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Development of an activity-based probe for amyloid β-hydrolyzing antibodies

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ABSTRACT

We report developing an activity-based probe containing an amyloid β peptide (Aβ) 17–27 and an electrophilic phosphonate diester at the C-terminus. A probe containing an electrophilic moiety is able to react with the nucleophiles on an antibody or an antibody with proteinase activity. The probe reacted with an Aβ specific monoclonal antibody and formed a covalent complex. The covalent binding also occurred specifically when the probe reacted with serum containing anti-Aβ antibodies. These results suggest that the probe would serve as a powerful tool to isolate Aβ specific antibodies that are capable of Aβ hydrolysis activity.

Antibodies (Abs) capable of hydrolyzing peptides and proteins are potentially useful agents for therapeutics, through the specific elimination of pathogenic peptides and the essential proteins in microorganisms. To date, Abs hydrolyzing antigens such as vasoactive intestinal polypeptide, thyroglobulin, prothrombin, HIV gp120, helicobacter pylori urease, TNF-α, and amyloid β peptides (Aβ) have been reported.[1–6] The Abs possessing peptidase activity that employ serine protease-like hydrolysis mechanism have been described.[7] Inhibitors for serine protease that consistently inhibit the hydrolyzing activity of Abs and serine protease-like catalytic triads have been identified by site-directed mutagenesis studies and crystallography.[8] Such Abs are considered useful for therapeutic applications to treat human diseases.[4,5,6a]

Approximately 26 million humans have Alzheimer's disease (AD) worldwide. No truly effective medicine has been approved for AD therapy. Progressive cerebral deposition of Aβ in the brain is one of the pathological hallmarks of AD and thought to play an important role in the neurodegenerative events causing AD.[9] The Aβ depositions consist of 39–43 residue peptides including the neurotoxic Aβ40 and Aβ42 peptides produced by the alternative cleavage of the β-amyloid precursor protein by β- or γ-secretase within the brain. Aβ is considered to be an attractive target molecule for AD therapy. Numerous efforts have been directed toward development of molecules that can reduce Aβ concentration in the brain, such as, monoclonal Abs and β- or γ-secretase inhibitors, using mice overexpressed mutant human APP genes that generate an age-associated increase in Aβ as well as cognitive decline.[10,11] Abs are perhaps the most promising molecules, since Abs capable of binding and hydrolyzing Aβ are candidates as immunotherapeutic agents for AD because of their ability to inactivate multiple Aβ molecules per Ab molecule.

We discovered Abs that hydrolyze the Lys16–Leu17 and Lys28–Gly29 bond of Aβ by serine protease-like mechanism, inhibit Aβ aggregation and protect neuronal cells from Aβ-induced toxicity.[6b] However, development of homogeneous Abs with catalytic rate constants sufficient for therapy has not been achieved until now. Fundamentally, probes that can specifically bind to the active site of Abs with hydrolyzing activity could be applied to the isolation of desired Abs from a phage or yeast library which would provide one avenue for AD immunotherapy.[7,12] Here, we report the design, synthesis and Ab reactivity characteristics of a biotinylated probe **1** consisting of a phosphonate diester warhead and a peptide derived from Aβ as an Ab recognition point (Fig. 1).

Peptidyl (α-aminoalkyl) phosphonate esters[13] have been shown to be very stable and specific irreversible inhibitors of trypsin-like enzymes[14,15] and activity–based probes for trypsin-family serine protease and antibodies capable of hydrolyzing Aβ.[16,17] Diphenyl amino(4-aminobutyl)methanephosphonate (Lys^P-$(OPh)_2$) derivatives are active site-directed irreversible inhibitors of trypsin-like enzymes.[18] The positively charged amino group adjacent to the

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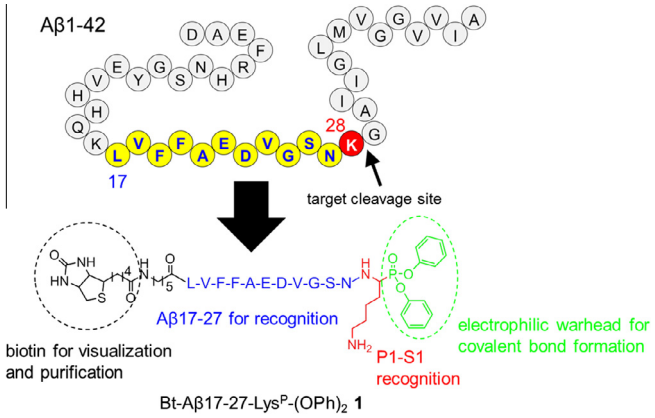
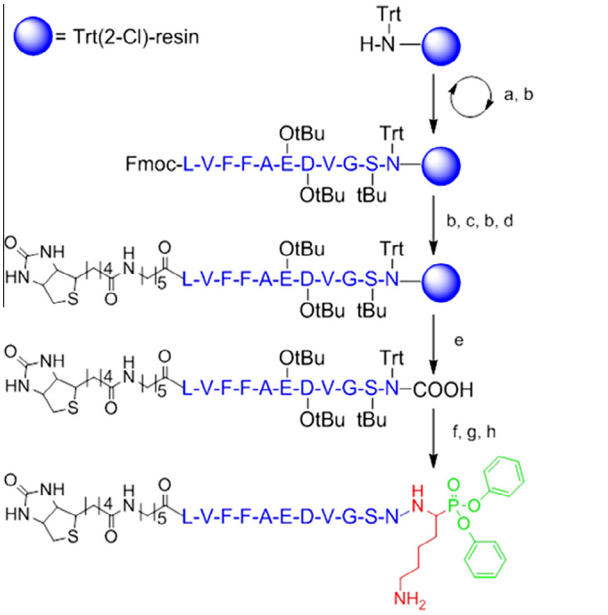


Figure 1. Design and chemical structure of Bt-Aβ17-27-Lys^{P}-(OPh)$_2$ **1**.



Scheme 1. Synthesis of Bt-Aβ17-27-Lys^{P}-(OPh)$_2$ **1**. *Reagents and conditions*: (a) Fmoc-AA-OH, DIEA, NMP, coupling reagent (HBTU, PyBOP or HATU), HOBt; (b) 20% piperidine/DMF; (c) Fmoc-NH-(CH$_2$)$_5$-COOH, PyBOP, DIEA, NMP; (d) biotin, HATU, HOBt, DIEA, NMP; (e) 30% HFIP/DCM; (f) H-Lys^{P}(Boc)-(OPh)$_2$, HATU, HOBt, DIEA, NMP; (g) 5% phenol/TFA; (h) HPLC purification.

phosphonate diester group serves as an analogue of Lys28 of Aβ. The Lys28–Gly29 bond is the major specific cleavage target by Abs[6b] because: (1) most natural proteolytic Abs cleave Lys/Arg–X bonds (X represents an amino acid residue); (2) hydrolyzing this bond leads to inhibition of the formation of Aβ aggregates and protection of neuronal cells from Aβ-induced toxicity. With regard to the peptide component, Abs can recognize peptides as short as 4–5 amino acids. High affinity binding, however, is usually accomplished with slightly longer peptides (more than 10 residues).[19] Furthermore, the Lys16–Leu17 bond is the minor cleavage site of natural proteolytic Abs.[6b] Consequently, the undecapeptide corresponding to Aβ residues 17–27 was connected to an α-amino group located in the phosphonate ester (Fig. 1). Biotin was incorporated to the N-terminus to permit visualization and purification of the molecule and the Aβ-derived peptide was intended to deliver the phosphonate moiety to the active site of the Ab with hydrolyzing activity.

The common stepwise peptide elongation by coupling of the protected amino acid to the phosphonate esters was not utilized in order to circumvent probable hydrolysis of the terminal phosphonate ester moiety due to repeated exposure strong deprotection conditions. Instead, compound **1** was prepared by condensation of the protected peptide fragment and the α-amino alkyldiphenylphosphonate (see Scheme 1). Briefly, the biotinylated protected peptide was synthesized by Fmoc-based solid-phase synthesis on 2-chlorotrityl chloride resin followed by cleavage with 30% HFIP/CH_2Cl_2.[20] Synthesis of H-Lys^{P}(Boc)-(OPh)$_2$ and compound **2** (Fig. 2) can be done through the Oleksyszyn 3-components reaction.[18,21,22] The protected peptide was coupled with H-Lys^{P}(Boc)-(OPh)$_2$ using HATU as a condensing reagent followed by fully deprotection by TFA to afford **1**, which was purified by HPLC.

To evaluate the reactivity of the probe with the active site nucleophiles, the serine proteinase trypsin was used because cleavage of a peptide or protein by Abs with hydrolyzing activity occurs on the C terminal side of basic amino acids. Incubation time-dependent inhibitions of trypsin-catalyzed Boc-Glu(OBzl)-Ala-Arg-MCA (EAR-MCA) hydrolysis by **1** and **2** were observed, suggesting that the mode of inhibition is irreversible (Fig. 2A).[23] Apparent first order inactivation rate constants (kapp) and apparent second order inactivation rate constants (kapp/[I]) were obtained for the probes against trypsin (**1** kapp = 0.036 ± 0.005 min^{-1}, kapp/[I] = 6.0 ± 0.9 M^{-1} s^{-1}; **2** kapp = 0.018 ± 0.001 s^{-1}, kapp/[I] = 9370 ± 385 M^{-1} s^{-1}). The addition of a peptide moiety decreased the reactivity of **1** toward trypsin, suggesting that the peptide moiety would be sterically-disfavored in the interaction between trypsin and **1**. A previous study indicated that the biotinylated phosphonate diester formed a binary complex with trypsin.[16] As expected, trypsin formed a complex with **1** that was determined by Western blotting with streptavidin detection of the biotin tag (Fig. 2B). In contrast, trypsin inactivated with **2**, an inhibitor of a serine protease, or by heating failed to form a complex with **1**. These results suggest that **1** has characteristics of a probe for serine protease-like enzymes.

After determining the ability of **1** to form a covalent bond with a nucleophile in the active site, we assessed the specificity of **1** against anti-Aβ Abs. The covalent bond formations of **1** with three different Abs were studied: (1) the monoclonal IgG against Aβ, BAM 90.1 that is produced by immunization with a synthetic Aβ13–28, conjugated to KLH, and the Ab epitope resides within amino acid 20–23; (2) the monoclonal IgG against protein C, as a control; and (3) antiserum from an Aβ17–42-immunized rabbit which reacts with Aβ17–42 and Aβ1–40. As shown in Figure 3A, the covalent adduct of **1** with the anti-Aβ monoclonal Ab was detected as a band at 150 kD by SDS–PAGE followed by Western blotting using streptavidin–HRP detection reagent. While no covalent complex formation was observed when **1** was incubated with irrelevant monoclonal Ab (monoclonal anti-protein C Ab), indicating that specificity of **1** would be sufficient to qualify as a reagent to isolate an Ab possessing Aβ-hydrolyzing activity from the phage or yeast library. Furthermore, probe **1** did not react with other proteins in rabbit antiserum to Aβ detectably, suggesting that it is less likely to attach to a surface protein on the phage due to specific covalent binding (Fig. 3B). Additionally, the peptide moiety in **1** seems to prohibit interaction between **1** and serine proteases that react with phosphonate diester in serum. Taking into account these observations, compound **1** is likely to be a suitable probe for Abs having the combination of a serine protease-like catalytic mechanism along with the ability to recognize Aβ.

In conclusion, we have designed and synthesized an activity-based probe containing Aβ17–27 that can covalently bind to monoclonal anti-Aβ Abs. Electrophoresis and western blot experiments confirmed the formation of the probe–Ab complex. To test the capability of specific binding, the probe was applied successfully for the formation of the covalent complex with anti-Aβ Abs in serum. Such a probe can be applied not only for isolating Ab with Aβ-hydrolyzing activity but also for identifying Aβ-hydrolyzing Abs