Stereochemical Analysis of (Hydroxyethyl)urea Peptidomimetic Inhibitors of γ-Secretase

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Received June 4, 2004

(Hydroxyethyl)urea peptidomimetics systematically altered at positions P2−P3′ with hydrophobic D-amino acids were synthesized. An all D-amino acid containing analogue was identified that effectively blocked γ-secretase activity in a cell-free system (IC_{50} = 30 nM). Systematic alteration of the stereocenters of a potent compound revealed interdependence between the various positions. Although typically less potent than their L-peptidomimetic counterparts, selected all D-amino acid containing analogues were equipotent to their counterparts in a cell-based assay when incubated for extended times.

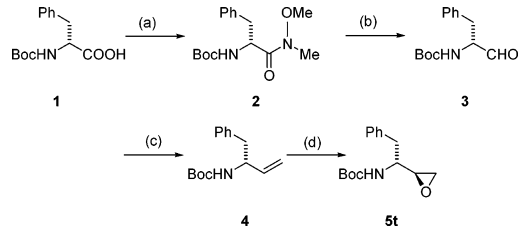
Introduction

Converging lines of evidence implicate the 39−43 amino acid amyloid β-peptide (Aβ) in the etiology of Alzheimer's disease (AD). Aβ, the primary protein component of amyloid plaques, is derived by sequential processing of amyloid precursor protein (APP) via β- and γ-secretases. Thus, these proteases are important targets for drug design.[1] β-Secretase has been identified as a novel membrane-bound aspartyl protease, which sheds the ectodomain of APP and generates a 99-residue (C99) membrane-associated C-terminal fragment (CTF). The remnant C99 gets cleaved by γ-secretase, which catalyzes hydrolysis within the transmembrane domain and is a founding member of a new family of intramembrane-cleaving proteases. Genetics, knockout studies, pharmacological profiling, mutagenesis, affinity labeling, and biochemical isolation point to the multipass membrane protein presenilin as the catalytic component of a novel aspartyl protease.[1] Other members of this protease complex include nicastrin, Aph-1, and Pen-2.[2]

Small organic inhibitors have played a prominent role in revealing the nature and biological roles of γ-secretase. Inhibitors of γ-secretase, including difluoro ketones and alcohols,[3] hydroxyethylenes,[4] benzodiazepines,[5] and helical peptides,[6] have served as useful molecular probes for characterizing and elucidating the mechanism of action of this protease. The readily accessible (hydroxyethyl)urea peptidomimetics (Scheme 3) in particular have been used by our laboratory for the affinity isolation of γ-secretase,[7] identification of the protease components,[8] and as active site labeling reagents to elucidate the mechanisms of other γ-secretase inhibitors.[9]

We recently reported (hydroxyethyl)urea peptidomimetics systematically altered in five positions (P2, P1′, P2′, P3′, and P4′) with small, medium, and large hydrophobic L-amino acids (Ala, Val, Leu, and Phe) and identified low nanomolar inhibitors of γ-secretase.[10] This study also confirmed the well-known loose sequence specificity of the enzyme, noted in various mutagenesis studies and by the fact that γ-secretase has a number of substrates (e.g., Notch, Erb-B4, *N*-cadherin).[2] Thus, we considered the possibility that γ-secretase could tolerate D-amino acids as well as L-amino acids in transition-state analogue inhibitors.

Scheme 1[a]



[a] Reagents and conditions: (a) *N,O*-dimethylhydroxylamine HCl, DIPEA, BOP, 4−5 h; (b) $LiAlH_4/Et_2O$, 0 °C, 30 min; (c) (i) CH_3PPh_3Br, KN($SiMe_3$), −78 °C, 30 min, (ii) 4 h, (iii) 40−45 °C, 12 h; (d) *m*-CPBA, CH_2Cl_2, 12 h.

Chemistry

The key intermediate [*R*(2*R**,3*S**)]-threo epoxide **5t** was prepared as previously described,[11] starting from Boc-protected D-phenylalanine instead of L-phenylalanine (Scheme 1). Briefly, this procedure involves formation of a Weinreb amide, LAH reduction to the α-aminoaldehyde, Wittig reaction to the alkene, and finally stereoselective oxidation using *m*-CPBA. The diastereomeric [*S*(2*R**,3*R**)]-erythro epoxide derived from L-phenylalanine was commercially available (Sigma-Aldrich). To access the [*R*(2*S**,3*S**)]-erythro epoxide (**5e**), a previously reported method[12] was modified (Scheme 2). This method involves stereoselective reduction of the α-haloketone, followed by base-induced ring closure. Briefly, the Boc-protected amino acid was converted to the corresponding α-amino bromomethyl ketone in a one-pot three-step sequence through mixed anhydride and diazoketone intermediates. We then surveyed reducing agents ($NaBH_4$/EtOH, L-selectride, 2-methyl-CBS-oxazaborolidine) to improve the diastereoselectivity. With (*S*)-2-methyl-CBS-oxazaborolidine, we obtained the highest diastereoselectivity (97% de), as determined by NMR and HPLC. Separation of isomers was followed by transformation of the chiral bromohydrin into the corresponding epoxide by treatment with base (NaOMe/MeOH).

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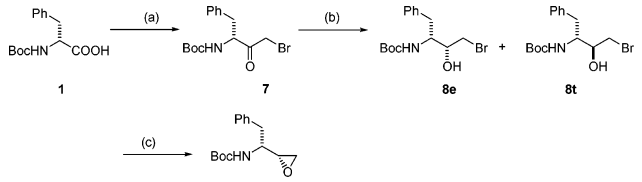
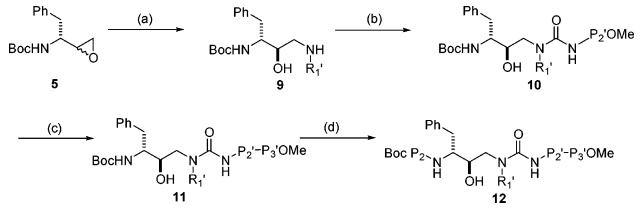
10.1021/jm049566e CCC: $27.50
Published on Web 11/16/2004

Scheme 2[a]



[a] Reagents and conditions: (a) (i) *N*-methylmorpholine, THF, $ClCO_2CH_2CHMe_2$, (ii) CH_2N_2, Et_2O, (iii) 48% HBr (aq); (b) (*S*)-2-methyl-CBS-oxazaborolidine/toluene, boranemethyl sulfide/THF, 0 °C, 1 h; (c) (i) flash chromatography, (ii) NaOMe/MeOH, 2 h.

Scheme 3[a]



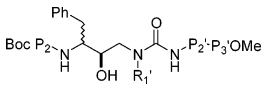
[a] Reagents and conditions: (a) R1′NH_2, *i*-PrOH, reflux, 12 h; (b) OCN−P_2′−OMe, 6 h; (c) (i) LiOH/dioxane, (ii) H_2N-P_3′-OMe, HATU, DIPEA, DMF, 8 h; (d) (i) TFA/CH_2Cl_2, (ii) BocHN-P_2-OH, HATU, DIPEA, DMF, 8 h.

Following the method of Getman et al.,[13] who developed (hydroxyethyl)urea peptidomimetics as inhibitors of HIV protease, these epoxides were opened with several different alkylamines (Scheme 3) in good yield (80−90%) by refluxing in 2-propanol for 16 h. The amino alcohols **9** were then treated with isocyanates (in turn obtained from α-amino methyl esters and phosgene) to yield the (hydroxyethyl)urea **10** in virtually quantitative yield. The resultant (hydroxyethyl)ureas **10** were extended to accommodate P3′ on the C-terminus (**11**) through methyl ester hydrolysis with LiOH in aqueous dioxane and subsequent coupling with α-amino esters using HATU in the presence of diisopropylethylamine (DIPEA) in DMF. Toward P2 variants **12**, the Boc group was removed with TFA/CH_2Cl_2 (1:1), followed by coupling with Boc-protected amino acids using HATU and DIPEA in DMF. In an attempt to increase stability, the methyl ester of the C-terminus was replaced with *tert*-butyl ester by using the corresponding *tert*-butyl ester of the P3′ amino acid.

Biological Evaluation and Discussion

On the basis of our previous results from L-amino acid containing (hydroxyethyl)urea peptidomimetics[10] and reports suggesting loose sequence specificity of γ-secretase, we synthesized **13**, an all D-amino acid containing analogue, and noted that this compound retained reasonable inhibitory potency in cell-free and cell-based assays, compared with the all L-peptidomimetic **14** (Table 1). To follow-up on this interesting observation, we made systematic changes in **13** from P2 to P3′ to probe the S2−S3′ pockets of γ-secretase for their ability to accommodate D-amino acid residues. All compounds were tested for their effect on Aβ in Chinese hamster ovary (CHO) cells stably transfected with human APP (cell line 7W).[3] Briefly, stock concentrations of peptide analogues were made in DMSO and added to media to reach a final concentration of 1% DMSO; positive controls contained 1% DMSO only. The compounds were incubated with cells for 4 h, media was centrifuged, and supernatant was checked for released Aβ by ELISA. To determine direct inhibition of γ-secretase, compounds were examined using detergent-soluble membrane preparations from HeLa cells (as a source of the protease) and an APP-based recombinant substrate, C100Flag.[14] C100Flag is the endogenous APP-derived γ-secretase substrate C99 plus an N-terminal methionine start site and a C-terminal Flag epitope. Inhibitory potencies were determined using an Aβ ELISA (Tables 1−3) and in some cases further confirmed by anti-Flag Western blot (Supporting Information). All tested compounds inhibited Aβ production from C100Flag, with potencies from 30 nM to 12 μM, indicating that they block γ-secretase directly. Selected compounds also inhibited the γ-secretase proteolysis of a Notch-based substrate, N100Flag (Supporting Information). Differences in the potencies of inhibitors for lowering Aβ formation were observed between the cell-based and cell-free assays, which might be due to the differences in cell permeability and/or metabolic stability.

Table 1. IC_{50} Values of (Hydroxyethyl)urea Analogues Containing D-Amino Acids in Cell-Based and Cell-Free Assays

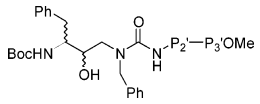


compd	P2	P1	R1′	P2′	P3′	IC_{50} (cell-free)[a] μM	IC_{50} (cell-based)[a] μM
13		D	Bz	D-Leu	D-Leu	0.20 ± 0.01	3.00 ± 0.5
14		L	Bz	L-Leu	L-Leu	0.03 ± 0.02	0.60 ± 0.1
15		D	Bz	D-Leu	D-Ala	2.00 ± 1.00	7.00 ± 1.0
16		D	Bz	D-Leu	D-Val	0.10 ± 0.05	2.00 ± 0.8
17		D	Bz	D-Leu	D-Phe	0.80 ± 0.20	3.00 ± 0.4
18		D	Bz	D-Ala	D-Val	3.00 ± 0.80	10.0 ± 1.0
19		D	Bz	D-Val	D-Val	2.00 ± 0.60	12.0 ± 0.7
20		D	Bz	D-Phe	D-Val	9.00 ± 1.00	24.0 ± 2.0
21		D	Me	D-Leu	D-Val	7.00 ± 1.00	12.0 ± 1.0
22		D	*i*-Pr	D-Leu	D-Val	4.00 ± 0.80	8.00 ± 2.0
23		D	*i*-Bu	D-Leu	D-Val	2.00 ± 1.00	5.00 ± 0.6
24	D-Ala	D	Bz	D-Leu	D-Val	12.0 ± 2.00	30.0 ± 3.0
25	D-Val	D	Bz	D-Leu	D-Val	3.00 ± 0.60	7.00 ± 1.0
26	D-Leu	D	Bz	D-Leu	D-Val	0.90 ± 0.05	2.00 ± 0.7
27	D-Phe	D	Bz	D-Leu	D-Val	2.00 ± 0.50	8.00 ± 1.0
28		D	Bz	D-Leu		6.00 ± 1.00	15.0 ± 2.0

[a] Values are the mean ± SD of three sets of experiments.

Modification of the P3′ D-leucine of **13** with D-alanine, D-valine, and D-phenylalanine revealed steric tolerance for substituents ranging from methyl through benzyl (Table 1). Nevertheless, an apparent preference for D-valine (**16**) led us to fix this position in the next round of synthesis. Similar alterations in the P2′ position led to the observation of a clear preference for D-leucine in cell-based and cell-free assays (compare **16** with **18**−**20**). This is in contrast to the L-peptidomimetic series in which L-alanine, L-valine, and L-leucine were well tolerated in the P2′ position; only L-phenylalanine incorporation results in a substantial loss of potency.[10] With P2′ fixed as D-leucine and P3′ fixed as D-valine, the achiral P1′ position was then examined, and a clear preference for a benzyl substituent was observed in the cell-free assay (compare **16** with **21**−**23**). This preference was also seen in the cell-based assay, but not as strongly. Although benzyl was also preferred in the L-peptidomimetic series, in this case the *i*-butyl-containing analogue was equipotent.[10] Extension of the best compound (**16**) into the P2 position (**24**−**27**) resulted in a loss of activity in all cases. This contrasts to findings

Table 2. IC_{50} Values of (Hydroxyethyl)urea Stereoisomers



compd	P1	OH	P2′	P3′	IC_{50} (cell-free)[a] μM	IC_{50} (cell-based)[a] μM
29	D	*R*	L-Leu	L-Val	0.01 ± 0.01	0.40 ± 0.10
31c	L	*R*	L-Leu	L-Val	0.01 ± 0.01	0.40 ± 0.20
30	L	*R*	D-Leu	D-Val	0.03 ± 0.01	0.60 ± 0.04
32	D	*R*	L-Leu	D-Val	0.08 ± 0.01	0.80 ± 0.20
33	L	*R*	L-Leu	D-Val	0.60 ± 0.10	1.00 ± 0.30
34	D	*R*	D-Leu	L-Val	0.80 ± 0.05	8.00 ± 1.00
35	L	*R*	D-Leu	L-Val	0.06 ± 0.01	0.80 ± 0.05
36	D	*S*	D-Leu	D-Leu	2.00 ± 0.40	8.00 ± 0.80
37	D	*S*	D-Leu	D-Val	3.00 ± 1.00	10.0 ± 2.00

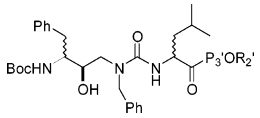
[a] Values are the mean ± SD of three sets of experiments.

in the L-peptidomimetic series in which extension with L-valine resulted in a substantial improvement of activity.[10] Truncation of **16** by removal of the P3′ position, however, resulted in a clear loss of activity (compare **16** with **28**).

Although **16** displayed reasonable potency for blocking γ-secretase (IC_{50} of 100 nM in solubilized membranes and 2 μM in cells), this activity is substantially lower than that seen with its all L-amino acid containing counterpart **31c** (called WPE-III-31c in our initial report of this compound) (Table 2).[7] To define the specific positions responsible for this difference, **16** was systematically altered at each stereocenter (Table 2). Interestingly, a clear stereochemical preference was not seen in P1, P2′, or P3′, suggesting interdependency between these positions. L-Valine in the P3′ position can be favorable (**31c** vs **33**) or unfavorable (**16** vs **34**), L-leucine in the P2′ position can be favorable (**29** vs **34**) or unfavorable (**30** vs **33**), and L-phenylalanine in the P1 position can be favorable (**30** vs **16**) or unfavorable (**32** vs **33**). Taken together, these results suggest that the conformation of the peptidomimetic backbone when bound in the γ-secretase active site can be different depending on the stereochemistry of the various residues. In other words, the stereochemical identity of a particular position may lead to alteration of the backbone conformation to allow accommodation of the substituents in the other positions. This study also identified **30** (IC_{50} of 30 nM in solubilized membranes and 600 nM in cells) (Table 2), with all D-amino acids (the P1 position is part of a pseudopeptide) as a potent inhibitor comparable to all L-peptidomimetic **31c**.

To investigate further the effect of the hydroxyl group stereochemistry on inhibitory activity of (hydroxylethyl)-ureas, analogues **36** and **37** (Table 2) were tested. The inhibitory potency of these compounds suggested a preference for (*R*)-hydroxyl group (**36** and **37** in Table 2 compared to **13** and **16** in Table 1). Finally, the effectiveness of selected compounds on inhibiting Aβ production in cells was examined at two different times, 4 and 24 h. Although L-peptidomimetic **31c** was 5 times more effective than its D-peptidomimetic counterpart **16** over 4 h, these two compounds were essentially equipotent over 24 h. Similar effects were seen with L-peptide analogue **14** and its D-peptidomimetic counterpart **13** and also when the C-terminal methyl ester was replaced with *tert*-butyl ester (Table 3; **38** and **39**). C-terminal modification of analogues **13** and **14** provided compounds **40** and **41**, which were similarly potent at either time point. These observations suggest that the D-peptidomimetics are in general more slowly metabolized by the cells than the L-peptide analogues. All compounds are less potent over 24 h, but the D-peptidomimetics generally retain their effectiveness better than the L-peptide analogues.

Table 3. IC_{50} Values of All D- and All L-Amino Acid Containing (Hydroxyethyl)ureas, Varied at the C-Terminus after 4 and 24 h of Incubation in Cell-Based Assays



compd	P1	P_2'	P_3'	R_2'	IC_{50} (cell-based, 4 h)[a] μM	IC_{50} (cell-based, 24 h)[a] μM
16	D	D	D-Val	Me	2.0 ± 0.80	17.0 ± 1.0
31c	L	L	L-Val	Me	0.4 ± 0.20	18.0 ± 2.0
13	D	D	D-Leu	Me	3.0 ± 0.50	9.00 ± 0.5
14	L	L	L-Leu	Me	0.6 ± 0.10	9.40 ± 0.2
38	L	L	L-Val	*t*-Bu	0.3 ± 0.04	6.00 ± 1.0
39	D	D	D-Val	*t*-Bu	3.0 ± 0.50	6.20 ± 0.6
40	L	L	L-Leu	*t*-Bu	0.4 ± 0.10	5.00 ± 1.0
41	D	D	D-Leu	*t*-Bu	0.6 ± 0.02	7.00 ± 0.5

[a] Values are the mean ± SD of three sets of experiments.

Taken together, the stereochemical analysis of these transition-state analogue peptidomimetics demonstrate that γ-secretase can tolerate D-amino acid residues, the stereochemical preferences in the peptidomimetics are interdependent, a secondary hydroxyl group with *R* stereochemistry is preferred, and in general all D-amino acid containing analogues retain potency better over time than L-peptidomimetics. These results suggest that the conformation of the bound peptidomimetic depends on the stereochemical identity of the various positions and that D-peptidomimetics might be appropriate leads for further drug development.

Experimental Section

General. *tert*-Butyl [*S*(*R**,*R**)]-(−)-(1-oxiranyl-2-phenylethyl)carbamate was purchased from Sigma (St. Louis, MO). All amino acids, HATU, and BOP were from Novabiochem (San Diego, CA). All solvents were anhydrous and used as supplied by Aldrich (Milwaukee, WI). Silica gel column chromatography was performed using Merck silica gel 60 ASTM (70−230 mesh). Final purification of peptides was carried out on a Shimadzu HPLC system using a reverse-phase Vydac C18 (218TP) semipreparative column (12 μm, 10 mm i.d.). Purity of peptides (Supporting Information) was checked with analytical HPLC (Vydac C18, 5 μm, 4.6 mm i.d) in two different solvent systems (methanol/water and acetonitrile/water) using a gradient program and found to be >99% pure. ^{1}H NMR spectra were recorded on a Varian Mercury 200 MHz spectrometer, and chemical shifts (Supporting Information) are expressed in ppm relative to tetramethylsilane as an internal standard. Mass spectra (Supporting Information) were acquired using MALDI-TOF mass spectroscopy (Applied Biosystems Voyager System 4036).

General Procedure. Synthesis of *tert*-Butyl [*R*(*R,*S**)]-(−)-(1-Oxiranyl-2-phenylethyl)carbamate (5t).** The starting compound **5t** was prepared as in Scheme 1 using standard protocols.[11] To a stirred solution of Boc-protected D-phenylalanine (5 mmol), BOP (6 mmol), and DIPEA (1 mL) at 0 °C in CH_2Cl_2 (8−10 mL) under a nitrogen atmosphere was added *N,O*-dimethylhydroxylamine hydrochloride (6 mmol) in diisoprpopylethylamine (DIPEA) (1 mL). The mixture was stirred for 4−5 h until completion of reaction (checked by TLC in hexane/EtOAc 2:1). The reaction mixture was diluted with 100

mL of CH_2Cl_2, and the organic layer was washed sequentially with 1 N HCl (3×), saturated $NaHCO_3$, and finally with brine. The organic layer was dried over Na_2SO_4 and evaporated to a colorless oil. The product was purified by flash chromatography using hexane/EtOAc 4:1 to yield 80−85% of Weinreb amide **2**. Compound **2** (1 mmol) was then reduced to α-(*tert*-butoxycarbonyl)aminoaldehyde **3** by dissolving in diethyl ether (20 mL) and slowly adding $LiAlH_4$ (5 mmol). After reduction for 30 min, the product was hydrolyzed with a solution of $KHSO_4$ (3.5 mmol) in water (10 mL). The product was extracted from the aqueous layer with ether (3 × 50 mL), and the organic layer was washed with 1 N HCl, saturated $NaHCO_3$, and brine. Drying (Na_2SO_4), filtering, and evaporating provided the corresponding protected aminoaldehyde (74−93% yield), which was used without further purification. For alkene **4**, 1,1,1,3,3,3-hexamethyldisilazane (1 mL, 1.1 mmol) was added dropwise to a 0 °C suspension of potassium hydride (35% dispersion in oil, 1.1 mmol) in anhydrous THF/DMSO (14 mL/3 mL) under dry N_2. After being stirred at 0 °C for 1 h, the resulting solution was added via cannula to a 0 °C flask containing methyltriphenylphosphonium bromide (1.1 mmol). The mixture was stirred vigorously for 1 h and then cooled to −78 °C. At −78 °C, a THF solution of the aldehyde (1 mmol) prepared above was added via cannula over 20 min. After being stirred at −78 °C for another 10 min, the mixture was allowed to slowly warm to room temperature (4 h) and then heated to 40−45 °C for 12 h. The mixture was then cooled to room temperature, and the reaction was quenched with methanol (200 μL), followed by aqueous Rochelle salts (10 mL of saturated solution and 100 mL of H_2O). The mixture was then extracted with EtOAc (2 × 150 mL). The combined extracts were washed with water and brine. Drying (Na_2SO_4) and evaporating provided the crude product, which was purified by flash chromatography on silica gel (ether/hexane) to give alkene **4** in 45−50% yield.

The resulting olefin (1 mmol) was stirred in CH_2Cl_2 with *m*-chloroperoxybenzoic acid (4 mmol) under a nitrogen atmosphere. When the reaction was complete by TLC analysis, the mixture was diluted with ether, washed sequentially with ice-cold 10% Na_2SO_3, saturated $NaHCO_3$, and brine. Drying and evaporating provided the white crystalline epoxide **5t** in 60% yield with 99% de {[α] −2°(*c* 0.6, CH_3OH)}. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.39−1.42 (s, 9H), 2.56−2.6 (m, 1H), 2.69 (dd, 1H), 2.82−3.14 (m, 3H), 4−4.16 (m, 1H), 4.44−4.52 (br, m, 1H), 7.2−7.36 (m, 5H)

Synthesis of *tert*-Butyl [*R*(*S,*S**)]-(−)-(1-Oxiranyl-2-phenylethyl)carbamate (5e).** Synthesis of the erythro epoxide was carried out according to the method described by Albeck et al.[12] with slight modifications. Briefly, a solution of Boc-protected D-phenylalanine **1** (1 mmol) and *N*-methylmorpholine (1.1 mmol) in dry THF (5 mL) under argon atmosphere was cooled to −15 °C. Isobutyl chloroformate (1 mmol) was added, and after 5 min the reaction mixture was quickly filtered and added to a precooled (−15 °C) ethereal solution of diazomethane (2 mmol). The reaction mixture was stirred for 2 h at 0 °C, and 1 equiv of 48% aqueous HBr was added. The reaction was continued for another 15−20 min, and the resultant bromoketone **7** was purified using flash chromatography (elution with 3:1 of ether/hexane). For bromohydrin **8**, a solution of (*S*)-2-methyl-CBS-oxazaborolidine (1.0 M in toluene, 0.1 mL, 0.1 mmol) and boranemethyl sulfide (2.0 M in THF, 0.05 mL, 0.1 mmol) in anhydrous THF (2 mL) was treated simultaneously with a solution of the α-bromoketone **7** in THF (1 mL) and boranemethyl sulfide (2.0 M in THF, 0.33 mL, 0.66 mmol) at 0 °C under argon over 20 min. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. The mixture was then cooled to 0 °C before 1 mL of methanol was added carefully (*CAUTION:* gas evolution!!). The reaction mixture was then concentrated in vacuo (Me_2S was trapped and oxidized with household bleach), and the residue was dissolved in 20 mL of EtOAc. The solution was washed with 1 N of HCl (3 × 5 mL) and water (2 × 5 mL), dried over Na_2SO_4, filtered, and concentrated. The product was purified by column chromatography on silica gel using hexane/ethyl acetate (90:10) as eluant to give **8e** as a white solid. The N-protected bromohydrin (0.3 mmol) was dissolved in methanol (5 mL), and 1 mL of 0.3 M NaOMe in MeOH was added. After 1.5 h of stirring, water (10 mL) was added and the solution was extracted with 10 mL of CH_2Cl_2. The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness. Flash chromatography afforded the pure product **5e** in 85% yield with 97% de. [α] +6° (*c* 0.6, CH_3OH). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.39−1.40 (s, 9H), 2.66−2.72 (m, 1H), 2.8 (dd, 1H), 2.82−3 (m, 3H), 3.72−3.78 (m, 1H), 4.42−4.48 (br, m, 1H), 7.2−7.36 (m, 5H).

Synthesis of (Hydroxyethyl)urea Analogues[7] (11). To a solution of oxirane **5** (1 equiv) in 2-propanol was added 20 equiv of primary amine, and the reaction mixture was refluxed under dry nitrogen for 12 h. However, for the reaction leading to analogue **21**, methylamine was bubbled into the solution to saturation and the reaction was carried out in a pressured vessel. The solution was diluted with ethyl acetate and washed consecutively with water, aqueous 1 N HCl, and saturated $NaHCO_3$, and the organic phase was dried over Na_2SO_4, filtered, and concentrated to provide the amino alcohol **9**. The isocyanates of P2′ amino acids were made separately by stirring amino acid ester (HCl salt) in a mixture of CH_2Cl_2 and $NaHCO_3$ for 20 min followed by addition of phosgene solution (20% in toluene) into the settled CH_2Cl_2 layer of the mixture. After an additional 30 min of stirring, the organic phase was separated, dried over Na_2SO_4, filtered, and concentrated. The (hydroxyethyl)amine **9** was then coupled with the isocyanate methyl ester in a minimal amount of CH_2Cl_2 for 5−6 h. The reaction mixture was concentrated, and the (hydroxyethyl)urea **10** was purified using flash chromatography. For C-terminal extension, the methyl ester functionality was hydrolyzed with LiOH (0.5 M) in aqueous dioxane for 2 h. After evaporation of dioxane, water and CH_2Cl_2 were added. The pH of the aqueous solution was reduced to 2 with aqueous 1 N HCl, and the organic layer was separated and washed with brine, dried over Na_2SO_4, and concentrated. The resultant carboxylic acid was then coupled to the P3′ α-amino alkyl ester in a minimum amount of DMF in the presence of HATU and DIPEA for 8 h. The reaction mixture was diluted with CH_2Cl_2 and washed with aqueous 1 N HCl, saturated $NaHCO_3$, and finally brine. After concentration, the crude product **11** was purified by flash chromatography, followed by preparative HPLC on a C18 column. All final compounds and their intermediates were characterized and assessed for purity by ^{1}H NMR (200 MHz), mass spectrometry (MALDI-TOF), and analytical HPLC in two different solvent systems (Supporting Information).

Synthesis of N-Terminal Extended (Hydroxyethyl)-urea Analogues (12). *tert*-Butyloxycarbonyl (Boc) protection was removed from peptidomimetic **11** by treating with TFA (trifluoroacetic acid) in CH_2Cl_2 (3:7) for 30−40 min. The reaction mixture was diluted with CH_2Cl_2, and the pH was increased to 7 with a saturated solution of $NaHCO_3$. The organic layer was extracted and washed with brine, dried with Na_2SO_4, and evaporated to provide the free amine. Coupling with Boc-protected amino acids was carried out as described above.

Biological Evaluation. Cell Lines, Compound Treatments, and ELISAs.[3] Compounds were tested in CHO cells stably transfected with the 751 amino acid splice variant of APP (7w cells). Cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 200 μg/mL G418 (Gibco BRL). Stock concentrations of the peptide analogues in DMSO were added to DMEM to reach the final concentrations with 1% DMSO. Positive controls contained 1% DMSO alone. After 4 or 24 h, the medium was removed and centrifuged at 3000*g* for 5 min, and the supernatant was stored at −80 °C until the assays were carried out. Sandwich ELISAs for Aβ40 and Aβ42 were performed using capture antibodies 2G3 (to Aβ40 residues 33−40) for the Aβ40 species and 21F12 (to Aβ42 residues 33−42) for the Aβ42 species. The reporter antibody was biotinylated 3D6 (to Aβ residues 1−5) in each assay. Horseradish peroxidase-avidin binding to the reporter

antibody was detected using 3,3′,5,5′-tetramethylbenzidine (Pierce) and measuring at 455 nm.

In Vitro γ-Secretase Assays.[7] Solubilized γ-secretase was prepared essentially as described by Li et al.[14] except that membranes were washed in 0.1 M Na_2CO_3, pH 11.3, to remove peripheral membrane proteins before solubilization in 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). To monitor inhibitory action of γ-secretase activity, the compounds and DMSO were incubated with the solubilized preparation (at 0.2 mg of protein/mL) of γ-secretase with a recombinant Flag-tagged APP-based substrate (C100Flag)[14] or Notch-based substrate (N100Flag)[7] at 37 °C for 0 or 4 h. The reactions were stopped by adding 0.5% SDS and boiling for 5 min. The samples were centrifuged, and the supernatant solutions were assayed for Aβ peptides by sandwich ELISA. In some cases, the C-terminal cleavage products were detected by Western blot (Supporting Information) using anti-Flag antibody M2 (Sigma). The Aβ40- and Aβ42-related products from γ-secretase-mediated processing of C100Flag possess a Met at the N-terminus and are thus defined as M-Aβ40 Aβ(x-40) and M-Aβ42 Aβ(x-42), respectively. The capture antibodies were 2G3 (to Aβ40 residues 33−40) for the x-40 species and 21F12 (to Aβ42 residues 33−42) for the x-42 species. The reporter antibody was biotinylated 266 (to Aβ residues 13−28) in each assay.

Acknowledgment. We thank Bing Zheng and Jennifer Strahle for carrying out Aβ ELISAs and Ilya Buldyrev for assistance with cell cultures. This work was supported by NIH Grant NS41355 to M.S.W.

Supporting Information Available: Characterization by ^{1}H NMR, MS, and HPLC in two different solvent systems and Western blot results of C100Flag and N100Flag. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049566E