A Substrate-Based Difluoro Ketone Selectively Inhibits Alzheimer's γ-Secretase Activity

Michael S. Wolfe,*,† Martin Citron,‡,§
Thekla S. Diehl,‡ Weiming Xia,‡
Isaac O. Donkor,† and Dennis J. Selkoe‡

Department of Pharmaceutical Sciences, University of Tennessee, Memphis, Tennessee 38163, and Center for Neurologic Diseases, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts 02115

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Amyloid plaques, invariantly associated with Alzheimer's disease (AD), contain amyloid β-protein (Aβ) as the primary protein component.[1] The 39−43 amino acid Aβ is derived from the β-amyloid precursor protein (APP), an integral membrane protein of unknown function.[2] Recent findings particularly implicate the more hydrophobic and highly insoluble 42 amino acid variant ($A\beta_{42}$) in amyloid plaque formation and in the pathogenesis of AD.[3−5] Although $A\beta_{42}$ accounts for only about 10% of total Aβ secreted from cells (roughly 90% is the 40 amino acid variant $A\beta_{40}$), $A\beta_{42}$ is the major protein component of the diffuse, largely nonfibrillar plaques which precede the development of the dense, fibrillar neuritic plaques characteristic of AD.[6,7] Genetic evidence strongly implicates Aβ in general and $A\beta_{42}$ in particular in the etiology of AD: all mutations linked to familial early-onset AD (FAD) examined to date result in increased production of $A\beta_{42}$ or of both $A\beta_{40}$ and $A\beta_{42}$.[3−5]

Aβ is formed from APP through two protease activities.[8] First, β-secretase cleaves APP at the Aβ N-terminus, resulting in a soluble, secreted APP derivative (β-APP_s) and a 12 kDa membrane-retained C-terminal fragment (CTF) (Figure 1). The latter is further processed to Aβ by γ-secretases, which cleave within the single transmembrane region. Other APP molecules can be cleaved by α-secretase within the Aβ region, thus precluding Aβ formation. This α-secretase cleavage, which occurs more commonly than β-secretase cleavage, also leads to release of a soluble derivative of APP (α-APP_s) and retention of a 10 kDa CTF. The CTF resulting from α-secretase cleavage can also serve as a substrate for the γ-secretases to yield a 3 kDa protein (p3), an N-terminally truncated version of Aβ. As yet, none of these APP-processing secretases have been isolated, nor have they been assigned to a specific protease class (although α-secretase is likely a metalloprotease[9]).

Recent studies suggest that different protease activities, dubbed γ(40)- and γ(42)-secretases, are responsible for the C-terminal heterogeneity of Aβ.[10,11] Four peptide aldehydes, all originally designed to inhibit the cysteine protease calpain, are the only γ-secretase inhibitors reported to date,[10−12] and no compounds selective for inhibiting $A\beta_{42}$ production more than that of $A\beta_{40}$ have been described. At the time of this writing, there have been no reports describing the design of γ-secretase inhibitors. Thus, while γ-secretases are considered important new targets for inhibitor design,[13,14] very little information on the structural requirements for inhibiting these enzymes is available. In this report, we describe a substrate-based difluoro ketone that inhibits γ-secretase activity in APP-transfected cells.

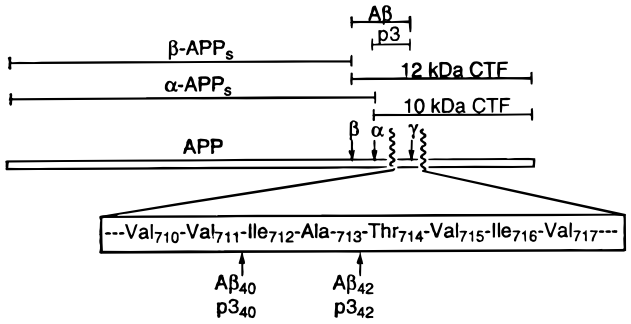

Figure 1. Sites of APP processing by α-, β-, and γ-secretases and the resulting proteolytic fragments. Heterogeneity of Aβ and p3 at the C-terminus is due to differential processing of the 12 and 10 kDa C-terminal fragments (CTFs) by γ-secretases.

The design of γ-secretase inhibitors at this time is challenging, because the proteases are unidentified. While it has been suggested that γ-secretases are aspartyl proteases,[15−17] the fact that the reported γ-secretase inhibitors are all calpain inhibitors suggests that the γ-secretases may be cysteine proteases. We initially designed peptidomimetic **1** (Scheme 1) based on the APP γ(42)-secretase cleavage site (Figure 1). This compound contains an (*S*)-4-amino-3-oxo-2,2-difluoropentanoyl moiety as a stable pseudodipeptide replacement for the scissile Ala-Thr bond. Although target compound **1** does not contain the P1′ side chain, related difluoro ketone peptide analogues without P1′ side chains potently inhibit aspartyl proteases (renin[18] and HIV protease[19]) and serine proteases (chymotrypsin[20] and porcine pancreatic elastase[21]). The difluoro ketone moiety is readily hydrated in aqueous media, and this hydrate is thought to mimic the tetrahedral intermediate formed in aspartyl protease catalysis. For serine proteases, the active site serine attacks the keto form of the analogue, resulting in a stable, covalent enzyme−inhibitor complex. A similar mechanism can be envisioned for cysteine protease inhibition, although there are no reports confirming this. There is at least one report, however, of fluoromethyl ketone peptide analogues that potently inhibit a cysteine protease.[22] Hence, without clear knowledge of the class of protease into which γ-secretases fall, difluoro ketone-type pseudodipeptides incorporated into the context of the appropriate APP sequence would appear to have a reasonable chance of inhibiting these enzymes. Peptidomimetics of this type would be expected to have important advantages over the few previously reported γ-secretase inhibitors: they are specifically designed toward γ-secretases and not toward calpain, and they allow exploration

* To whom correspondence should be addressed. Tel: (901) 448-7533. Fax: (901) 448-6828. E-mail: mwolfe@utmem1.utmem.edu.
† University of Tennessee.
‡ Harvard Medical School.
§ Present address: Amgen, Inc., Thousand Oaks, California 91320-1789.

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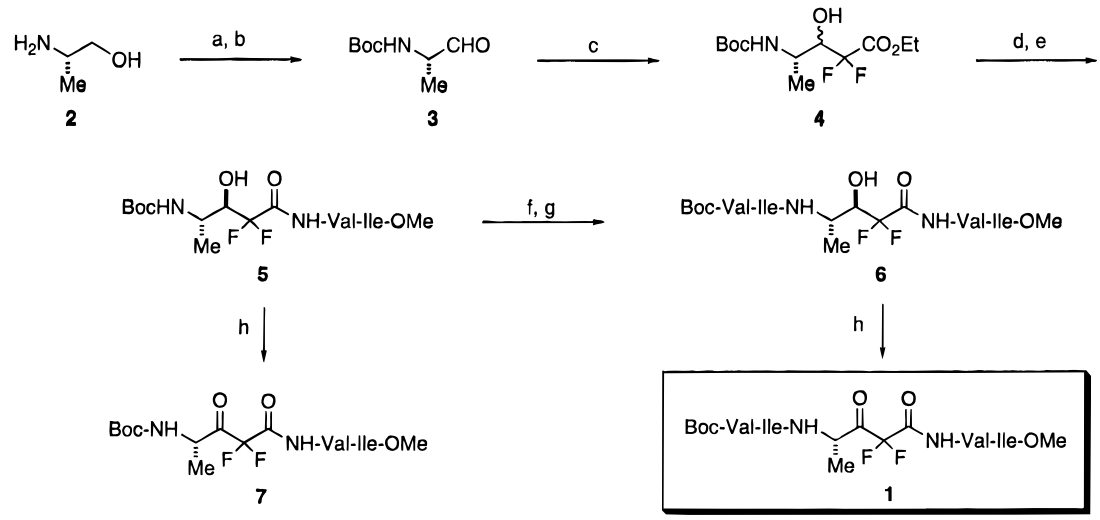
Scheme 1[a]



[a] (a) Boc_2O, $KHCO_3$; (b) DMSO, oxalyl chloride, Et_3N; (c) $BrCF_2CO_2Et$, Zn, reflux; (d) 1 equiv of NaOH, then H^+; (e) H_2N-Val-Ile-OMe, DIPC, HOBT; (f) TFA; (g) Boc-Val-Ile-OH, DIPC, HOBT; (h) Dess−Martin oxidation.

of binding sites downstream from the cleavage site (i.e., P2′, P3′, etc.).

Peptide analogue **1** was designed based on the Ala_{713}−Thr_{714} APP cleavage site leading to Aβ_{42} (i.e., it was specifically designed as a γ(42)-secretase inhibitor). Flanking residues were included to increase binding potency as well as specificity. The synthetic procedure for realizing this compound is shown in Scheme 1. Commercially available (*S*)-2-amino-1-propanol (L-alaninol, **2**) was protected as a *tert*-butoxycarbamate (Boc) and oxidized under Swern conditions to produce aldehyde **3** in 75% yield for the two steps. Although many amino aldehydes are easily racemized or otherwise unstable, aldehyde **3** was remarkably stable and obtained in enantiopure form (>95% ee) via recrystallization from hexane and ether.[23] This aldehyde was then converted to difluoro alcohol **4** in 60% yield via a Reformatsky reaction with ethyl bromodifluoroacetate under refluxing conditions. A similar transformation has been reported with L-leucinal in which the more thermodynamically stable 3*R*,4*S* diastereomer was formed,[18] presumably via chelation control. When this transformation is carried out with L-alaninal, the smaller methyl substituent does not provide high stereoselectivity, and the difluoro alcohol **4** is formed as a 4:1 mixture of diastereomers. The major diastereomer was initially assigned as 3*R*,4*S* by analogy with the result from L-leucinal and proven correct through crystallographic analysis of a later synthetic intermediate (vide infra).

Compound **4** was hydrolyzed with a slight excess of hydroxide and then coupled to H_2N-Val-Ile-OMe using diisopropylcarbodiimide (DIPC) and 1-hydroxybenzotriazole (HOBT) to form **5** in 66% yield after chromatography. Peptide analogue **5** was then isolated as a single diastereomer after recrystallization from ether/pentane, the stereochemical assignment confirmed via X-ray crystallography. Compound **5** was N-deprotected with trifluoroacetic acid and coupled to BocHN-Val-Ile-OH to provide the penultimate product **6** in 58% yield. Alcohol **6** was oxidized with Dess−Martin periodinane reagent[24,25] in virtually quantitative yield to afford final compound **1**. The N- and C-terminal protecting groups were retained in the design of **1** to enhance permeability and localization to membranes in the cell-based assay employed.

The ^1H NMR spectra of difluoro ketone **1** illustrated the tendency of this compound to form a hydrate: addition of D_2O to a DMSO-d_6 solution of **1** resulted in dramatic decreases of the peaks corresponding to the ketone and increases in the peaks corresponding to the hydrate. In most cases, overlap between ketone and hydrate peaks was considerable, but the methyl peak of the pseudopeptide portion of the molecule proved diagnostic: this peak is a doublet (J = 7.0 Hz) at 1.25 ppm in the ketone form of **1** and a doublet (J = 6.8 Hz) at 1.08 ppm in the hydrate form.

Compound **1**, alcohol precursors **5** and **6**, as well as the oxidized counterpart of **5** (difluoro ketone **7**) were tested for their ability to selectively inhibit the production of Aβ_{40} and Aβ_{42} according to the protocol described fully in ref 10. Human embryonic kidney 293 cells were stably transfected with a plasmid construct carrying a double mutant form of human APP. This double mutation, K595N/M596L, occurs immediately prior to the β-secretase cleavage site and is found in certain Swedish families that are prone to FAD.[8] The double mutation leads to increased levels of both Aβ_{40} and Aβ_{42}[26] and thus allows for easier detection of these APP metabolites. The transfected cells were pulse-labeled with [^{35}S]-methionine for 2 h and then chased for 2 h in media with or without compound.[10] Secreted APP cleavage products in the conditioned media were then immunoprecipitated with specific antibodies, and the immunoprecipitates were analyzed by PAGE and fluorography.

At 200 μM, compounds **5**, **6**, and **7** did not decrease either Aβ or p3 production. In contrast, **1** completely blocked Aβ_{40} and Aβ_{42} as well as p3$_{40}$ and p3$_{42}$ formation at this concentration (Figure 2A). At lower concentrations (25−100 μM), **1** was surprisingly more effective against Aβ_{40} than Aβ_{42} production (Figure 2B). The 10 and 12 kDa C-terminal APP fragments (i.e., γ-secretase substrates) were dramatically increased in the presence of **1** (Figure 3A). However, this compound did not decrease production of α-APP$_s$ or β-APP$_s$ (Figure 3B), indicating that α- and β-secretase activities were not

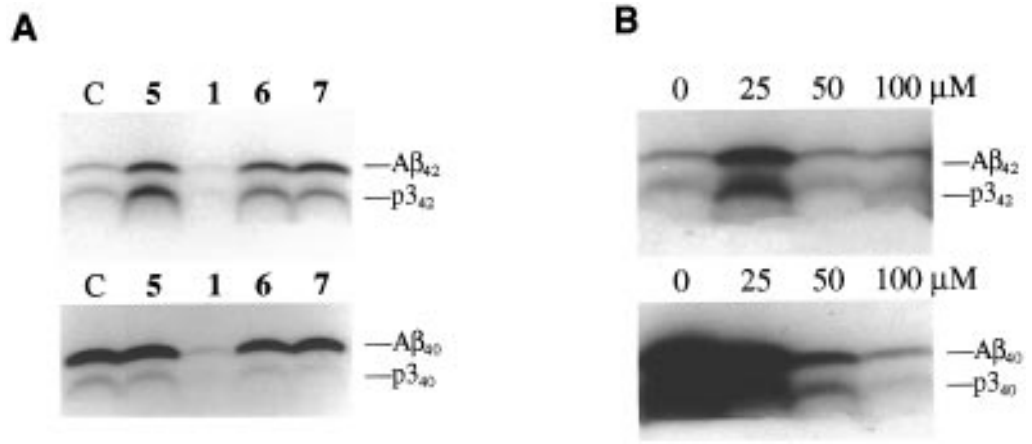


Figure 2. Fluorographic SDS−PAGE analysis of Aβ secretion from transfected cells. Panel A: Effect of **1** and **5**−**7** on Aβ and p3 production. Immunoprecipitation of media performed with antibody 21F12 (specific for Aβ_{42} and p3$_{42}$) or with 2G3 (specific for Aβ_{40} and p3$_{40}$) either in the absence (lane C) or presence of 200 μM compound. Panel B: Effect of **1** on Aβ and p3 production at lower concentrations. Antibodies and their specificities are described in ref 10 and citations therein.

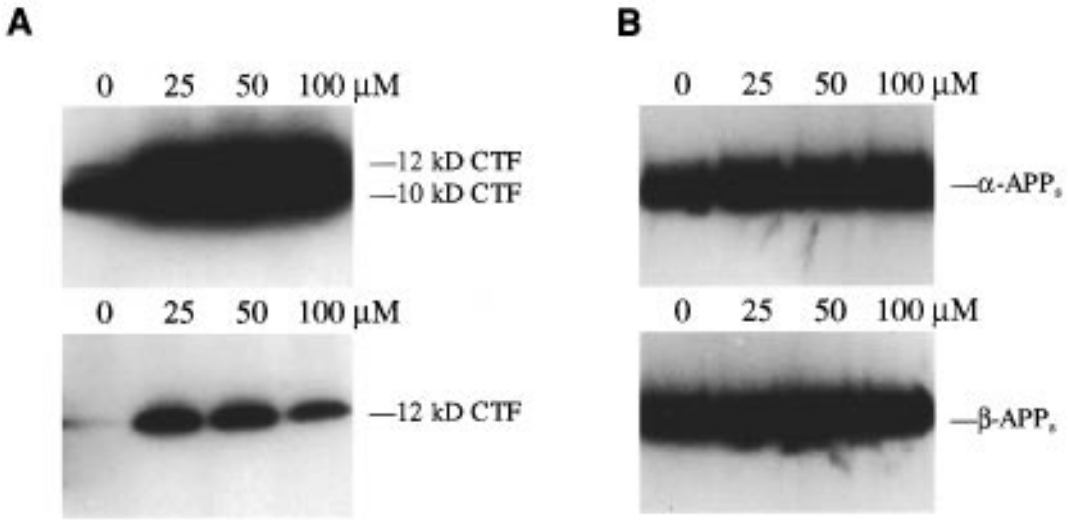


Figure 3. Fluorographic SDS−PAGE analysis of APP α- and β-secretase cleavage products from transfected cells. Panel A: Effect of **1** on APP 10 and 12 kDa C-terminal fragments. Immunoprecipitation performed with antibody C7 (specific for full-length APP and its C-terminal fragments) (upper panel) or with antibody 1282 (specific for the 12 kD C-terminal fragments) (lower panel). Panel B: Effect of **1** on APP$_s$. Immunoprecipitation performed with antibody 1736 (specific for α-APP$_s$) or with 192sw (specific for β-APP$_s$). Antibodies and their specificities are described in ref 10 and citations therein.

inhibited. Thus, this difluoro ketone peptidomimetic appears to selectively inhibit Aβ synthesis at the γ-secretase level. Furthermore, the differential effect of **1** on Aβ_{40} versus Aβ_{42} further supports the hypothesis that these Aβ variants result from different proteases. That the ketone functionality is critical for this activity is indicated by the lack of activity of the alcohol counterpart **6** (Figure 2A). Also, at least part of the N-terminal dipeptide is essential, because the truncated difluoro ketone **7** is likewise devoid of activity. Analysis of the effects of **1** on Aβ production 2 h after its removal from the medium indicated that this compound is a reversible inhibitor of γ-secretase activity: Aβ and p3 levels were higher than control, apparently due to the buildup of the precursor 10 kDa and 12 kDa C-terminal APP fragments prior to compound removal (data not shown).

Compound **1** was further tested for inhibition of Aβ production in APP-transfected Chinese hamster ovary cells using a rapid and quantitative double antibody ("sandwich") ELISA.[27] Total Aβ secreted from media was captured by monoclonal antibody 266 (specific for Aβ peptide residues 13−28) and detected via Aβ-specific biotinylated monoclonal antibody 3D6 (recognizing Aβ residues 1−5) followed by reaction with an alkaline phosphatase−avidin conjugate and incubation with fluorogenic substrate, 4-methylumbelliferyl phosphate. Using this assay, the IC$_{50}$ of **1** for total Aβ inhibition was 13 ± 5 μM (average of three independent experiments). The potency of **1** is thus comparable to the best reported peptide aldehyde (IC$_{50}$ ~5 μM).[11] Because the reported γ-secretase inhibitors were all originally designed as calpain inhibitors, we tested **1** as an inhibitor of this cysteine protease. Difluoro ketone **1** was quite ineffective against calpain II (Sigma) in a purified enzyme assay,[28] with an IC$_{50}$ of about 100 μM. By this criterion, **1** is a selective γ-secretase inhibitor in comparison with the reported peptide aldehyde inhibitors.

The γ-secretases, which catalyze the final step in Aβ biosynthesis from APP, are thought to be important new therapeutic targets.[13,14] Only four compounds have been previously reported as inhibitors of these pro-

teases: all are peptide aldehydes, and all are calpain inhibitors.[10−12] Identifying other types of inhibitors is critical for creating molecular probes for studying these proteases and for generating drug leads. We have demonstrated that a substrate-based difluoro ketone is a specific inhibitor of Aβ biosynthesis in APP-transfected cells. While this inhibition occurs at the γ-secretase level, it is unclear whether this compound and the reported peptide aldehydes interact directly with γ-secretases. Clarification of this issue will have to await the development of a purified enzyme assay. These results again support the existence of pharmacologically distinct γ-secretases cleaving at amino acids 40 and 42; thus far, selective inhibitors of γ(40)-secretase activity have been easier to identify. Currently, we are modifying prototype **1** in various ways to determine structural requirements for activity, to reverse the selectivity in favor of Aβ_{42} reduction, and to create new molecular tools for AD research.

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Supporting Information Available: Synthetic procedures and characterization for all compounds as well as crystallographic data on **5** (11 pages). Ordering information is given on any current masthead page.

References

(1) Glenner, G. G.; Wong, C. W. Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885−890.
(2) Kang, J.; Lemaire, H.; Unterbeck, A.; Salbaum, J. M.; Masters, C. L.; Grzeschik, K. H.; Multhaup, G.; Beyreuther, K.; Muller-Hill, B. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **1987**, *325*, 733−736.
(3) Hardy, J. The Alzheimer family of diseases: Many etiologies, one pathogenesis? *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2095−2097.
(4) Selkoe, D. J. Alzheimer's disease: Genotypes, phenotype, and treatments. *Science* **1997**, *275*, 630−631.
(5) Lamb, B. T. Presenilins, amyloid-β and Alzheimer's disease. *Nature Med.* **1997**, *3*, 28−29.
(6) Roher, A. E.; Lowenson, J. D.; Clarke, S.; Wolkow, C.; Wang, R.; Cotter, R. J.; Reardon, I.; Zurcher-Neely, H. A.; Heinrikson, R. L.; Ball, M. J.; Greenberg, B. D. Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.* **1993**, *268*, 3072−3083.
(7) Iwatsubo, T.; Odaka, A.; Suzuki, N.; Mizusawa, H.; Nukina, N.; Ihara, Y. Visualization of Aβ42(43) and Aβ40 in senile plaques with end-specific monoclonals: Evidence that an initially deposited species is Aβ42(43). *Neuron* **1994**, *13*, 45−52.
(8) Selkoe, D. J. Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease. *Annu. Rev. Cell Biol.* **1994**, *10*, 373−403.
(9) Roberts, S. B.; Ripellino, J. A.; Ingalis, K. M.; Robakis, N. K.; Felsenstein, K. M. Non-amyloidogenic cleavage of the β-amyloid precursor protein by an integral membrane metalloendopeptidase. *J. Biol. Chem.* **1994**, *269*, 3111−3116.
(10) Citron, M.; Diehl, T.; Gordon, G.; Biere, A. L.; Seubert, P.; Selkoe, D. J. Evidence that the 42- and 40-amino acid forms of amyloid β protein are generated from the β-amyloid precursor protein by different protease activities. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13170−13175.
(11) Klafki, H.-W.; Abramowski, D.; Swoboda, R.; Paganetti, P. A.; Staufenfiel, M. The carboxyl termini of β-amyloid peptides 1−40 and 1−42 are generated by distinct γ-secretase activities. *J. Biol. Chem.* **1996**, *271*, 28655−28659.
(12) Higaki, J.; Quon, D.; Zhong, Z.; Cordell, B. Inhibition of β-amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron* **1995**, *14*, 651−659.
(13) Schenk, D. B.; Rydel, R. E.; May, P.; Little, S.; Panetta, J.; Lieberburg, I.; Sinha, S. Therapeutic approaches related to amyloid-β peptide and Alzheimer's disease. *J. Med. Chem.* **1995**, *38*, 4141−4154.
(14) Brennan, M. Bringing back memories: Expanding understanding of Alzheimer's disease drives development of new drugs. *Chem. Eng. News* **1997**, Jan. 20, 29−35.
(15) Evin, G.; Cappai, R.; Li, Q.-X.; Culvenor, J. G.; Small, D. H.; Beyreuther, K.; Masters, C. L. Candidate γ-secretases in the generation of the carboxyl terminus of the Alzheimer's disease βA4 amyloid: Possible involvement of cathepsin D. *Biochemistry* **1995**, *34*, 14185−14192.
(16) Ladror, U. S.; Snyder, S. W.; Wang, G. T.; Holzman, T. F.; Krafft, G. A. Cleavage at the amino and carboxyl termini of Alzheimer's amyloid-β by cathepsin D. *J. Biol. Chem.* **1994**, *269*, 18422−18428.
(17) Saftig, P.; Peters, C.; von Figura, K.; Craessaerts, K.; Van Leuven, F.; De Strooper, B. Amyloidogenic processing of human amyloid precursor protein in hippocampal neurons devoid of cathepsin D. *J. Biol. Chem.* **1996**, *271*, 27241−27244.
(18) Thaisrivongs, S.; Pals, D. T.; Kati, W. M.; Turner, S. R.; Thomasco, L. M.; Watt, W. Design and synthesis of potent and specific renin inhibitors containing difluorostatine, difluorostatone, and related analogues. *J. Med. Chem.* **1986**, *29*, 2080−2087.
(19) Dreyer, G. B.; Metcalf, B. W.; Tomaszek, T. A., Jr.; Carr, T. J.; Chandler, A. C., III; Hyland, L.; Fakhoury, S. A.; Magaard, V. W.; Moore, M. L.; Strickler, J. E.; Debouck, C.; Meek, T. D. Inhibition of human immunodefiency virus 1 protease in vitro: Rational design of substrate analogue inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9752−9756.
(20) Parisi, M. F.; Abeles, R. H. Inhibition of chymotrypsin by fluorinated α-keto acid derivatives. *Biochemistry* **1992**, *31*, 9429−9435.
(21) Takahashi, L. H.; Radhakrishnan, R.; Rosenfield, R. E., Jr.; Meyer, E. F., Jr.; Trainor, D. A. Crystal structure of the covalent complex formed by a peptidyl α,α-difluoro-β-keto amide with porcine pancreatic elastase at 1.78-Å resolution. *J. Am. Chem. Soc.* **1989**, *111*, 3368−3374.
(22) Angliker, H.; Anagli, J.; Shaw, E. Inactivation of calpain by peptidyl fluoromethyl ketones. *J. Med. Chem.* **1992**, *35*, 216−220.
(23) Hamada, Y.; Shioiri, T. New methods and reagents in organic synthesis. 29. A practical method for the preparation of optically active N-protected α-amino aldehydes and peptide aldehydes. *Chem. Pharm. Bull.* **1982**, *30*, 1921−1924.
(24) Ireland, R. E.; Liu, L. An improved procedure for the preparation of the Dess-Martin periodinane. *J. Org. Chem.* **1993**, *58*, 2899.
(25) Linderman, R. J.; Graves, D. M. An efficient procedure for the oxidation of fluorinated carbinols. *Tetrahedron Lett.* **1987**, *28*, 4259−4262.
(26) Citron, M.; Oltersdorf, T.; Haass, C.; McConlogue, K.; Hung, A. Y.; Suebert, P.; Vigo- Pelfrey, C.; Lieberburg, I.; Selkoe, D. J. Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature* **1992**, *360*, 672−674.
(27) Johnson-Wood, K.; Lee, M.; Motter, R.; Hu, K.; Gordon, G.; Barbour, R.; Khan, K.; Gordon, M.; Tan, H.; Games, D.; Lieberburg, I.; Schenk, D.; Seubert, P.; McConlogue, L. Amyloid precursor protein processing and Aβ_{42} deposition in a transgenic mouse model Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1550−1555.
(28) Buroker-Kilgore, M.; Wang, K. K. W. A Coomassie Brilliant Blue G-250-based colorometric assay for measuring activity of calpain and other proteases. *Anal. Biochem.* **1993**, *208*, 387−392.
(29) Hamada, Y.; Shibata, M.; Sugiura, T.; Kato, S.; Shioiri, T. New methods and reagents in organic synthesis. 67. A general synthesis of derivatives of optically pure 2-(1-aminoalkyl)-thiazole-4-carboxylic acids. *J. Org. Chem.* **1987**, *52*, 1252−1255.

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