Peptide Cleavage

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Cleavage Agents for Soluble Oligomers of Amyloid β Peptides**

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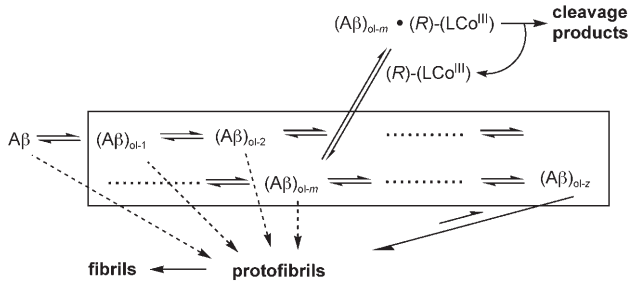
Alzheimer's disease (AD) is characterized by neuronal loss and the presence of senile plaques containing amyloid β (Aβ) peptides in the brain.[1] Aβ peptides are primarily composed of amyloid β-40 ($A\beta_{40}$) and amyloid β-42 ($A\beta_{42}$) peptides, which contain 40 and 42 amino acid residues, resepctively.[2] Even when the concentration of $A\beta_{42}$ peptides is much lower than that of $A\beta_{40}$, $A\beta_{42}$ peptides are the predominant components in the plaques.[3] An increase in the $A\beta_{42}/A\beta_{40}$ ratio is associated with familial forms of early onset AD.[4]

The association process of $A\beta_{40}$ or $A\beta_{42}$ peptides involves the formation of several oligomers, protofibrils, and fibrils as summarized in Scheme 1.[5,6] Here, the species placed in the rectangle are the soluble oligomers that possess unique[7] structures. The conversion of large assemblies such as protofibrils and fibrils into smaller ones is slow, and the formation of the large assemblies is irreversible or partially irreversible.[6] Immediately after peptide production, the $A\beta_{40}$ peptide produces monomers, dimers, trimers, and tetramers in rapid equilibrium, whereas the $A\beta_{42}$ peptide preferentially forms pentamers or hexamers.[6] In addition, the $A\beta_{42}$ peptide forms oligomers and fibrils considerably faster than does the $A\beta_{40}$ peptide.[8,9] A more recent study indicated that the $A\beta_{42}$ peptide exists in solution as a binary mixture of the monomer and high-molecular-mass oligomers, whereas a monomer–dimer equilibrium exists for the $A\beta_{40}$ peptide.[10]



Scheme 1. Formation of various assemblies of the $A\beta_{40}$ or $A\beta_{42}$ peptides and cleavage of the assemblies.

Soluble oligomers of the $A\beta_{42}$ peptide, instead of the monomer or insoluble amyloid fibrils, are proposed to be responsible for synaptic dysfunction in the brains of patients with AD.[11] Several lines of evidence have been reported in support of the role of soluble oligomers, such as the dodecamer, of the $A\beta_{42}$ peptide as the intermediate neurotoxic species in the pathology of AD.[5,12–14]

As a therapeutic option to alleviate $A\beta_{42}$-induced neurotoxicity, various attempts have been made to lower the level of the $A\beta_{42}$ assembly in the brain.[15–19] One way to do this is to inhibit the action of either β-secretase or γ-secretase which produces Aβ peptides from β-amyloid precursor proteins.[11] One could attempt to prevent oligomerization of the $A\beta_{42}$ peptide by the use of $A\beta_{42}$ immunization.[15,16] Small molecules with high affinity toward the $A\beta_{42}$ peptide may suppress its oligomerization.[17] Enhancement of the activity of proteases known to degrade the $A\beta_{42}$ peptide is an alternative way to suppress its oligomerization in the brain.[18]

A novel method discovered in this study to reduce the level of $A\beta_{42}$ oligomers is to cleave the $A\beta_{42}$ peptide included in an oligomer: As shown in Scheme 1, where the cleavage agent is indicated as (*R*)-(LCo^{III}), cleavage of the $A\beta_{42}$ peptide included in a target oligomer reduces the concentration of the target oligomer, thereby leading to a decrease in the concentrations of other oligomers which are readily transformed into the target oligomer. Reduction of the concentration of the oligomers slows down the formation of protofibrils and fibrils. Herein we present the design, synthesis, and activity of four agents that effectively cleave $A\beta_{42}$ oligomers.

We discovered that target-selective artificial proteases can be designed for proteins using myoglobin or peptide deformylase as the target proteins.[20–23] As the catalytic center for the target-selective artificial proteases, the Co^{III} complex of cyclen ([Co^{III}cyclen]) is one of the most preferred in view of its ability to hydrolyze[21–25] peptide bonds and the inertness of Co^{III} complexes to exchange.

The combinatorial library of candidates of agents for the cleavage of soluble oligomers of the $A\beta_{42}$ peptide was constructed by exploiting [Co^{III}cyclen] as the cleavage center in this study. Several aromatic moieties are known to possess affinity for β-amyloid plaques when appropriately derivatized, and have been used as essential parts of imaging agents for β-amyloid plaques.[26] To facilitate the selective

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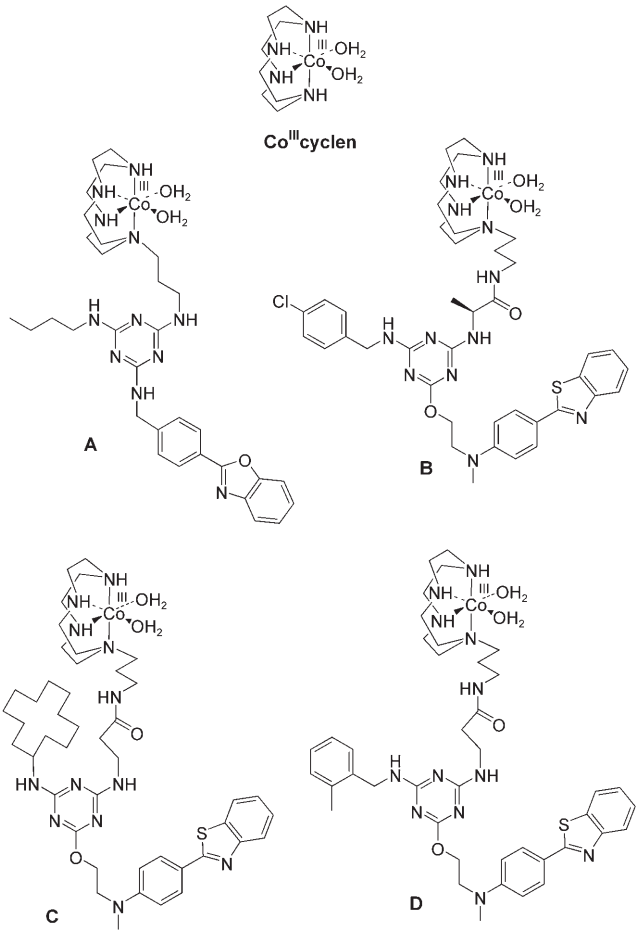
recognition of soluble oligomers of the $A\beta_{42}$ peptide, those aromatic moieties were employed as auxiliary binding components in the combinatorial library. A chemical library containing 888 compounds was constructed (see the Supporting Information).

The activity of the candidates as cleavage agents was checked by MALDI-TOF MS after incubating each library member with 4.0 μM of the $A\beta_{42}$ peptide at pH 7.50 (0.050 M phosphate) and 37 °C for 24 h. The activity for cleavage of the $A\beta_{42}$ assembly was judged by the appearance of peptide fragments. After repeated screening, **A**–**D** were selected and



synthesized on larger scales as described in the Supporting Information. Whether **A**–**D** could cleave the $A\beta_{40}$ peptide was also examined by incubating **A**–**D** with 4.0 μM of the $A\beta_{40}$ peptide at pH 7.50 and 37 °C: **A** and **B** were found to effect cleavage, whereas **C** and **D** were inactive.[27]

The aggregation behavior of the Aβ peptides is known to be sensitive to the experimental conditions.[10] We have, therefore, carried out the following filtration experiments under the experimental conditions to obtain quantitative information on the rate of decrease in the amount of monomer and/or small oligomers (see the Supporting Information).

To ensure generation of the monomeric form of the $A\beta_{40}$ or $A\beta_{42}$ peptides, the synthetic Aβ peptide was treated with NaOH before introducing it into pH 7.50 medium.[28] The results (see the Supporting Information) of filtration through a membrane with a cut-off molecular weight (M_w) of 10000 indicate that most (> 80 %) of the $A\beta_{42}$ peptide (M_w 4514) passes through the filter immediately after transfer from a 1 mM NaOH environment to a pH 7.50 medium. Since the amount of dimer or trimer is not significant compared to that of the monomer,[6,10] the amount of $A\beta_{42}$ peptide that passes through the filter represents the amount of monomer. During the filtration step, which takes about 10 minutes, small amounts of large oligomers may form, which accounts for the incomplete passage through the filter. Thus, the $A\beta_{42}$ peptide appears to exist mostly as the monomer immediately after exposure to the reaction buffer. Within 3 hours, about two-thirds of the initially added $A\beta_{42}$ peptide forms aggregates that cannot pass through the filter. About 10 % of the initially added $A\beta_{42}$ peptide remains as the monomer even after 36 hours, in agreement with literature results.[10] On the other hand, more than 90 % and about 50 % of the initially added $A\beta_{40}$ peptide (M_w 4330) passes through the membrane immediately and after 24 h, respectively, after addition of the stock solution to the pH 7.50 buffer. The monomer, the dimer, and, to some extent, the trimer of the $A\beta_{40}$ peptide are expected to pass through the membrane. The results agree with the previous observations that the $A\beta_{40}$ peptide produces small oligomers in equilibrium with the monomer and that the $A\beta_{40}$ peptide is converted into protofibrils and fibrils considerably more slowly than is the $A\beta_{42}$ peptide.[8–10]

MALDI-TOF mass spectra obtained after incubation of the $A\beta_{40}$ or $A\beta_{42}$ peptides (4.0 μM) with **A**–**D** (see the Supporting Information) showed the presence of $A\beta_{1-20}$ and $A\beta_{1-21}$ peptides as some of the cleavage products. Here, the Aβ fragments are named according to the amino acid sequence of the $A\beta_{42}$ peptide. The structures of the cleavage products were confirmed by MALDI LIFT-TOF/TOF MS.[29] Since the intensity of a MALDI-TOF MS signal does not indicate its relative concentration, some oligopeptide fragments may be present in significant concentrations without showing strong MALDI-TOF MS signals.

Unless noted otherwise, the cleavage reaction was initiated by adding the stock solution of the $A\beta_{40}$ or $A\beta_{42}$ peptides to the buffer solution containing one of the cleaving agents **A**–**D**. The product solution obtained by the reaction of the $A\beta_{40}$ or $A\beta_{42}$ peptides (4.0 μM) with **A**–**D** was filtered through a membrane with a cut-off molecular weight of 10000 to remove aggregates of the two peptides, and the resulting solution was subjected to HPLC separation to isolate the oligopeptide fragments. The oligopeptide fragments were hydrolyzed under alkaline conditions, and the resulting amino acids were quantified with fluorescamine (see the Supporting Information). The amounts of oligopeptide fragments obtained by the action of **A**–**D** were estimated as mol % of the initially added amount of the $A\beta_{40}$ or $A\beta_{42}$ peptides and are defined as the cleavage yields. The cleavage yields measured after reaction with various initial concentrations (C_o) of **A**–**D** after 36 h at 37 °C and pH 7.50 are plotted against $\log C_o M^{-1}$ in Figure 1. Plateau values of the cleavage yields are 10–30 %, and significant yields were observed at a C_o value of 100 nM. As analyzed in the Supporting Informa-

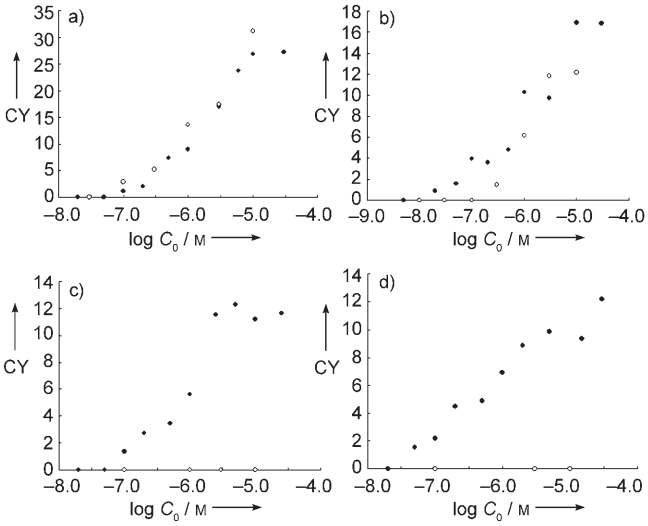


Figure 1. Plot of the cleavage yield (CY, mol%) against log C_o/M for the cleavage of $A\beta_{40}$ (○) or $A\beta_{42}$ (●) (4.0 μM) by **A** (a), **B** (b), **C** (c), and **D** (d) measured after reaction for 36 h at 37°C and pH 7.50. In the present study, the estimated values of the cleavage yields are the average of results of 4 or 6 measurements carried out with different reaction mixtures, respectively, and their relative standard deviations are 5–15%.

tion, the efficiency for cleavage of the $A\beta_{42}$ peptide by **A**–**D** is expected to be much higher in patients with AD.

To examine the effect of aggregation of the $A\beta_{40}$ or $A\beta_{42}$ peptides prior to exposure to the cleavage agent, either the $A\beta_{40}$ or $A\beta_{42}$ peptide was incubated in the buffer solution for various periods of time before treating the solution with **A** (see the Supporting Information). Little cleavage was observed when **A** was added to the reaction mixture after preincubation of the $A\beta_{42}$ peptide for 24 h, probably because of extensive polymerization of the peptide leading to the formation of protofibrils or fibrils. When **A** was added to the reaction mixture after preincubation of the $A\beta_{42}$ peptide for 3–6 h, the amounts of products formed by cleavage of the peptide were not much smaller than that obtained with **A** added without preincubation of the peptide. This result stands in contrast with the considerable reduction in the amount of $A\beta_{42}$ monomer during the initial 3–6 hours. Thus, the fragments are formed mainly by cleavage of oligomers instead of monomer, protofibrils, or fibrils.

The addition of **A** after preincubation of the $A\beta_{40}$ peptide for 24 h leads to considerable cleavage, while preincubation for longer periods reduces the cleavage yield. This observation is consistent with the slower formation of protofibrils and fibrils by the $A\beta_{40}$ peptide compared with that from the $A\beta_{42}$ peptide. In addition, it reveals that the protofibrils or fibrils of the $A\beta_{40}$ peptide are not the main source of the fragments. Since the yield for cleavage of the $A\beta_{40}$ peptide by **A** does not decrease considerably by preincubation for 3–18 h, the monomer of $A\beta_{40}$ is not the main source of the fragments, in view of the results of the filtration experiment.

To examine the progress of the cleavage reaction, the cleavage yield was measured by treating the $A\beta_{40}$ or $A\beta_{42}$ peptides with **A** for various periods of time at 37°C and pH 7.50. The results (Supporting Information) reveal that the yield for cleavage of the peptides by **A** does not increase appreciably upon reaction for more than 24 h; this result is likely due to consumption of the target oligomer through both the cleavage by **A** and formation of protofibrils and fibrils.

At present, the identity of the oligomers of the $A\beta_{40}$ or $A\beta_{42}$ peptides cleaved by **A**–**D** is unknown. Nevertheless, reduction of the concentration of the target oligomer would decrease the concentrations of other oligomers, since the oligomers are in equilibria with one another.

Agents **A** and **B** cleaved both the $A\beta_{42}$ and $A\beta_{40}$ peptides, even though they were selected from the combinatorial library by screening against the $A\beta_{42}$ peptide. The β-amyloid precursor protein is cleaved by secretases mainly to produce the $A\beta_{40}$ peptide throughout life, and this peptide carries out physiological functions.[30] Excessive cleavage of the $A\beta_{40}$ peptide may, therefore, interfere with its normal functions. In the brains of patients with AD, however, the level of soluble $A\beta_{40}$ peptide is 30–40 times higher than those of nondemented elderly controls.[31] Partial cleavage of soluble oligomers of $A\beta_{40}$ during cleavage of the $A\beta_{42}$ peptide may not cause considerable side effects for patients with AD.

Many more cleavage agents for the $A\beta_{42}$ oligomers can be synthesized by combining a Co^{III}–ligand complex and a binding auxiliary with affinity for the $A\beta_{42}$ oligomers. After performing proper in vivo tests, some of the synthetic cleavage agents may be found suitable for therapeutic treatment of patients with AD.

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