# Inhibitors Designed for Presenilin 1 by Means of Aspartic Acid Activation

Preliminary Communication

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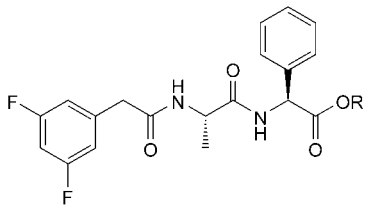
$\gamma$-Secretase, a multiprotein aspartic protease crucial to *Alzheimer*'s dementia, is not available for NMR experiments and has, so far, escaped crystallization. A positional scan of the aspartic protease by reactive probes may provide the necessary structural information for drug development. We describe here the synthesis of acid-labile compounds based on the known inhibitor DAPT (**1**), *e.g.*, the N-terminally functionalized diazo compound **4** or the C-terminally acid-labile (cyclopropylmethyl)ester **11**, which were designed to react in the specific acidic active-site environment of the aspartic protease presenilin 1. The acid-labile DAPT analogues **11**–**13**, indeed, displayed strong inhibition in a cell-free $\gamma$-secretase assay.

**Introduction.** – 'Alzheimer'*s disease* (AD) *is a devastating illness that robs humans of their ability to remember, to think and to understand, all the things we cherish most about being human*' (*P. F. Chapman*). The observed loss of neurons in AD is accompanied by the formation of plaques composed primarily of the neurotoxic amyloid $\beta$-peptide (A$\beta$). A rational approach to a successful, causal therapy is based on the detailed understanding of A$\beta$ formation, deposition, and the inflammatory consequences [1]. Decisive functions were assigned to the aspartic protease $\beta$-secretase (BACE) and to the $\gamma$-secretase and its catalytic subunits, the presenilins (PS), as the responsible proteases that generate A$\beta$ from the $\beta$-amyloid precursor protein (APP).

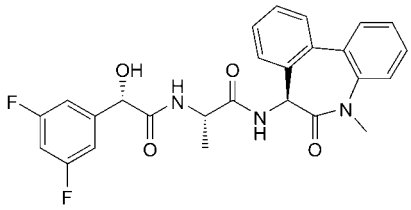
The primary goal of our programme is to establish the structures of presenilins 1 and 2 as aspartic proteases by characterization of the active complexes and their differentiation from the *Notch* pathway [2]. This requires selective inhibition of presenilins, preferentially by irreversible, nonpeptidic, cell-penetrating small molecules. The structure/activity relationship of the peptidic inhibitors DAPT (LY374973; **1**)[1]) and LY411575 (**2**), both originally developed at *Eli Lilly/Elan* [3], which are known inhibitors of $\gamma$-secretase, provides a good starting point for such endeavors. Although DAPT is a competitive inhibitor of the known transition-state analogue **3** (L-685,458) [4][5], the orientation of DAPT within the active site is still unknown. Unfortunately, $\gamma$-secretase, which consists of presenilin and at least three further proteins [6], is not available for NMR experiments. Furthermore, $\gamma$-secretase has, so far, escaped crystallization. However, a positional scan by reactive probes may fill this

[1]) DAPT is an acronym for '*N*-[*N*-(3,5-**di**fluorophenylacetyl)-L-**a**lanyl]-*S*-**p**henylglycine ***t****ert*-butyl ester' (=*tert*-butyl (2*S*)-({*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl}amino)(phenyl)acetate).

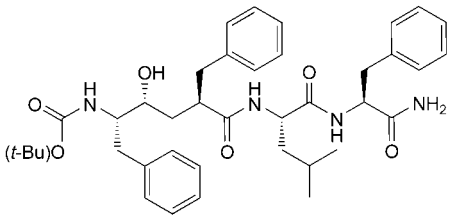
gap in structural information. Thus, we describe here reactive compounds designed to recognize the specific acidic environment of the aspartic protease presenilin 1.



**1** R = *t*-Bu (DAPT; LY374973)
**1a** R = H



**2** (LY411575)



**3** (L-685,458; *Merck*)

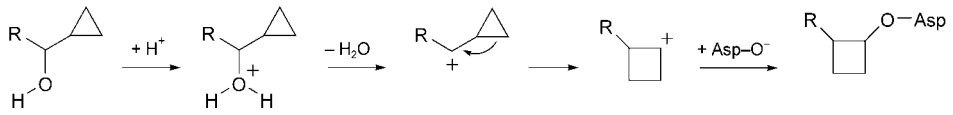
**Results and Discussion.** – *General Considerations.* In our first approach, we have chosen to study diazo compounds (see below), although *diazo ketones* are more commonly used as inhibitors for cysteine (Cys) proteases. In the latter case, the catalytically active SH (mercapto) function undergoes a nucleophilic substitution with a diazo ketone, which results in irreversible alkylation. Despite their well-documented nucleophilicity [7–10], diazo compounds are rarely used as peptidic nucleophiles. However, the protonation of a diazotized moiety of the type $N_2$–CHR by an aspartic acid within the active site should deliver a highly reactive $RCH_2^+$ carbocation after loss of $N_2$, which then could alkylate important catalytic functions.

We embarked on a programme directed at creating such reactive cations, especially at the N-terminus of DAPT, because the configuration of the additional OH group in **2** is important for inhibition ($IC_{50} < 1$ nM [11]), an observation that points at a crucial hydrophilic interaction at the active site. We further reasoned that this OH group may interact with the Asp residues of the active site. However, since the precise orientation of the DAPT molecule in the active site is not known, we also designed reactive probes for the C-terminus. And, in case the C-terminus of DAPT is, indeed, 'sandwiched' between the two Asp residues, we wanted these chemical probes to tell us more about their environment.

Although the pH dependence and the individual pH-optima of $\beta$-secretase and the presenilins are well-documented, the local pH usually remains unknown. This is due to the peculiarities of the aspartic protease environment: the protease provides a nonaqueous envelope for the distinct assembly of the substrate, a COOH and a $COO^-$ function, and coordinated $H_2O$. This arrangement defies pH measurement and pH definition. However, this elusive pH may be replaced by a relative pH derived *via* pH-

sensitive, reactive probes. The stability of such probes in aqueous solution at different pH values can be related to the cleavage by presenilin to obtain the relative pH at the active site. Therefore, an $H^+$-catalyzed ring enlargement according to *Scheme 1* (cyclopropyl → cyclobutyl) was implemented into DAPT-analogous structures. This rearrangement relays the cation and can be terminated by the irreversible alkylation of aspartate (*Scheme 1*).

Scheme 1. *Rearrangement of Protonated Cyclopropylmethanols to Cyclobutyl Cations as a Semi-Quantitative Test for the Acid Sensitivity of Potential Presenilin Inhibitors*




*Synthesis.* The N-terminally diazotized DAPT analogue **4** was derived from Z-protected glycinylalanine (**5**) and phenylglycine *tert*-butyl ester (**6**), which were coupled by the EDAC/HOBt method to afford **7** (*Scheme 2*)[2]). The Z group was removed reductively, and the free base **8** was purified. Then, the diazotization to **4** was accomplished by careful reaction of **8** with (*t*-Bu)O–N=O in the presence of 0.25 equiv. of AcOH in refluxing $CHCl_3$ for 20 min. Thereby, excess AcOH or residual HCl led to reduced yields due to formation of the acetate-derived depsipeptide **9** and the chlorinated compound **10**, respecitvely, as by-products. Fortunately, AcOEt or DMSO solutions of **4** were stable for more than a week at room temperature, which was sufficient to perform biochemical assays.

We next prepared a series of C-terminally functionalized DAPT analogues. Exposure of DAPT (**1**) [3][4] to trifluoroacetic acid (TFA) in $CH_2Cl_2$ afforded the corresponding free acid, *i.e.*, (2*S*)-({*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl}amino)-(phenyl)acetic acid (**1a**), which was readily coupled to various cyclopropyl-substituted, acid-labile alcohols by means of the EDAC/HOBt method (see *General Procedure* in the *Exper. Part*). Compounds **11**–**15** were thus obtained in high purity (> 95%)[3]), but, unfortunately, mostly in moderate yields only (23, 28, 38, and 73% for **11**, **12**, **13**, and **15**, respectively). Alternative coupling reagents were tested, *e.g.*, PyBop and PyBroP[4]), but did not give rise to better yields.

Since the (cyclopropylmethyl)amide **14** was isolated in trace amounts only, an alternative synthesis was envisaged. Boc-Phg-Gly-OH[5]) was condensed with 1-cyclopropylmethanamine in the presence of PyBroP, which afforded Boc-Phg-NHR (R = cyclopropylmethyl) in 97% yield. Quantitative Boc deprotection with 10% TFA in $CH_2Cl_2$, followed by EDAC/HOBt condensation of the resulting free acid with Z-Ala-OH, then afforded **16** in 28% yield. Finally, reductive removal of the Z group,

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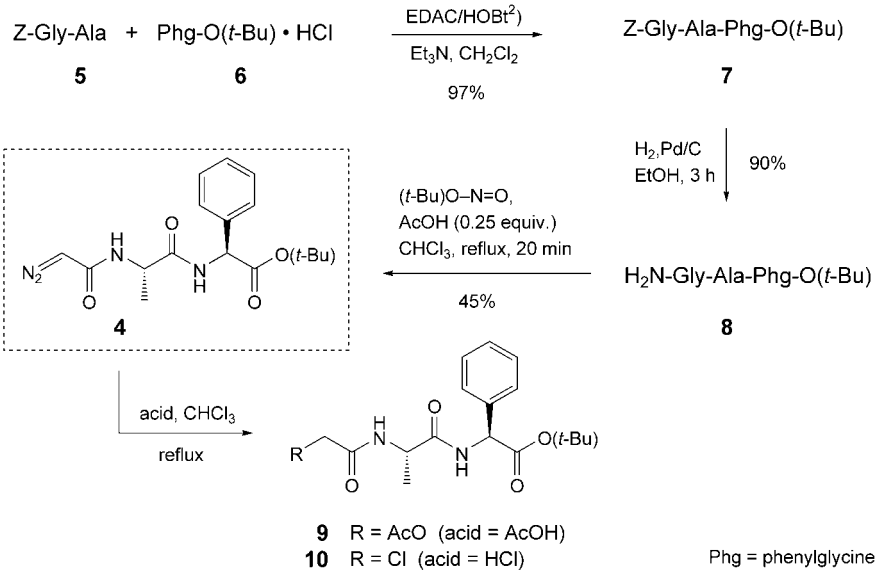
[2]) Abbreviations: Z = (benzyloxy)carbonyl; EDAC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HOBt = 1-hydroxy-1*H*-1,2,3-benzotriazole.

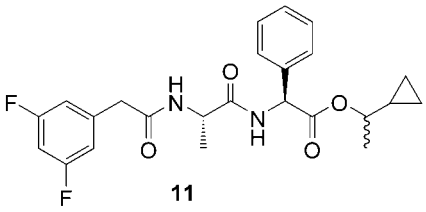
[3]) No efforts were made at this preliminary stage to separate the diastereoisomers of compounds **11** and **15**.

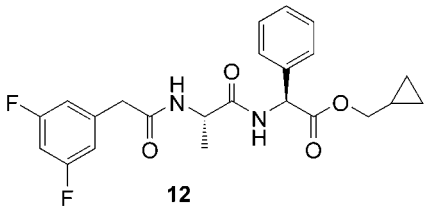
[4]) PyBop and PyBroP are '(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate' and 'bromotrispyrrolidinophosphonium hexafluorophosphate', resp.

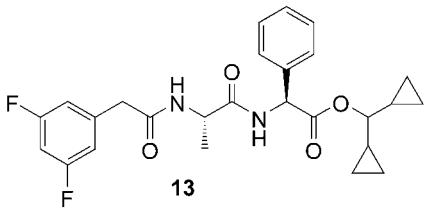
[5]) Phg = phenylglycine.

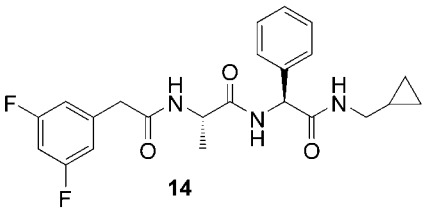
Scheme 2. *Synthesis of the Diazotized DAPT Analog* **4**

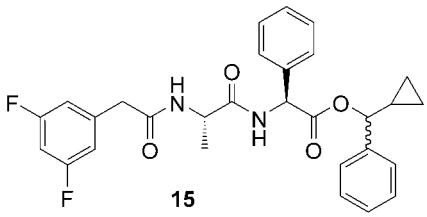


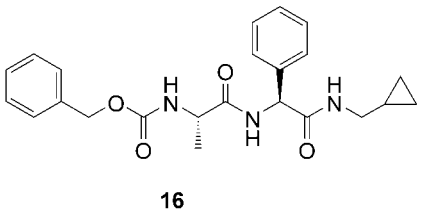


**11**


**12**


**13**


**14**


**15**


**16**

followed by amide formation of the resulting free amine with (3,5-difluorophenyl)-acetic acid under EDAC/HOBt conditions, afforded **14** in 78% yield.

*Inhibitor Properties.* The lability of the cyclopropylmethyl ester functions of compounds **11**–**15** towards acid-catalyzed rearrangement (see *Scheme 1*) was found to decrease in the order **13**, **15** > **11** > **12** ≫ **14**, **16**. This was confirmed by cleavage experiments in $CH_2Cl_2$ in the presence of varying concentrations of AcOH and TFA. At a concentration of 10% TFA, compounds **11**–**14** were cleaved within 30 minutes, whereas the control compound **16** was found to be stable under these conditions.

All compounds (except for **14**) were evaluated by means of a previously published method [12] for their ability to inhibit $\gamma$-secretase. Preliminary experiments indicated that some of these compounds are potent $\gamma$-secretase inhibitors, with inhibition values in the nano- to micromolar range. The diazo compound **4** and the (cyclopropylmethyl)-amide **16** were inactive up to concentrations of 100 μM; compounds **11**–**13** were active in the low nanomolar range (*ca.* 10 nM), but neither reliable $IC_{50}$ values nor a conclusive ranking has been established yet. These problems seem to arise from a time-dependent inhibition mode, possibly due to irreversible alkylation or simple cleavage to the lesser-active acids. Currently, compounds **11**–**13** are being investigated mass spectroscopically to confirm the anticipated irreversible binding to the $\gamma$-secretase complex.

We thank *Christian Haass* and *Harald Steiner* for access to preliminary data, and the *Fonds der Chemischen Industrie* and the *Deutsche Forschungsgemeinschaft* (*DFG*) for generous support of this work.

## Experimental Part

*General.* IR Spectra: in $cm^{-1}$. $^1H$- and $^{13}C$-NMR Spectra: recorded at 300 and 75 MHz, resp., in ($D_6$)DMSO opr $CDCl_3$ at r.t.; chemical shifts $\delta$ in ppm. EI-MS (70 eV): in *m*/*z* (rel. intensity in percent).

*{*N*-[(Benzyloxy)carbonyl]glycyl}*-L-*alanine*-N-*[(1*S*)-2*-tert-*butoxy-2-oxo-1-phenylethyl]amide* (Z-Gly-Ala-Phg-O(*t*-Bu); **7**). EDAC (192 mg, 1 mmol) and HOBt hydrate[2]) (184 mg, 1.2 mmol) were added to a soln. of Z-Gly-Ala-OH (**5**; 280 mg, 1 mmol) in $CH_2Cl_2$ (10 ml), and the resulting mixture was stirred at r.t. for 10 min. Then, Phg-O(*t*-Bu)·HCl (**6**; 219 mg, 0.9 mmol) and $Et_3N$ (152 mg, 1.5 mmol) were added, and the mixture was stirred for 24 h. $CH_2Cl_2$ (20 ml) was added, and the soln. was washed with 0.1N aq. HCl soln. (3 × 30 ml), 0.1N aq. NaOH soln. (3 × 30 ml), and brine (30 ml), and dried ($Na_2SO_4$). The solvent was removed *in vacuo* to yield **7** as a colorless solid (412 mg, 97 %), which required no further purification. $^1H$-NMR (300 MHz, ($D_6$)DMSO): 8.65 (*d*, NH); 8.00 (*d*, NH); 7.42–7.31 (*m*, 10 arom. H); 5.21 (*d*, CH); 5.02 (*s*, $CH_2$); 4.49–4.44 (*m*, CH); 3.64–3.58 (*m*, $CH_2$); 1.35 (*s*, *t*-Bu); 1.24 (*d*, Me).

*Glycyl*-L-*alanine*-N-*[(1*S*)-2*-tert-*butoxy-2-oxo-1-phenylethyl]amide* (**8**). Compound **7** (412 mg, 0.88 mmol) was dissolved in EtOH (15 ml) and treated with Pd on activated carbon (150 mg). The flask was evacuated, filled with $H_2$, and the mixture was stirred at r.t. for 150 min (TLC control). The suspension was filtered, and the solvent was removed *in vacuo* to yield **8** as a colorless solid (302 mg, 90%), which required no further purification. $^1H$-NMR (300 MHz, ($D_6$)-DMSO): 8.76 (*d*, NH); 8.10 (*d*, NH); 7.44–7.40 (*m*, 5 arom. H); 5.28 (*d*, CH); 4.55–4.51 (*m*, CH); 3.12 (*s*, $CH_2$); 1.40 (*s*, *t*-Bu); 1.31 (*d*, Me).

N-*(Diazoacetyl)*-L-*alanine*-N-*[(1*S*)-2*-tert-*butoxy-2-oxo-1-phenylethyl]amide* (**4**). Compound **8** (302 mg, 0.9 mmol) was dissolved in $CHCl_3$ (5 ml) and treated with *tert*-butylnitrite (111 mg, 1.08 mmol) and AcOH (13.5 mg, 0.22 mmol). The mixture was heated to reflux for *ca.* 20 min. Then, $CHCl_3$ (10 ml) was added, and the soln. was washed with 0.1N aq. HCl soln. (3 × 20 ml), 0.1N aq. NaOH soln. (3 × 20 ml), and brine (20 ml), dried ($Na_2SO_4$), and evaporated *in vacuo* to yield crude **4** (264 mg) as a yellow oil, which was crystallized from $Et_2O$ (140 mg, 45%). Pale yellow solid. M.p. 160–162° ($Et_2O$; dec.). IR ($CHCl_3$): 2105 ($N_2C$). $^1H$-NMR (300 MHz, ($D_6$)DMSO): 8.45 (*d*, NH); 7.66 (*d*, NH); 7.26–7.20 (*m*, 5 arom. H); 5.22 (*d*, CH); 5.07 (*d*, CH); 4.38–4.33 (*m*, CH); 1.21 (*s*, *t*-Bu); 1.1 (*d*, Me). $^{13}C$-NMR (75 MHz, ($D_6$)DMSO): 172.3, 169.3, 164.5 (3 C=O); 136.3, 128.5, 128.1, 127. 6 (Ph); 81.0 ($Me_3C$); 57.0, 47.8, 46.1, 27.4 ($Me_3C$), 18.7 (Me).

*General Procedure* (*GP*) *for EDAC/HOBt Couplings*[2]). A soln. of the N-terminally protected amino acid or peptide[6]) (2.9 mmol, 1 equiv.), EDAC (1 equiv.), and HOBt (1 equiv.) in anh. $CH_2Cl_2$ (20 ml) was stirred for 10 min at r.t. Then, $Et_3N$ (0.693 ml, 5 mmol, 1.7 equiv.) and either a *C*-terminally protected amino acid or (for ester formation) the corresponding alcohol (1 equiv.) were added. The mixture was stirred for *ca.* 22 h at r.t., extracted with 0.1N aq. NaOH soln. (3×), dried ($Na_2SO_4$), and evaporated *in vacuo*. The resulting colorless oil was purified by HPLC ($SiO_2$; hexane/AcOEt 1:1).

*1-Cyclopropylethyl (2*S*)-({*N*-*[(3,5-Difluorophenyl)acetyl]*-L-*alanyl}amino)(phenyl)acetate* (**11**). Prepared in 23% yield (1:1 diastereoisomer mixture) according to the *GP*. HPLC: $t_R$ 5.293 min. $^1$H-NMR (300 MHz, $CDCl_3$): 7.47–7.19 (*m*, 5 arom H); 6.76–6.63 (*m*, 3 arom. H); 6.15 (*d*, NH); 5.41 (*d*, CH); 4.52–4.48 (*m*, CH); 4.14–4.05 (*m*, CH); 3.48 (*d*, $CH_2$); 1.32 (*d*, Me); 1.25 (*d*, Me); 1.16–1.09 (*m*, 2 $CH_2$); 0.85–0.79 (*m*, CH). EI-MS: 444.5 (22, $M^+$), 331 (26), 127 (26), 106 (100), 44 (63).

*Cyclopropylmethyl (2*S*)-({*N*-*[(3,5-Difluorophenyl)acetyl]*-L-*alanyl}amino)(phenyl)acetate* (**12**). Prepared in 28% yield according to the *GP*. HPLC: $t_R$ 5.292 min. $^1$H-NMR (300 MHz, $CDCl_3$): 7.36–7.26 (*m*, 5 arom. H); 7.19 (*d*, NH); 6.84–6.69 (*m*, 3 arom. H); 6.34 (*d*, NH); 5.49 (*d*, CH); 4.58–4.54 (*m*, $CH_2$); 4.21–4.12 (*m*, CH); 3.46 (*d*, $CH_2$); 1.39 (*d*, Me); 1.30–1.18 (*m*, 2 $CH_2$); 0.88–0.85 (*m*, CH). EI-MS: 430.5 (31, $M^+$), 331 (42), 127 (32), 106 (100), 44 (87).

*Dicyclopropylmethyl (2*S*)-({*N*-*[(3,5-Difluorophenyl)acetyl]*-L-*alanyl}amino)(phenyl)acetate* (**13**). Prepared in 38% yield according to the *GP*. HPLC: $t_R$ 5.206 min. $^1$H-NMR (300 MHz, $CDCl_3$): 7.41–7.23 (5 arom. H); 6.81–6.70 (3 arom. H); 5.52 (*d*, CH); 5.13 (*d*, CH), 4.19–4.12 (*m*, CH); 3.46 (*d*, $CH_2$); 1.71 (*d*, Me); 1.38–1.26 (*m*, $CH_2$); 1.01 (*d*, $CH_2$); 0.88–0.85 (*m*, CH); 0.54–0.48 (*m*, $CH_2$); 0.31–0.27 (*m*, $CH_2$). EI-MS: 471.0 (2, $M^+$), 453 (22), 311 (20), 44 (84), 91 (100).

N-*{(1*S*)-2-[(Cyclopropylmethyl)amino]-2-oxo-1-phenylethyl}*-L-*alanine*-N-*[(3,5-difluorophenyl)acetyl]-amide* (**14**). Obtained in trace amounts only *via* the *GP*. However, *via* the alternative route, starting from Boc-Phg-Gly-OH, described in the text, and later on following the EDAC/HOBt protocol, 77% of 14 were obtained. EI-MS: 430 (100, $M^+$), 412 (15), 365 (45), 359 (100).

*Cyclopropyl(phenyl)methyl (2*S*)-({*N*-*[(3,5-Difluorophenyl)acetyl]*-L-*alanyl}amino)(phenyl)acetate* (**15**). Prepared in 73% yield (1:1 diastereoisomer mixture) according to the *GP*. HPLC: $t_R$ 5.30 min. EI-MS: 507.6 (9, $M^+$); 496 (100), 410 (47), 290 (25), 107 (48), 91 (98).

N-*(Benzyloxycarbonyl)*-L-*alanine*-N-*({(1*S*)-2-[(cyclopropylmethyl)amino]-2-oxo-1-phenyl}ethyl)amide* (**16**). Prepared in 45% yield according to the *GP*. HPLC: $t_R$ 5.24 min. $^1$H-NMR (300 MHz, $CDCl_3$): 7.21–7.10 (10 arom. H); 6.09 (*d*, NH); 5.39 (*d*, CH); 5.02 (*s*, $CH_2$); 4.19–4.08 (*m*, CH); 3.04–2.90 (*m*, $CH_2$); 1.29 (*d*, Me); 0.74–0.72 (*m*, CH); 0.32–0.29 (*m*, $CH_2$); 0.05–0.01 (*m*, $CH_2$). EI-MS: 409.0 (63, $M^+$), 384 (84), 338 (100).

*Bioassay.* γ-Secretase activity was monitored by *de novo* production of AICD (= APP intracellular domain) *in vitro*. Membrane fractions of HEK-293 cells, stably transfected with Swedish mutant APP, were prepared as described in [5], except that membranes were directly resuspended in assay buffer (150 mM sodium citrate, pH 6.4; 1× protease inhibitors (Complete, *Roche*)) without any further washing step. To allow generation of AICD, samples were incubated at 37° for 2 h. Control samples were kept on ice. Where indicated, γ-secretase inhibitors were added to the samples. After termination of the assay reactions on ice, samples were subjected to ultracentrifugation (1 h at 4°, $10^5 g$). The soluble fractions were subjected to SDS-PAGE on 10–20% *Tris-Tricine* gels (*Invitrogen*), and AICD was analyzed by immuno-blotting with antibody 6687, using enhanced chemiluminescence (ECL; *Amersham Pharmacia Biotech*).

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6) For the synthesis of **11**–**15**, peptide acid **1a** (= (2*S*)-({*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl}amino)-(phenyl)acetic acid) was used.

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