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Design of substrate transmembrane mimetics as structural probes for γ-secretase

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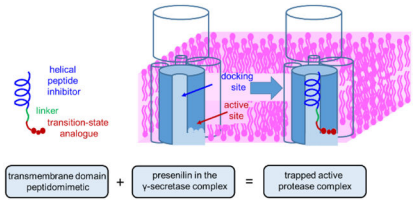
Abstract

γ-Secretase is a membrane-embedded aspartyl protease complex central in biology and medicine. How this enzyme recognizes transmembrane substrates and catalyzes hydrolysis in the lipid bilayer is unclear. Inhibitors that mimic the entire substrate transmembrane domain and engage the active site should provide important tools for structural biology, yielding insight into substrate gating and trapping the protease in the active state. Here we report transmembrane peptidomimetic inhibitors of the γ-secretase complex that contain an N-terminal helical peptide region that engages a substrate docking exosite and a C-terminal transition-state analog moiety targeted to the active site. Both regions are required for stoichiometric inhibition of γ-secretase. Moreover, enzyme inhibition kinetics and photoaffinity probe displacement experiments demonstrate that both the docking exosite and the active site are engaged by the bipartite inhibitors. The solution conformations of these potent transmembranemimetic inhibitors are similar to those of bound natural substrates, suggesting these probes are preorganized for high-affinity binding and should allow visualization of the active γ-secretase complex, poised for intramembrane proteolysis, by cryo-electron microscopy.

Graphical Abstract



γ-Secretase is a membrane-embedded aspartyl protease complex, with presenilin as the catalytic component,[1] that hydrolyzes >90 known substrates,[2–3] including the amyloid

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Supporting Information

Detailed methods and results for (1) synthesis and characterization of hydroxyethylurea tripeptidomimetic building blocks and all HPI-TSA inhibitor peptidomimetics, (2) γ-secretase inhibition assays, (3) photoaffinity labelling experiments, (4) inhibition of γ-secretase by series **10**–**16** (P2' = Ala); (5) LC-MS/MS study of proteolysis of peptides **8** and **12** by γ-secretase, (6) 1D- and 2D- NMR studies of **6** and **9**, and (7) spectra of synthetic intermediates and final compounds are available in supporting information (95 pages).

precursor protein (APP) of Alzheimer's disease and the Notch family of developmental signaling receptors. How this enzyme recognizes substrate transmembrane domains and carries out intramembrane proteolysis has been mysterious. Advances in cryo-electron microscopy paved the way to the first detailed structure of the ~230 kDa complex,[4] comprised of membrane proteins nicastrin, Aph-1 and Pen-2 along with presenilin. Most recently, structures of γ-secretase bound to Notch and APP substrates were reported,[5–6] providing important insights into substrate recognition. Nevertheless, the active site was disabled through mutagenesis, and the substrates were artificially crosslinked to presenilin. To date, the enzyme has not been trapped in its active state, and the lateral gating pathway of substrate into the active site remains unclear.

To address this problem, we aimed to develop substrate TMD mimetics as chemical probes for structural analysis of γ-secretase. These probes would trap the active enzyme at the transition state, allowing acquisition of high-resolution snapshots of substrate recognition with the protease poised for catalysis of TMD cleavage. We and others previously reported peptidomimetic transition-state analogue inhibitors (TSAs) of γ-secretase[7–9] and use of these as probes for active site binding pockets.[10–13] We have also reported helical peptide inhibitors (HPIs) that interact with a substrate docking exosite distinct from but proximal to the active site.[14–15] We recently demonstrated that substrate TMD is sufficient for high-affinity binding (K_m < 100 nM)[16] and therefore sought peptide-based inhibitors that would mimic the entire TMD and interact with both the docking site and the active site. Specifically, we worked to couple an HPI to a TSA through a variable linker (Fig. 1).

We chose a pentapeptide TSA with a hydroxyethylurea moiety and spanning residues P2 through P3' (TSA **1**, see Table 1) that showed optimal activity in a cell-based assay for inhibiting γ-secretase-mediated production of the amyloid β-peptide (Aβ) from APP substrate.[13] Residues P1', P2', and P3' are especially important for substrate recognition and processing.[17] In a purified enzyme assay, TSA **1** displayed an IC_{50} of 41 nM (Table 1). HPI **2**, containing helix-inducing α-aminoisobutyric acid (Aib) residues spaced apart to arrange the Aib residues along one face of the helix and presenting APP TMD residues to the enzyme along the rest of the helix,[14] showed comparable activity (IC_{50} of 58 nM). We aimed to connect these two compounds between HPI C-terminus and TSA N-terminus with intervening linkers of varying lengths. Coupling in this manner, with the TSA on the C-terminus of the TMD mimetic, is consistent with γ-secretase initially cleaving APP TMD on the C-terminal end three residues from the membrane-cytosol interface.[18–19] To access these highly hydrophobic HPI-TSA conjugates, we generated hydroxyethylurea-containing tripeptide building blocks suitably protected for solid-phase peptide synthesis (Scheme S1). All synthesized peptides were purified to >95% by HPLC.

HPI-TSA conjugate **3**, containing no linker moiety, displayed an IC_{50} of 53 nM, with no improvement in potency compared to either TSA **1** or HPI **2** alone (Table 1). Inserting a 4-atom hydro-carbon linker gave **4**, with an IC_{50} of 12 nM, while inserting a 6-atom spacer provided **5**, with an IC_{50} of 10 nM, increasing potency 4-to-6-fold over TSA **1** or HPI **2**. Finally, extending the linker to a 10-atom spacer gave **6**, the most potent compound in the series, with an IC_{50} of 0.8 nM. This compound essentially titrates the enzyme, as the assay was done with 1 nM of purified γ-secretase.

Control peptide **7** containing TSA **1** and the 10-atom spacer alone was roughly twice as potent as **1**, showing that the linker contributes to binding to the enzyme. Control peptide **8**, with the transition-state mimicking isostere in **6** replaced with an amide bond, showed >20-fold reduced potency (IC_{50} = 18 nM). Moreover, MS analysis revealed that **8** was hydrolyzed by γ-secretase between the two Phe residues, validating the correct registry of binding and appropriate placement of the transition-state isostere in **6** (Fig. S1). When two internal *L*-Val residues of the potent **6** were exchanged with *D*-Val in compound **9**, inhibitory potency decreased nearly eight-fold, presumably due to disruption of the helical conformation in this part of the TMD mimetic. A similar series of TMD mimetics were also synthesized with P2' in the TSA moiety as Ala in place of Leu (**10–16**, Table S2), leading to identification of another stoichiometric inhibitor **16** (IC_{50} + 0.5 nM).

To confirm helicity of the HPI portion of **6** and disruption of this helix in **9**, we performed 2D NMR experiments. Rotating frame nuclear Overhauser effect spectroscopy (ROESY) reveals spatial proximity between NH↔NH and C_αH↔NH protons in peptides. NH↔NH cross-peak intensities between residues 5↔6, 6↔7, 7↔8 were found to be higher in **6** than in **9**, suggesting that the HPI region of **6** is more helical than that of **9** (Fig. S3). In the C_αH↔NH region of **6**, medium-range NOEs indicated coupling between distal residues (e.g., 1↔4, 2↔5, 4↔7, 6↔9; Fig. S4). Such cross couplings are absent in **9**, again suggesting that the HPI region of **6** is more helical than that of **9**. Thus, the integrity of the helical peptide moiety is apparently important for maintaining stoichiometric inhibition of γ-secretase.

Enzyme inhibition kinetics experiments were carried out for the two most potent HPI-TSA inhibitors (**6** and **16**). Both compounds showed noncompetitive inhibition (Figs. S5, S6). Both HPI **2** and TSA **17** (identical to **1** but with C-terminal methyl ester) likewise showed noncompetitive inhibition on their own (Fig. S7), as has been reported before with other γ-secretase inhibitors.[20] To determine whether the HPI and TSA moieties of **6** bind both docking and active sites on the enzyme, we performed cross-competition assays between two inhibitors.[21] In these experiments, TSA **17** and HPI **2** did not compete with each other for binding, consistent with binding to distinct sites (active site for **17**, docking site for **2**) (Fig. 2a). However, HPI-TSA inhibitor (**6**) competed with both TSA **17** and HPI **2** for binding (seen as parallel lines in Fig. 2b,c), indicating that **6** occupies both docking site and active site on the enzyme.

To verify this, we performed competition experiments with biotinylated photoaffinity probes for γ-secretase:[15, 22] active-site-directed photoprobe TSA-Bpa-Bt based on TSA **1** and docking-site-directed photoprobes HPI-Bpa-Bt based on HPI **2**. We have previously shown that 10-residue HPIs do not inhibit labeling of presenilin by TSA-Bpa-Bt, whereas TSAs do not inhibit labeling by 10-residue HPI-Bpa-Bt.[15] Solubilized lysates from γ-secretase-expressing human embryonic kidney 293 cells were incubated with each photoprobe in the presence and absence of the HPI-TSAs inhibitors and irradiated at 350 nM. Photolabelled proteins were pulled down with streptavidin beads, and the ~32 kDa band of the presenilin-1 (PS1) N-terminal fragment (NTF) subunit was detected by immunoblot. Lack of competition between TSA and HPI was confirmed (Fig. 3a, b). Decreased photolabelling of the PS1 NTF

γ-secretase subunit by either probe was observed in the presence of HPI-TSA inhibitors **6** and **16** (Fig. 3c,d, lane 1 vs 2 and 3) and in a concentration-dependent manner (Fig. S8). These results indicate that stoichiometric inhibitors **6** and **16** interact with both active and docking sites on γ-secretase, as they compete with the binding of both photoprobes.

Finally, the conformation of these new inhibitors, determined using NMR constraints for **6**, resembles the bound conformation of substrate in the new cryo-EM structures (Fig. 4), with a helical N-terminal region and an unfolded or extended C-terminal region. Thus, the new potent HPI-TSA inhibitors appear to be preorganized for ideal binding to γ-secretase and trapping the enzyme complex as it would be when poised for intramembrane proteolysis. Moreover, after initial substrate proteolysis, γ-secretase carries out successive carboxypeptidase trimming in intervals of three amino acids of the N-terminal cleavage product that contains most of the substrate TMD,[19] and this trimming activity is deficient in γ-secretase complexes with Alzheimer-causing presenilin mutations.[23–24] These new TMD mimetic inhibitors, with three P' residues in the TSA moiety, should capture the enzyme as it would carry out this trimming reaction. Cryo-EM analysis of these new probes in complex with γ-secretase is in progress. Variations on these TMD inhibitors are under development to trap other stages of substrate interaction with γ-secretase (e.g., lateral gating, initial proteolysis). Together, these conjugates should be important structural probes for gaining insights into the mechanism of intramembrane proteolysis by γ-secretase and how mutations in the enzyme cause Alzheimer's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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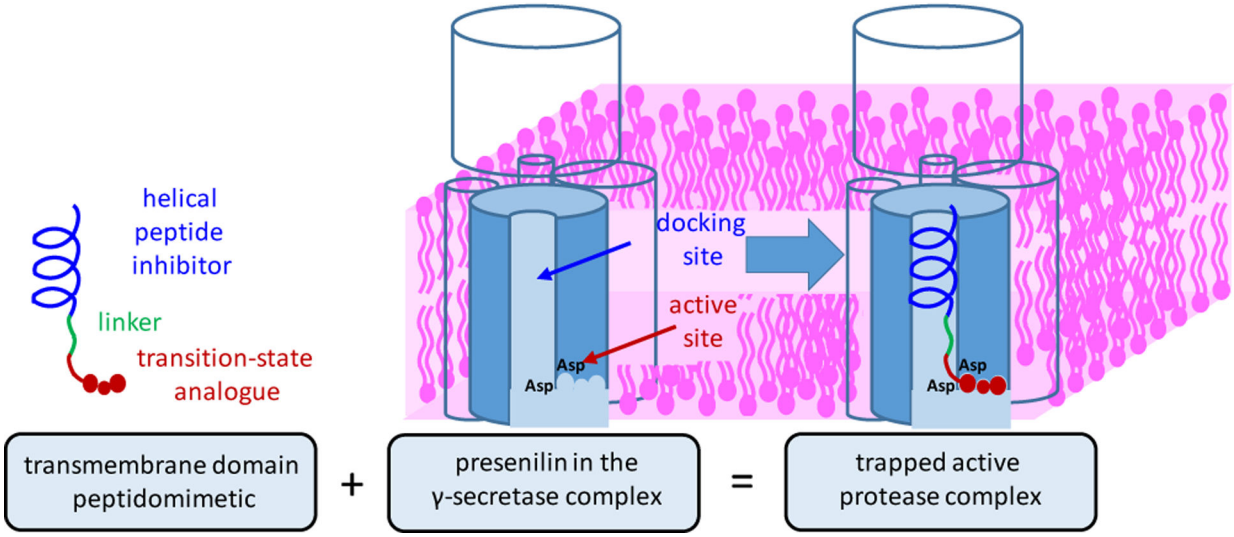

Figure 1. Design of inhibitors that mimic the transmembrane domain of γ-secretase substrates.

Helical peptide inhibitors (HPIs) directed to the substrate docking exosite were conjugated through a variable linker to transition-state analogue inhibitors (TSAs) directed to the active site. Presenilin (blue-grey) and other components of the γ-secretase complex (outlined) are shown schematically in the absence and presence of a hybrid HPI-TSA inhibitor.

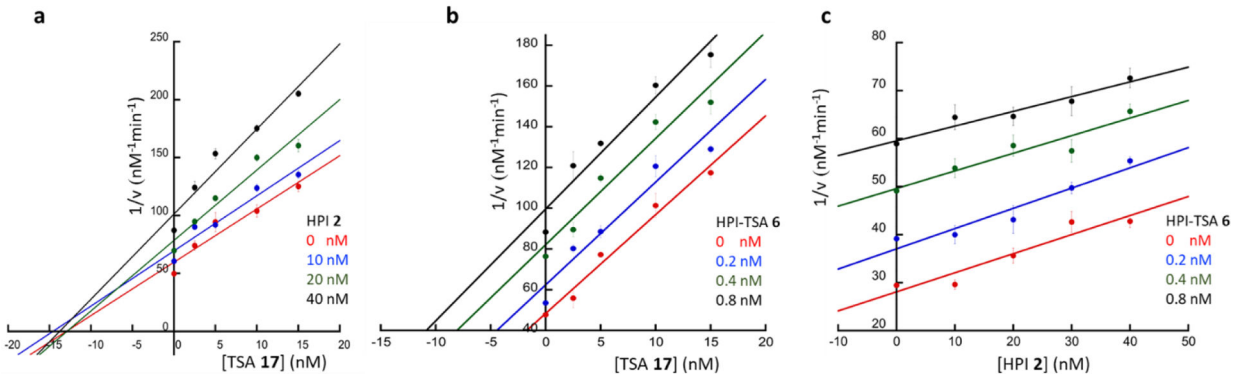

Figure 2. Cross-competition kinetic experiment between two inhibitors.

(**a**) TSA **17** and HPI **2** at [**2**] = 0 nM (●), 10 nM (●), 20 nM (●), and 40 nM (●). These two inhibitors do not compete, as lines converge near the x-axis. (**b**) TSA **17** and HPI-TSA **6** at [**6**] = 0 nM (●), 0.2 nM (●), 0.4 nM (●), and 0.8 nM (●). Parallel lines indicate cross-competition. (**c**) HPI **2** and HPI-TSA **6** at [**6**] = 0 nM (●), 0.2 nM (●), 0.4 nM (●), and 0.8 nM (●). Again, parallel lines indicate cross-competition, and HPI-TSA **6** competes with both TSA **17** and HPI **2**.

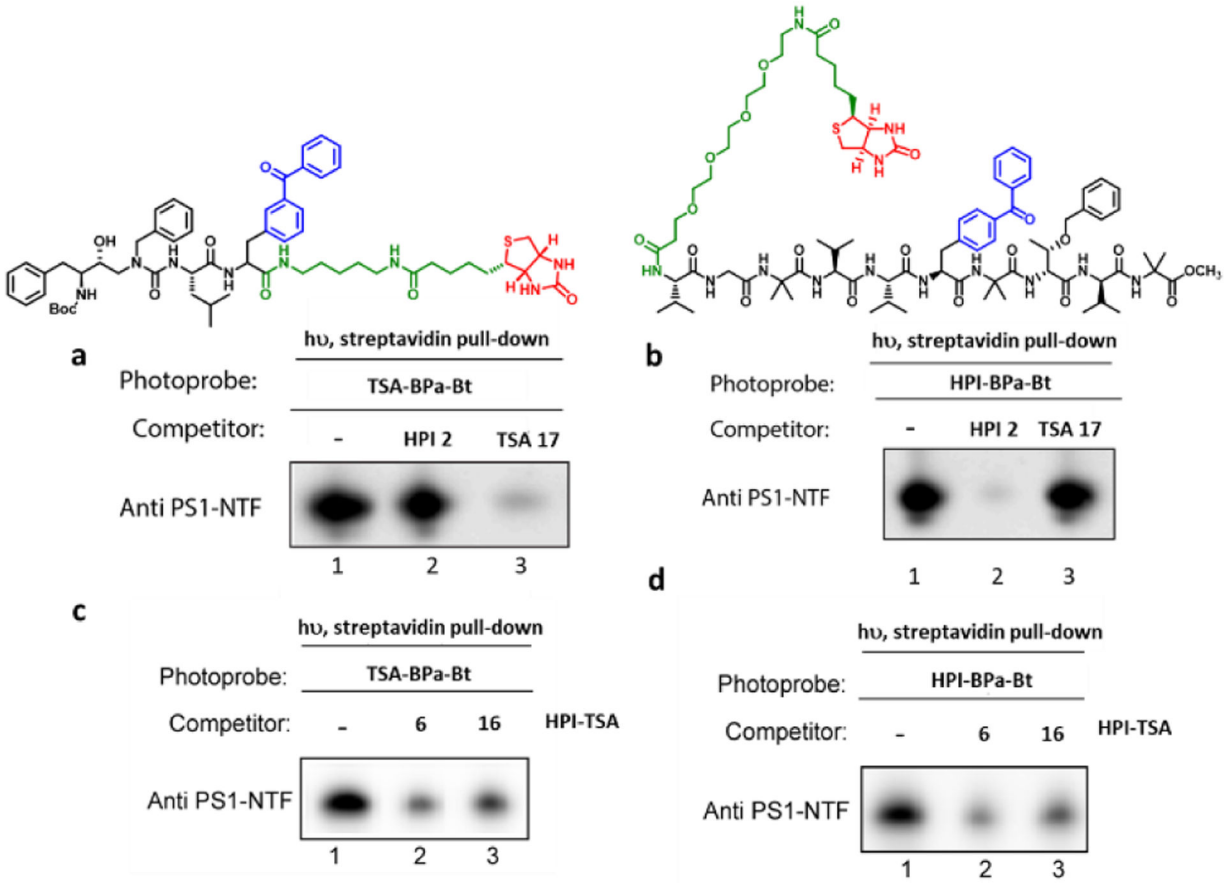

Figure 3: Competition of HPI-TSAs with photoaffinity probes for γ-secretase.

Photoprobes TSA-Bpa-Bt (left) and HPI-Bpa-Bt (right) covalently label presenilin-1 (PS1) N-terminal fragment (NTF) at the active site and docking site, respectively. (**a**) TSA (**17**) but not HPI (**2**) decreased the labelling of PS1 NTF by the TSA photoprobe. (**b**) HPI (**2**) but not TSA (**17**) decreased the labelling PS1 NTF by the HPI photoprobe. (**c, d**). HPI-TSAs **6** and **16** decreased labelling by both TSA photoprobe (**c**) and HPI photoprobe (**d**).

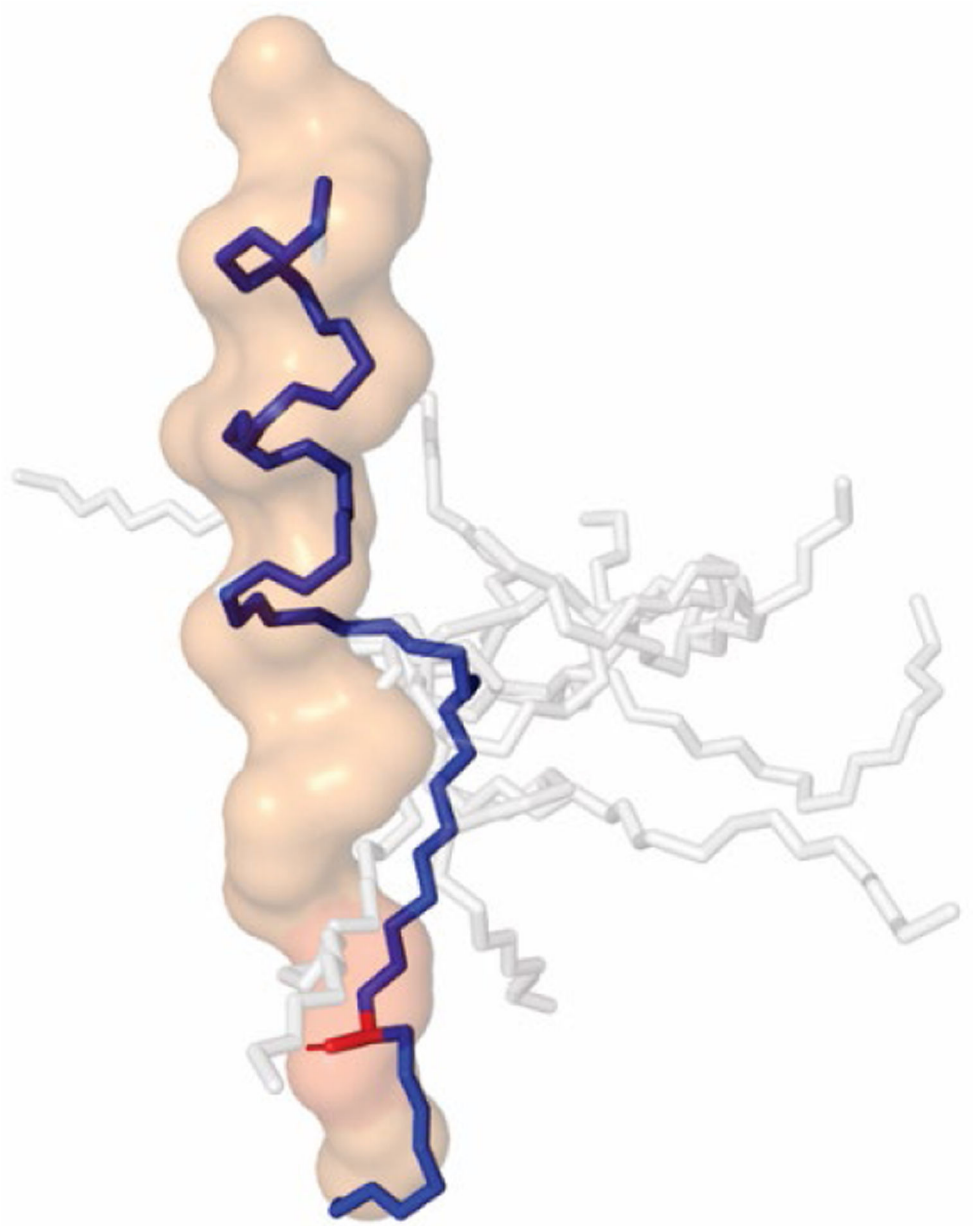

Figure 4: Solution conformation of designed HPI-TSA conjugate 6 resembles that of APP TMD substrate bound to γ-secretase.

NMR constraints were used to determine low-energy conformations of **6**. The top 10 conformers are shown as sticks, with the conformer closest to that of the bound substrate in blue. Structure rendered in Pymol using PDB file 6IYC for bound APP substrate, shown as surface outline. Transition-state mimicking hydroxyl group (red) of **6** overlaps with the scissile amide bond in the extended region of APP substrate when the helical moiety is aligned with the helical region of bound APP substrate.

Table 1.

Inhibition of γ-secretase by helical peptide/transition-state analogue conjugates.

Cmpd	Helical Peptide	Linker	Transition-State Analogue[a]	IC_{50}[b]
	APP transmembrane residues 707–717:		--------*Optimized TSA*----------	
	---Val-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Ile----		----P2 - P1 - P1′- P2′-P3′-----	
1			Boc-Val-Phe-ψ-Phe-Leu-Val-NH_2	41 ± 4
2	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-OCH_3			58 ± 6
3	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-	-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	53 ± 1
4	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-	-$NH(CH_2)_2CO$-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	12 ± 2
5	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-	-$NH(CH_2)_4CO$-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	10 ± 1
6	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-	-$NH(CH_2)_8CO$-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	0.80 ± 0.03
7	-	Boc$NH(CH_2)_8CO$-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	16 ± 1
8	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Phe-Val-Aib-	-$NH(CH_2)_8CO$-	-Val-Phe - Phe-Leu-Val-NH_2	18 ± 3
9	Boc-Val-Gly-Aib-^{D}Val-^{D} Val-Ile-Aib-Phe-Val-Aib-	-$NH(CH_2)_8CO$-	-Val-Phe-ψ-Phe-Leu-Val-NH_2	6 ± 2

[a] ψ represents hydoxyethylurea replacement of peptide backbone;

[b] concentration that inhibits 50% activity of 1 nM purified γ-secretase