of the target protein.[13] Synthesis of the probes **18a**,**b** started from the benzophenone derivative **13**[6] as shown in Scheme 3. Incorporation of the amine functionality into **13** was performed by the Mitsunobu reaction of the Ns-protected ammonia **14**.[14] After removal of the THP group, the primary alcohol was subjected to the Mitsunobu reaction with the Ns-protected amino acid derivatives **15a**,**b**,[9] respectively. Deprotection of the Ns groups, conjugation with the resin **10**, and simultaneous removal of the alloc group and the allyl ester gave the polymer-bound amino acid derivatives **16a**,**b**. Stepwise incorporation of the ligand (DAP−OH, **2**) and the biotinyl cystamine amide **17**[9] and acidic cleavage from the resin provided the probes **18a**,**b**, respectively (**18a**, 77% from **13**; **18b**, 65% from **13**). Using a similar procedure

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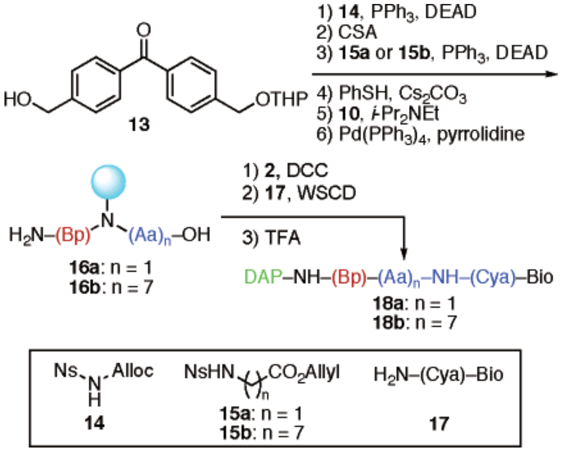
[12] (a) Hidai, Y.; Kan, T.; Fukuyama, T. *Tetrahedron Lett.* **1999**, *41*, 4711. (b) Hidai, Y.; Kan, T.; Fukuyama, T. *Chem. Pharm. Bull.* **2000**, *48*, 1570. (c) Kan, T.; Kobayashi, H.; Fukuyama, T. *Synlett* **2002**, 1338.

[13] Li H.; Liu, Y.; Fang, K.; Nakanishi, K. *Chem. Commun.* **1999**, 365.

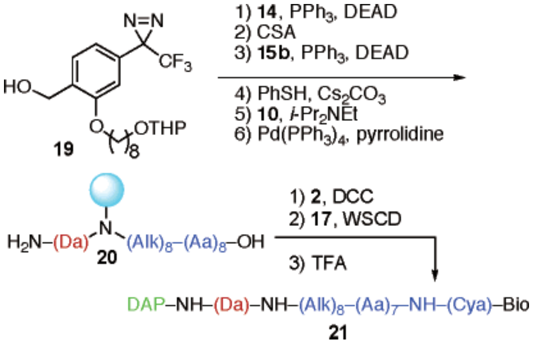
[14] Fukuyama, T.; Cheung, M.; Kan, T. *Synlett* **1999**, 1301.

Scheme 3. Synthesis of Probes **18a** and **18b**



for the preparation of **15**, we also obtained the diazirine-type probe **21**[9] efficiently as shown in Scheme 4 (**21**, 86% from **5**).

Scheme 4. Synthesis of Probe **21**



Since biochemical analysis of a membrane-bound protease is known to be challenging, photoaffinity labeling is one of the more effective methods for γ-secretase.[15] Thus, the photoaffinity labeling experiments were performed by the following sequence. After mixing a CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate)-solubilized lysate of the HeLa S3 cell with probes as shown in Figure 2 in the absence or presence of an excess amount of DAPT (**1**), the solution was irradiated with long-wave near-UV light. Subsequently, the biotinylated proteins were captured with streptavidin sepharose beads, separated by SDS-PAGE, and detected by an anti-PS1 CTF antibody.

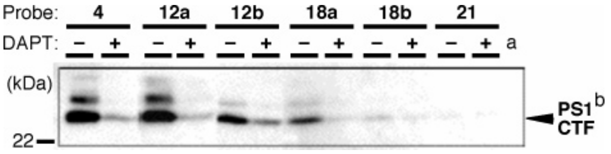


Figure 2. Results of SDS-PAGE of the photoaffinity labeling experiment by synthetic probes (**4**, **12a**, **12b**, **18a**, **18b**, and **21**). [a]−, absence of DAPT (**1**); +, presence of DAPT (**1**). [b]Detailed experimental conditions of labelings are described in ref 7.

Structures of the synthetic probes and inhibition activities[16] (IC_{50}) of Aβ 40 are shown in Figure 1. The results of photolabeling (Western-blot analysis) are also shown in Figure 2. Compared with **4** and **12a**, no distinct difference in labeling ability or inhibition activities was observed between the benzophenone and the diazirine photophores. However, the hydrophobic (alkyl) linker appeared to be more suitable than the hydrophilic (Peg) linker for this protease compared with **12a** and **12b**. Although detection of the labeled protein was achieved by utilizing probe **18a**, the similar disulfide-bridged probes **18b** and **21** were unsuccessful in labeling, even upon treatment with excess thiol. Furthermore, the longer probe **21** exhibited no inhibitory activity of γ-secretase at 10 mM. Presumably, the disulfide bond and/or the biotin group of **18b** and **21** blocks the binding with γ-secretase. Unfortunately, we could not discover the probes more effective than **4** in this work. However, these results helped confirm the importance of combinatorial synthesis of photoaffinity probes, which readily provide various probes.

In conclusion, we accomplished a convenient synthetic method for photoaffinity probes and evaluated their utilities. To our knowledge, there have been few or no such systematic structure and photoaffinity labeling ability relationship (SAR) studies of photoaffinity probes. The present investigation was carried out by utilizing the combination of our Ns strategy[10,11] and the novel resin **10**.[12] Additionally, this synthetic strategy would be applicable not only to photoaffinity probes[17] but also to fluorescein probes, thus making a great contribution to an area of chemical biology. Further investigation is underway in our laboratories.

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Supporting Information Available: Detailed experimental procedures and spectroscopic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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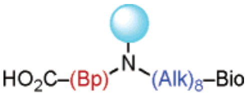
[15] Beher, D.; Fricker, M.; Nadin, A.; Clarke, E. E.; Wrigley, J. D. J.; Li, Y.-M.; Culvenor, J. G.; Masters, C. L.; Harrison, T.; Shearman, M. S. *Biochemistry* **2003**, *42*, 8133.

[16] Inhibitory potencies of probes on γ-secretase activity were analyzed by cell-based assays. For detailed procedures, see ref 17.

[17] Synthetic procedures for a similar probe intermediate **22**, which possessing reactive carboxylic acid functionality was also described in the Supporting Information.



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