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# Enhanced delivery of γ-secretase inhibitor DAPT into the brain *via* an ascorbic acid mediated strategy

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Inhibition of γ-secretase, one of the enzymes responsible for the cleavage of the amyloid precursor protein (APP) to produce pathogenic Aβ peptides, is an attractive approach for the treatment of Alzheimer's disease. We designed a γ-secretase inhibitor bearing an ascorbic acid moiety which allows a specific delivery of the drug to the brain. Through, on the one hand, Aβ peptide production measurements by specific *in vitro* assays (γ-secretase cell free assay and cell based assay on HEK 293 APP transfected cells) and on the other hand through pharmacokinetic studies on animal models, the new inhibitor shows a good pharmacokinetic profile as well as a potent γ-secretase inhibitory activity *in vitro*. From the obtained results, it is expected that drug **2** will be mainly delivered to the CNS with a low diffusion in the peripheral tissues. Consequently the side effects of this γ-secretase inhibitor on the immune cells could be reduced.

## Introduction

One of the main problems for the current therapies of brain diseases is the distribution of drugs to the central nervous system (CNS). In the past few years, most of the attempts to overcome these difficulties in crossing the blood brain barrier (BBB) have targeted the improvement of the lipophilic properties through the design of prodrugs. These prodrugs involve the conjugation of an active drug and a carrier *via* the formation of a cleavable bond in order to enhance the penetration of the drug through the hematoencephalic barrier.[1,2] Once passed into the CNS, the prodrug releases the active drug in the close neighbourhood of its biological target.

Other promising strategies have been recently investigated, such as: liposomes exploiting receptor mediated transcytosis system;[3] nanoparticles;[4] implantation of either genetically engineered cells secreting a drug or a polymeric matrix containing the drug;[5] neuroproteomic approaches and gene therapy;[6] chimeric peptide technology, where a non-transportable drug is conjugated to a BBB transport vector;[7] or chemical drug delivery systems which represent a systematic way of targeting active biological molecules to specific sites or organs based on predictable enzymatic activation.[8]

One of these later systems called redox chemical delivery system (RCDS) involves the linking of a drug to a lipophilic dihydropyridine carrier leading to a complex that readily permeates the BBB through a passive diffusion phenomenon. Once trapped inside the brain, the dihydropyridine moiety is enzymatically oxidized into the corresponding ionic pyridinium salt increasing the intracranial concentration of a variety of compounds, including neurotransmitters, while its ability to cross the BBB is very limited.[9,10]

Another chemical drug delivery system uses the biochemical properties of ascorbic acid (AA). Indeed, it has been observed that ascorbic acid which does not penetrate the BBB, is present in the brain when given orally.[11] This transportation of AA is based on its *in vivo* reversible metabolic conversion into its oxidized form dehydroascorbic acid (DHAA) which is rapidly transported into the CNS. This oxidized form is then reduced into AA and retained in the brain enhancing its CNS levels.[12,13] Several ways have been proposed to explain the AA entry into the CNS. Among them, the facilitated transport of AA previously metabolized into its oxidized form DHAA *via* glucose transporter GLUT1[12] or $Na^+$-dependent ascorbate transporter SVCT2[14,15] has been proposed.

Once in the brain, DHAA is hydrolysed[12] or reduced into AA which is accumulated into the cells to exert its biological activity.[16] It can then protect brain tissues in a wide variety of CNS pathologies[17] and eventually it may protect against cognitive impairment and reduce the risk for development of Alzheimer's disease (AD).[18] Selected derivatives bearing ascorbic acid as carrier on their scaffold could then be seen as potential AD drug candidates.

In the past years, important progress has been made in the understanding of the pathogenic mechanism of AD. The "amyloid hypothesis" has become the dominant theory in this field. It is now believed that Aβ accumulation in plaques or as partial soluble filaments initiates a pathological cascade leading to tangle formation,[19] neuronal dysfunction and possibly inflammation and oxidative damage, with neurodegeneration and dementia as final outcome.[20] Two enzymatic activities known as β- and γ-secretases which cleave the Aβ precursor protein (APP) to yield Aβ peptide are now the potential therapeutic targets. It is believed that lowering Aβ production will decrease the formation rate of senile plaques in AD patients.[21] Numerous very potent inhibitors of γ-secretase have been described so far. *N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-(*S*)-phenylglycine *tert*-butyl ester (DAPT) **1** (Fig. 1) has been shown to dose-dependently reduce Aβ levels in the brains of two different strains of APP-transgenic mice after only a single dose injection.[22,23] Nevertheless, various studies suggest caution in the testing of γ-secretase inhibitors in humans and unwanted

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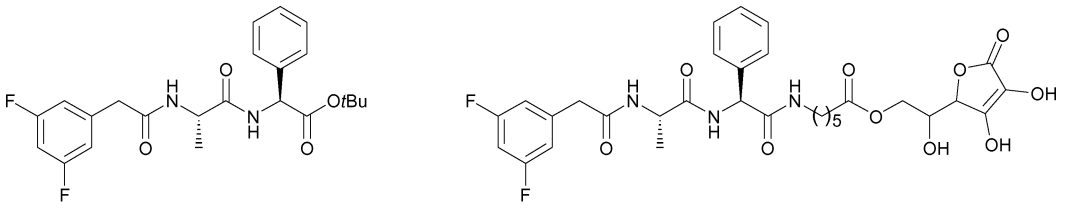
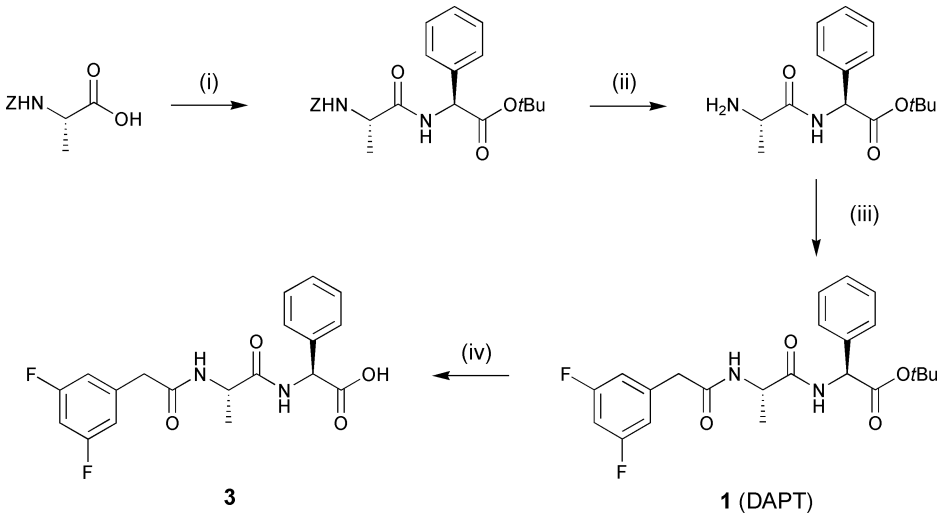



**Fig. 1**




**Scheme 1** Conditions and reagents: (i) phenylglycine *tert*-butyl ester hydrochloride, BOP, DIEA, $CH_2Cl_2$, rt; quantitative; (ii) $H_2$, $Pd(OH)_2/C$, MeOH, rt; 96%; (iii) 3,5-difluorophenylacetic acid, BOP, DIEA, $CH_2Cl_2$, rt; 88%; (iv) TFA, $CH_2Cl_2$, rt; 87%.

side-effects could be observed during long-term treatment in humans.[24,25] A possible way to overcome or at least to minimize these rather severe side-effects is to find drugs that are more rapidly and more effectively delivered to the CNS in order to reduce the concentration of the drug in the peripheral tissues by lowering the amount of drug to be administrated *in vivo*.

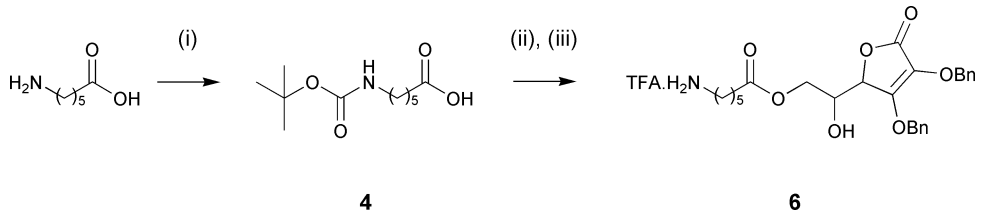
In this paper, we report the design, synthesis and biological evaluation of a new DAPT analog pseudopeptide derivative with γ-secretase inhibitory properties. As shown in Fig. 1, an ascorbic acid moiety was introduced on the scaffold of the target molecule in order to improve its BBB permeation. This derivative was assayed as γ-secretase inhibitor in cell free and cell based assays and their brain specific *in vivo* delivery was investigated.

## Chemical synthesis of the inhibitors

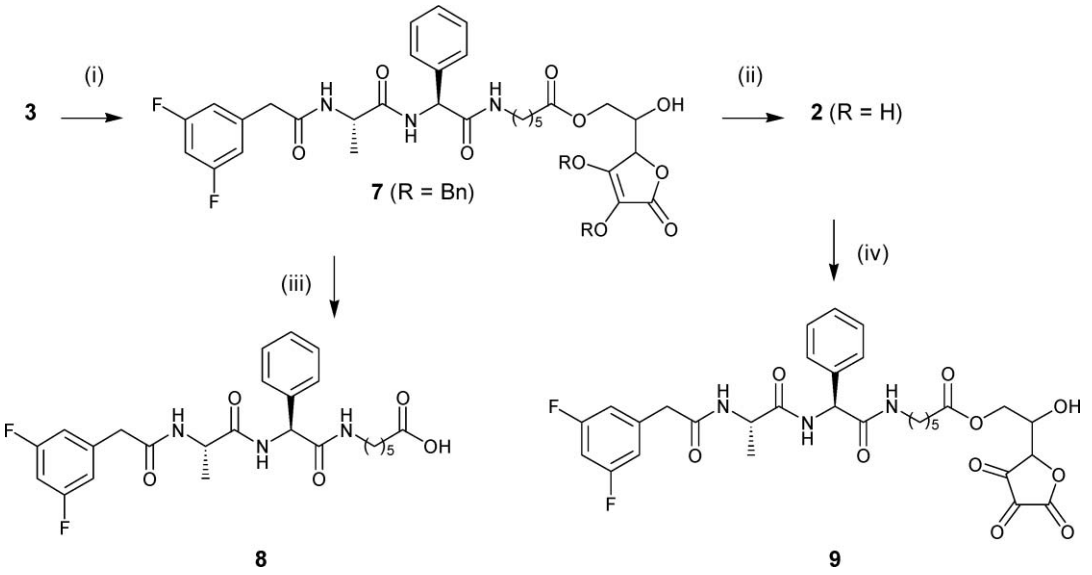
The target molecule is the result of a multi step synthetic sequence involving on the one hand a DAPT-based peptide-like derivative and on the other hand an ascorbic acid moiety, both entities being linked *via* a linear ω-amino acid spacer. Both moieties were synthesized separately and subsequently coupled in order to isolate the target ascorbic acid derivative **2**. DAPT **1** and DAPT-based peptide-like derivative **3** were synthesized as summarized in Scheme 1. DAPT **1** was easily prepared through a three-step classical solution-phase peptide synthesis involving a coupling–deprotection–coupling synthetic sequence. The corresponding free acid **3** was isolated in high yield and purity by removal of the DAPT *tert*-butyl ester protecting group.

The second synthon was obtained according to the synthetic sequence described in Scheme 2. 6-Aminocaproic acid was selected as linear ω-amino acid spacer. It was first ω-*N*-protected before esterification of the terminal carboxylic acid function. This esterification reaction was performed using 2,3-di-*O*-benzyl-L-ascorbic acid **5** as primary alcohol entity. This alcohol **5** was itself synthesized according to a well-documented three-step synthetic sequence.[15,26] The esterification reaction was performed by using BOP as coupling agent. The resulting ascorbate derivative was isolated in a moderate yield (57%) quite similar to those described by using DCC–DMAP as coupling system for the synthesis of some ascorbate derivatives.[15] The *N*-Boc protecting group was removed in acidic conditions and the resulting TFA salt **6** was quantitatively isolated. This TFA salt was then acylated by the previously described acid derivative **3** as summarized in Scheme 3. The use of BOP as coupling agent allowed isolation of the desired acylated compound **7** in a good yield (73%). Finally, the target molecule **2** was obtained after removal of the benzyl protecting groups on the ascorbic acid moiety by catalytic hydrogenolysis.

In order to compare the influence of ascorbic acid as transporter through the BBB, we synthesized the non ascorbic




**Scheme 2** Conditions and reagents: (i) $Boc_2O$, 1 M NaOH, dioxane–$H_2O$ (v/v, 2 : 1), quantitative; (ii) 2,3-di-*O*-benzyl-L-ascorbic acid **5**,[15,26] BOP, DIEA, $CH_2Cl_2$, rt; 57%; (iii) TFA, $CH_2Cl_2$, rt; quantitative.




**Scheme 3** Conditions and reagents: (i) **6**, BOP, DIEA, $CH_2Cl_2$, rt; 73%; (ii) $H_2$, $Pd(OH)_2/C$, MeOH, rt; 90%; (iii) LiOH, $CaCl_2$, iPrOH–$H_2O$ (v/v, 7 : 3), rt; 58%; (iv) $H_2O_2$, MeOH, rt.

analog of the target molecule **2**. This analog **8** was simply obtained through a smooth hydrolysis in basic conditions of the ester derivative **7**.[27]

### *In vitro* and *in vivo* biological studies

**Biological stability.** Ascorbic acid was introduced to the scaffold of our target molecule **2** *via* a hydrolysable ester bond. Prior to any *in vivo* study, it was of interest to investigate the stability of the synthesized derivative in various biological conditions. This study was performed on both rat liver and brain extracts, by using the chromophoric properties of the ascorbic acid residue ($\lambda_{max} = 266$ nm, $\varepsilon \approx 10\,000$ L mol$^{-1}$ cm$^{-1}$ ($H_2O$)) while the non ascorbic carboxylic derivative **8** presents no absorbance at the same wavelength. UV analysis was used to monitor the hydrolysis rate of the target molecule. This study was performed as described in the Experimental section. Compound **2** was first incubated in both biological media, separated from the crude mixture, purified and then quantified by comparison with a calibration curve. It was found that only 20% of the starting material survived the biological processes after 3.5 hours of incubation at 37 °C in rat liver extract, while after 15 hours of incubation in rat brain extract, more than 50% of **2** was recovered indicating that this compound is quite resistant to hydrolysis and other biological modifications.

It is necessary to take these results into account and more precisely the low stability of **2** into liver extract as far as the digestive track and the plasma stream are the main causes of drug degradation before CNS penetration. It is well known that ascorbic acid transport within the brain is a fast phenomenon in mice. Indeed, ascorbic acid derivative of nipecotic acid increased the latency of induced convulsions in mice.[15] This phenomenon was witnessed only 15 minutes after *i. p.* injection of the ascorbic prodrug. Similarly, one can imagine that the entry of derivative **2** into the brain will require only few minutes if injected in the blood stream. We determined that during the two first hours of incubation in rat liver extract only a small amount (5%) of **2** disappeared, indicating that compound **2** could be a good candidate for *in vivo* BBB permeation study.

***In vitro* γ-secretase cell free assay.** Compounds **2** and **8** were first tested for their ability to block specifically *in vitro* γ-secretase and compared to DAPT **1**. This *in vitro* assay was performed using solubilized γ-secretase prepared as described by Li *et al.*[28] This assay which uses solubilized γ-secretase and C100 flag as substrates reproduces both major cleavage events (giving rise to the $A\beta_{40}$ and $A\beta_{42}$ termini) that are ascribed to γ-secretase activity in cells. It should be recalled that to catalytically recover competent soluble γ-secretase the choice of detergent (CHAPSO or CHAPS) used for the membrane extraction is critical. This methodology is now widely used as standard *in vitro* γ-secretase assay.[29] Moreover, it is now well established that γ-secretase activity is catalysed by a Presinilin-1 (PS-1) containing macromolecular complexes. In this *in vitro* assay, the new tested analogues **2** and **8** exhibit similar inhibition of Aβ-peptide production ($IC_{50} \approx 1$ μM).

***In vitro* cell based assay.** Compounds **2** and **8** were also tested for their ability to inhibit Aβ peptide production in a cell based assay according to a well known procedure using APP transfected HEK 293 cells.[30] As it can be seen in Table 1, the potency of analogue **8** to inhibit the production of $A\beta_{40}$ and $A\beta_{42}$ peptides was found to be slightly upper than that for analogue **2**. The assay was validated by using DAPT **1** as reference inhibitor.

**BBB permeation evaluation.** BBB permeation studies were achieved with derivative **2**, its corresponding metabolite **8** and DAPT **1** as reference compound. The experiments were carried on anaesthetized healthy Sprague Dawley male rats, according to a well known methodology.[31] Solutions of each compound were prepared and kept in refrigerator in order to avoid any compound degradation reaction which could interfere with the demonstration of the efficiency of the concept. The samples were injected into rats through the jugular vein at a dose of 20 mg kg$^{-1}$. At various intervals of time, a blood sample was withdrawn from the eyeball and after treatment was analysed by HPLC. At the end of this blood sampling, the animal was decapitated and the brain was submitted to the appropriate treatment as described in the Experimental section before HPLC analysis. HPLC profiles

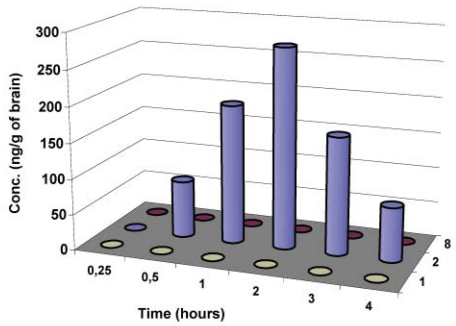
**Table 1** Cell free Aβ peptide production inhibitory activities

| Compound | Aβ production | | log *P*[d] |
|---|---|---|---|
| **1** (DAPT) | 50% (10 nM)[a,22] | | 3.07 |
| **2** | 36% (1 μM)[b] | 20% (1 μM)[c] | −0.63 |
| **8** | 42% (1 μM)[b] | 46% (1 μM)[c] | 2.19 |

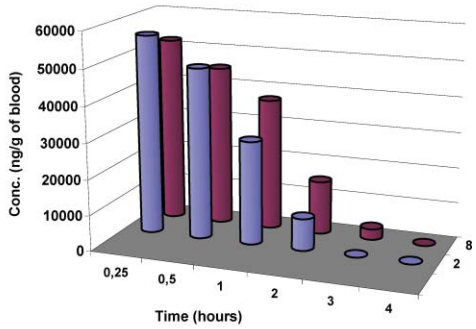
[a] Potency determination for the ability of compounds to reduce total Aβ production from HEK293 cells. [b] Potency determination to reduce $A\beta_{40}$ production. [c] Potency determination to reduce $A\beta_{42}$ production. [d] Log *P* determinations were performed using ACD (Advanced Chemistry Development, Inc.)/log *P* 1.0 base calculations.

of each type of samples were analysed in order to determine the relative permeation of the target molecule **2**, compared to that of the corresponding metabolite **8** and reference DAPT **1**. As it is known that ascorbic acid is susceptible to *in vivo* oxidation into DHAA, **2** was oxidized as described in the Experimental section by $H_2O_2$ into its DHAA analog **9** in order to identify its possible presence in the biological samples. The total amount of drug **2** in the brain was estimated as the amount of both ascorbic derivative **2** which has penetrated the brain in its intact form and its metabolites **8** and **9**. Compound **8** comes from enzymatic hydrolysis, while **9** is the result of enzymatic oxidation. These results are displayed on Fig. 2.

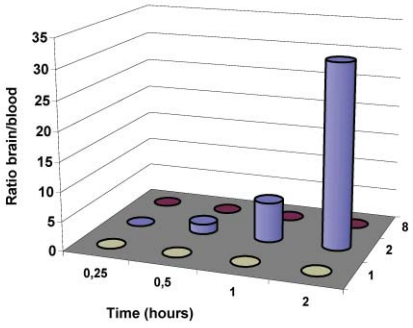
(a) Bioavailability in brain (ng/g of brain).




(b) Bioavailability in blood (ng/g of blood).




(c) Ratio between brain and blood.




$$R = \frac{concentration\ of\ drug\ in\ brain\ (ng\,/\,g\ of\ brain)}{concentration\ of\ drug\ in\ blood\ (ng\,/\,g\ of\ blood)} \times 10^3$$

**Fig. 2** Biological availability of the tested compounds in brain (a), in blood (b) and ratio between the concentrations in brain and blood.

## Discussion

It can be observed that compound **2** is remarkably delivered within the brain with an excellent pharmacokinetic profile. Indeed, it can be seen that after 1 hour, the concentration of compound **2** in the brain was around 200 ng $g^{-1}$ of brain, while during the same interval of time, the two other derivatives were not even detected (Fig. 2a). Optimal concentration of **2** in the brain was reached within 2 hours (280 ng $g^{-1}$ of brain). Consequently, the cumulative effect of compound **2** in the brain increases with time with minimal systemic concentration of the system. In the same experimental conditions, we observed that the reference compound DAPT **1** was not detected in the brain even after 4 hours while it has been reported that a level of 490 ng $g^{-1}$ of brain cortex of DAPT was achieved 3 hours after treatment.[22] Besides, levels greater than 100 ng $g^{-1}$ of brain were sustained through the first 18 hours. These differences between our results and those reported in the literature for the BBB permeation of DAPT could result from differences in the respective experimental protocols. Indeed, we injected our samples through the jugular vein at a dose of 20 mg $kg^{-1}$. Moreover, we used Sprague Dawley rats while Dovey *et al.*[22] performed subcutaneous injections on PDAPP transgenic mice at a dose of 100 mg $kg^{-1}$. In any case, in our experimental conditions, the results clearly show that the use of ascorbic acid BBB drug delivery system optimizes the nervous system drug uptake of the new γ-secretase inhibitor **2**. As indicated in the introduction of the paper, this observed improved BBB permeation property could reduce the diffusion of compound **2** into the peripheral tissues and consequently could lowered its side-effects on the immune cells or on neural cell differentiation.[25]

In addition to the determination of drug levels in the brain, the concentration of compounds **2** and **8** in the blood was also studied. As shown in Fig. 2b, the concentration levels of **2** and **8** in the blood decreased with time to completely disappear from the plasma after about 3 hours.

Analysis of the ratio between the drug levels in the brain and in the blood reinforces our findings about the far better BBB permeation for compound **2** compared to that of derivatives **1** and **8** (Fig. 2c).

Through *in vivo* experiments, we have shown that the use of ascorbic acid as drug carrier, appeared to be efficient since the obtained results are consistent with those expected according to the previous work reported by Manfredini *et al.*[15] Indeed, these studies revealed that the conjugation of a γ-secretase inhibitor to ascorbic acid *via* a linear ω-amino acid linker allows the delivery of the active drug to the brain with improvement of the γ-secretase inhibition activity.

In conclusion, we report herein the synthesis of a new DAPT-like pseudopeptide γ-secretase inhibitor whose design includes an ascorbic acid moiety as delivery system, which allows an efficient BBB permeation. Its favourable pharmacokinetic profile could be of interest *in vivo*. *In vivo* experiments on the Tg2576 mouse model of AD are currently under way to validate the pharmacological potential of such compound and to determine if the drug is an *in vivo* active γ-secretase inhibitor. It should be also postulated that such ascorbic acid mediated strategy could be applied for the numerous drugs directed to the CNS and particularly for AD drugs.

## Experimental

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without purification. All the protected amino acids and peptide coupling reagents were purchased from Bachem or Neosystem. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl immediately prior to use. Methylene dichloride ($CH_2Cl_2$) was distilled over $P_2O_5$ just prior to use. Dimethylformamide (DMF) was of anhydrous quality from commercial suppliers (Aldrich, Carlo Erba Reagents). $^1H$ Nuclear magnetic resonance spectra were recorded at 250 MHz on a Bruker AC-250 spectrometer. Chemical shifts are expressed as $\delta$ units (part per million) downfield from TMS (tetramethylsilane). Electrospray mass spectra were obtained on a Waters Micromass ZMD spectrometer by direct injection of the sample solubilized in acetonitrile. Microanalyses were carried out by Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4%

of the theoretical values. Analytical thin layer chromatography (TLC) and preparative thin layer chromatography (PLC) were performed using silica gel plates 0.2 mm thick and 1 mm thick respectively ($60F_{254}$ Merck). Preparative flash column chromatographies were carried out on silica gel (230–240 mesh, G60 Merck). The melting points were not determined because of the amorphous character of our synthesized compounds. UV spectra for biological stability studies were recorded on a Safas UV $mc^2$ spectrophotometer (Safas, Monaco).

### *N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycine *tert*-butyl ester (DAPT), 1

*N*-Carbobenzyloxycarbonyl-L-alanine (2.00 g, 1.0 equiv., 8.96 mmol) was dissolved in freshly distilled $CH_2Cl_2$ (30 mL) with 1.2 equiv. (4.75 g, 10.75 mmol) of BOP reagent. The reaction mixture was cooled to 0 °C and then 1.0 equiv. of DIEA (1.56 mL, 8.96 mmol) was added dropwise. The reaction mixture was stirred for 1 hour at room temperature and then once again cooled to 0 °C. A $CH_2Cl_2$ solution (30 mL) of 1.0 equiv. (2.18 g, 8.96 mmol) of phenylglycine *tert*-butyl ester hydrochloride and 2.0 equiv. (3.12 mL, 17.92 mmol) of DIEA was added dropwise. The solution was allowed to warm and stirred for 3 hours at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic layer was washed successively by using 5% aqueous citric acid (3 × 50 mL), brine (50 mL), 5% aqueous $NaHCO_3$ (3 × 50 mL) and brine (50 mL), was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 1 : 1) to give quantitatively the orthogonally protected dipeptide *N*-(*N*-carbobenzyloxy-L-alanyl)-L-phenylglycine *tert*-butyl ester (3.70 g) as a white solid (Found: C, 67.08; H, 7.05; N, 6.54. $C_{23}H_{28}N_2O_5$ requires C, 66.97; H, 6.84; N, 6.79%). $R_f$ 0.56 (EtOAc–hexane 1 : 1). $\delta_H$ (250 MHz, $CDCl_3$) 1.42 (12 H, broad s, C$H_3$ β Ala + C$H_3$*t*-Bu), 4.25–4.37 (1 H, m, C$H$ α Ala), 5.13 (2 H, s, -C$H_2$-O), 5.33 (1 H, broad s, N$H$), 5.43 (1 H, d, C$H$ α Phg, $^3J = 7.3$ Hz), 7.03 (1 H, broad s, N$H$), 7.28–7.36 (10 H, m, Ar$H$). ES/MS *m*/*z* 413 $(M + H)^+$, calcd for $C_{23}H_{28}N_2O_5$ 412.48 g mol$^{-1}$.

*N*-(*N*-Carbobenzyloxy-L-alanyl)-L-phenylglycine *tert*-butyl ester (3.70 g, 8.96 mmol) was dissolved in 10 mL of MeOH. 10% in weight of Pearlman's catalyst ($Pd(OH)_2$ over activated charcoal) was added to the previous solution and the resulting suspension was stirred overnight at room temperature under $H_2$ atmosphere. The solution was filtered on Celite and concentrated under reduced pressure to yield the *N*-deprotected dipeptide *N*-(L-alanyl)-L-phenylglycine *tert*-butyl ester (2.41 g, yield: 96%) as a white solid which was used without any further purification (Found: C, 64.51; H, 8.05; N, 9.84. $C_{15}H_{22}N_2O_3$ requires C, 64.73; H, 7.97; N, 10.06%). $R_f$ 0.17 (EtOAc). $\delta_H$ (250 MHz, $CDCl_3$) 1.34 (3 H, d, C$H_3$ β Ala, $^3J = 7.0$ Hz), 1.42 (9 H, s, C$H_3$*t*-Bu), 1.67 (2 H, broad s, N$H_2$), 3.48–3.60 (1 H, m, C$H$ α Ala), 5.47 (1 H, d, C$H$ α Phg, $^3J = 7.8$ Hz), 7.32–7.41 (5 H, m, Ar$H$), 8.19 (1 H, broad d, N$H$ Phg, $^3J = 7.8$ Hz). ES/MS *m*/*z* 279 $(M + H)^+$, calcd for $C_{15}H_{22}N_2O_3$ 278.35 g mol$^{-1}$.

3,5-Difluorophenylacetic acid (1.49 g, 1.0 equiv., 8.70 mmol) was dissolved in freshly distilled $CH_2Cl_2$ (20 mL) with 1.2 equiv. (4.60 g, 10.40 mmol) of BOP reagent. The reaction mixture was cooled to 0 °C and 1.0 equiv. of DIEA (1.20 mL, 8.70 mmol) was added dropwise. The reaction mixture was stirred for 1 hour at room temperature and then once again cooled to 0 °C. A $CH_2Cl_2$ solution (20 mL) of 1.0 equiv. (2.41 g, 8.70 mmol) of *N*-(L-alanyl)-L-phenylglycine *tert*-butyl ester and 2.0 equiv. (2.40 mL, 17.40 mmol) of DIEA was added dropwise. The solution was allowed to warm and stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic layer was washed successively by using 5% aqueous citric acid (3 × 50 mL), brine (50 mL), 5% aqueous $NaHCO_3$ (3 × 50 mL) and brine (50 mL), was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 1 : 1) to give DAPT **1** which was isolated as a white solid (3.40 g, yield: 88%) (Found: C, 63.75; H, 5.94; N, 6.75. $C_{23}H_{26}F_2N_2O_4$ requires C, 63.88; H, 6.06; N, 6.48%). $R_f$ 0.35 (EtOAc–hexane 1 : 1). $\delta_H$ (250 MHz, $CDCl_3$) 1.41 (12 H, broad s, $CH_3$ *t*-Bu + C$H_3$ β Ala, $^3J = 7.0$ Hz), 3.45 (2 H, s, -C$H_2$-C(O)-NH-), 4.54–4.65 (1 H, m, C$H$ α Ala, $^3J = 7.0$ Hz), 5.38 (1 H, d, C$H$ α Phg, $^3J = 7.3$ Hz), 6.46 (1 H, broad d, N$H$ Ala, $^3J = 7.0$ Hz), 6.68–6.85 (3 H, m, Ar$H$), 7.14 (1 H, d, N$H$ Phg, $^3J = 7.3$ Hz), 7.25–7.35 (5 H, m, Ar$H$). MS-ES *m*/*z* 433 $(M + H)^+$, calcd for $C_{23}H_{26}F_2N_2O_4$ 432.46 g mol$^{-1}$.

### *N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycine, 3

The *tert*-butyl ester DAPT **1** (1.0 equiv., 3.40 g, 7.66 mmol) was dissolved in $CH_2Cl_2$ (40 mL). Trifluoroacetic acid (5.9 mL, 10.0 equiv., 76.60 mmol) was then added and the reaction mixture was stirred overnight at room temperature. The solvent and excess of TFA were removed under reduced pressure and the resulting grey solid was triturated in $Et_2O$. The desired acid **3** was isolated after filtration as a white solid (2.50 g, yield: 87%) (Found: C, 60.45; H, 4.69; N, 7.25. $C_{19}H_{18}F_2N_2O_4$ requires C, 60.64; H, 4.82; N, 7.44%). $\delta_H$ (250 MHz, DMSO-$d_6$) 1.26 (3 H, d, C$H_3$ β Ala, $^3J = 7.0$ Hz), 3.51 (2 H, s, -C$H_2$-C(O)-NH-), 4.41–4.47 (1 H, m, C$H$ α Ala), 5.30 (1 H, d, C$H$ α Phg, $^3J = 7.5$ Hz), 6.97–7.50 (8 H, m, Ar$H$), 8.38 (1 H, d, N$H$ Phg, $^3J = 7.5$ Hz), 8.63 (1 H, d, N$H$ Ala, $^3J = 6.3$ Hz), 13.00 (1 H, broad s, COO$H$). MS-ES *m*/*z* 377 $(M + H)^+$, calcd for $C_{19}H_{18}F_2N_2O_4$ 376.35 g mol$^{-1}$.

### 6-*N*-(*tert*-Butyloxycarbonyl)-aminocaproic acid, 4

6-Aminocaproic acid (2.62 g, 1.0 equiv., 20.0 mmol) was dissolved in a dioxane–$H_2O$ (2 : 1) solution. The reaction mixture was cooled to 0 °C and a 1 M aqueous solution of NaOH (20 mL, 1.0 equiv., 20.0 mmol) was added, followed by $Boc_2O$ (4.80 g, 1.1 equiv., 22.0 mmol) which was added as a solid. The reaction mixture was stirred at room temperature for 3 hours. The solution was concentrated under reduced pressure. The basic aqueous residue was washed once with EtOAc (50 mL). The aqueous layer was acidified by aqueous 1 M HCl until pH 1 and then extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to afford the desired compound **4** as a colourless oil which slowly crystallized (4.06 g, quantitative). $R_f$ 0.71 (EtOAc). $\delta_H$ (250 MHz, $CDCl_3$) 1.09–1.55 (15 H, m, C$H_3$ Boc + -$CH_2$-(C$H_2$)$_3$-$CH_2$-COOH), 2.20 (2 H, t, -C$H_2$-COOH, $^3J = 7.5$ Hz), 3.86–3.02 (2 H, m, -NH-C$H_2$-), 4.40 (1 H, broad s, N$H$). MS-ES *m*/*z* 232 $(M + H)^+$, calcd for $C_{11}H_{21}NO_4$ 231.29 g mol$^{-1}$.

### 2,3-Di-*O*-benzyl-L-ascorbic acid, 5[15,26]

Ascorbic acid (1.00 g, 1.0 equiv., 5.70 mmol) was suspended in 10 mL of anhydrous acetone. A catalytic amount of acetyl chloride (21 μL, 0.05 equiv., 0.29 mmol) was then added and the slurry mixture was stirred overnight at room temperature. The solid was filtered, washed with EtOAc and dried under vacuum to afford the desired ketal as a white solid (0.72 g, yield: 58%) which was used without any further purification (Found: C, 50.15; H, 5.41. $C_9H_{12}O_6$ requires C, 50.00; H, 5.59%). $R_f$ 0.45 (EtOAc–hexane 4 : 1). $\delta_H$ (250 MHz, DMSO-$d_6$) 1.25 (6 H, s, 2 C$H_3$), 3.89 (1 H, dd, -O-C$H$H-CH(O)-, $^2J = 8.3$ Hz, $^3J = 6.3$ Hz), 4.11 (1 H, dd, -O-CH$H$-CH(O)-, $^2J = 8.3$ Hz, $^3J = 7.0$ Hz), 4.27 (1 H, td, -O-$CH_2$-C$H$(O)-CH-, $^3J = 7.0$ Hz, $^3J = 6.3$ Hz), 4.71 (1 H, d, -$CH_2$-CH(O)-C$H$(O)-, $^3J = 3.0$ Hz), 8.48 (1 H, broad s, O$H$), 11.30 (1 H, broad s, O$H$). MS-ES *m*/*z* 217 $(M + H)^+$, calcd for $C_9H_{12}O_6$ 216.19 g mol$^{-1}$.

The previous ketal (0.68 g, 1.0 equiv., 3.15 mmol) was suspended in anhydrous acetone (10 mL). $K_2CO_3$ (1.10 g, 2.5 equiv., 7.88 mmol) was then added and the heterogenous

mixture was refluxed. The insoluble ketal progressively dissolved in acetone whilst the temperature was rising. At reflux, one lot of benzyl bromide (0.90 mL, 2.3 equiv., 7.25 mmol) was added, and the solution was refluxed. The reaction mixture turned orange and after 2 hours it was allowed to cool to room temperature. The solution was concentrated under reduced pressure and the residue was diluted by $Et_2O$ (30 mL). The organic layer was washed with brine (30 mL) and the aqueous layer was extracted twice with $Et_2O$ (2 × 30 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography (EtOAc–hexane 1 : 4 then 1 : 1) to afford the dibenzyl derivative as a pale yellow solid (0.75 g, yield: 60%) (Found: C, 69.95; H, 6.28. $C_{23}H_{24}O_6$ requires C, 69.68; H, 6.10%). $R_f$ 0.23 (EtOAc–hexane 1 : 4), 0.67 (EtOAc–hexane 1 : 1). $\delta_H$ (250 MHz, $CDCl_3$) 1.39 (3 H, s, C*H*$_3$), 1.44 (3 H, s, C*H*$_3$), 4.03 (1 H, dd, -O-C*H*H-CH(O)-, $^2J$ = 8.5 Hz, $^3J$ = 6.8 Hz), 4.11 (1 H, dd, -O-CH*H*-CH(O)-, $^2J$ = 8.5 Hz, $^3J$ = 6.5 Hz), 4.28 (1 H, dt, -O-CH$_2$-C*H*(O)-CH-, $^3J$ = 6.8 Hz, $^3J$ = 3.3 Hz), 4.56 (1 H, d, -O-CH$_2$-CH(O)-C*H*-, $^3J$ = 3.3 Hz), 4.99 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 11.3 Hz), 5.02 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 11.3 Hz), 5.07 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.3 Hz), 5.12 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.3 Hz), 7.11–7.34 (10 H, m, Ar*H*). MS-ES *m/z* 397 (M + H)$^+$, calcd for $C_{23}H_{24}O_6$ 396.43 g mol$^{-1}$.

The tetra protected ascorbic acid derivative (0.72 g, 1.82 mmol) was dissolved in a mixture of 2 mL of THF and 2 mL of MeOH. An aqueous solution of HCl 2 M was added and the reaction mixture was stirred overnight at room temperature. The solution was concentrated under reduced pressure. The residue was diluted in EtOAc (10 mL) and washed with $H_2O$ (2 × 5 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to afford the desired compound **5** as a yellow oil which slowly solidified (0.64 g, quantitative) (Found: C, 67.63; H, 5.71. $C_{20}H_{20}O_6$ requires C, 67.41; H, 5.66%). $R_f$ 0.20 (EtOAc–hexane 1 : 1). $\delta_H$ (250 MHz, $CDCl_3$) 3.00 (2 H, broad s, 2 O*H*), 3.56–3.72 (2 H, m, -C*H*$_2$-OH), 3.80–3.84 (1 H, m, -CH-C*H*(OH)-CH$_2$OH), 4.56 (1 H, d, -C*H*-CH(OH)-CH$_2$OH, $^3J$ = 2.0 Hz), 5.06 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 11.3 Hz), 5.11 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.3 Hz), 5.14 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.8 Hz), 5.21 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 11.8 Hz), 7.07–7.25 (10 H, m, Ar*H*). MS-ES *m/z* 357 (M + H)$^+$, calcd for $C_{20}H_{20}O_6$ 356.37 g mol$^{-1}$.

### 6-Aminocaproyl 2,3-di-*O*-benzyl-L-ascorbic acid trifluoroacetic acid salt, 6

6-*N*-(*tert*-Butyloxycarbonyl)-aminocaproic acid **4** (0.80 g, 1.0 equiv., 3.46 mmol) was dissolved in freshly distilled $CH_2Cl_2$ (10 mL) with 1.2 equiv. (1.84 g, 4.15 mmol) of BOP reagent. The reaction mixture was cooled to 0 °C and then 1.0 equiv. of DIEA (600 μL, 3.46 mmol) was added dropwise. The reaction mixture was stirred for 1 hour at room temperature and then once again cooled to 0 °C. A $CH_2Cl_2$ solution (30 mL) of 1.1 equiv. (1.36 g, 3.81 mmol) of alcohol **5** and 2.0 equiv. (3.12 mL, 17.92 mmol) of DIEA was added dropwise. The solution was allowed to warm and stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic layer was washed successively by using 5% aqueous citric acid (3 × 50 mL), brine (50 mL), 5% aqueous $NaHCO_3$ (3 × 50 mL) and brine (50 mL), was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 2 : 3 then 1 : 1) to give the desired compound (1.13 g, yield: 57%) as a white solid (Found: C, 65.42; H, 7.05; N, 2.35. $C_{31}H_{39}NO_9$ requires C, 65.36; H, 6.90; N, 2.46%). $R_f$ 0.50 (EtOAc–hexane 1 : 1). $\delta_H$ (250 MHz, $CDCl_3$) 1.26–1.71 (15 H, m, C*H*$_3$ Boc + -NH-CH$_2$-(C*H*$_2$)$_3$-CH$_2$-C(O)-O-), 2.36 (2 H, t, -C*H*$_2$-C(O)-O-, $^3J$ = 6.3 Hz), 3.04–3.18 (2 H, m, -NH-C*H*$_2$-), 4.14 (1 H, broad s, -O-CH$_2$-C*H*(OH)-CH-), 4.23 (1 H, dd, -O-C*H*H-CH(OH)-CH-, $^2J$ = 11.5 Hz, $^3J$ = 4.8 Hz), 4.35 (1 H, dd, -O-CH*H*-CH(OH)-CH-, $^2J$ = 11.5 Hz, $^3J$ = 7.0 Hz), 4.61 (1 H, broad s, -CH(O*H*)-), 4.69 (1 H, d, -O-CH$_2$-CH(OH)-C*H*-, $^3J$ = 2.0 Hz), 5.14 (2 H, s, -O-C*H*$_2$-C$_6$H$_5$), 5.15 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 11.5 Hz), 5.24 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.5 Hz), 7.20–7.50 (10 H, m, Ar*H*). ES-MS *m/z* 570 (M + H)$^+$, calcd for $C_{31}H_{39}NO_9$ 569.64 g mol$^{-1}$.

The *N*-Boc derivative (1.13 g, 1.0 equiv., 2.0 mmol) was dissolved in $CH_2Cl_2$ (25 mL). The solution was cooled to 0 °C and trifluoroacetic acid (1.5 mL, 10.0 equiv., 20.0 mmol) was added dropwise. The resulting reaction mixture was stirred for 2 hours at room temperature. The solvent and excess of TFA were removed under reduced pressure. The residue was triturated in $Et_2O$ and the title compound **6** was quantitatively isolated as a white solid (1.15 g). $\delta_H$ (250 MHz, $CDCl_3$) 1.24–1.78 (6 H, m, H$_2$N-CH$_2$-(C*H*$_2$)$_3$-CH$_2$-C(O)-O-), 2.39 (2 H, t, -C*H*$_2$-C(O)-O-, $^3J$ = 6.5 Hz), 2.98–3.18 (2 H, m, H$_2$N-C*H*$_2$-), 4.20 (2 H, broad s, -O-C*H*H-C*H*(OH)-CH-, $^2J$ = 11.5 Hz, $^3J$ = 4.5 Hz), 4.39 (1 H, dd, -O-CH*H*-CH(OH)-CH-, $^2J$ = 11.5 Hz, $^3J$ = 6.5 Hz), 4.71 (1 H, d, -O-CH$_2$-CH(OH)-C*H*-, $^3J$ = 1.8 Hz), 5.03 (2 H, s, -C*H*$_2$-C$_6$H$_5$), 5.16 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^1J$ = 11.5 Hz), 5.22 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 11.5 Hz), 7.18–7.40 (10 H, m, Ar*H*). MS-ES *m/z* 470 (M + H)$^+$, calcd for $C_{26}H_{31}NO_7$ 469.53 g mol$^{-1}$ and for $C_{26}H_{31}NO_7.CF_3COOH$ 583.55 g mol$^{-1}$.

### 6-{*N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycyl}-aminocaproyl 2,3-di-*O*-benzyl-L-ascorbic acid, 7

The title compound was synthesized according to a similar procedure that described previously for the synthesis of DAPT **1** by using BOP as coupling agent. Compound **7** was isolated as a white solid (800 mg, yield: 73%) after flash chromatography (EtOAc) (Found: C, 65.23; H, 5.88; N, 4.95. $C_{45}H_{47}F_2N_3O_{10}$ requires C, 65.29; H, 5.72; N, 5.08%). $R_f$ 0.47 (EtOAc). $\delta_H$ (250 MHz, $CDCl_3$) 1.11–1.63 (9 H, m, -NH-CH$_2$-(C*H*$_2$)$_3$-CH$_2$-C(O)-O- + C*H*$_3$ β Ala, $^3J$ = 7.5 Hz), 2.18–2.33 (2 H, m, -C*H*$_2$-C(O)-O-), 2.93–3.30 (3 H, m, -NH-C*H*$_2$- + O*H*), 3.51 (2 H, broad s, -C*H*$_2$-C(O)-NH), 4.06–4.36 (4 H, m, -O-C*H*$_2$-C*H*(OH)-CH- + C*H* α Ala), 4.71 (1 H, d, -O-CH$_2$-CH(OH)-C*H*-, $^3J$ = 3.1 Hz), 5.05 (2 H, broad s, -O-C*H*$_2$-C$_6$H$_5$), 5.11 (1 H, d, -O-C*H*H-C$_6$H$_5$, $^2J$ = 10.9 Hz), 5.23 (1 H, d, -O-CH*H*-C$_6$H$_5$, $^2J$ = 10.9 Hz), 5.74 (1 H, d, C*H* α Phg, $^3J$ = 7.8 Hz), 6.42–6.81 (3 H, m, Ar*H*), 7.18–7.44 (15 H, m, Ar*H*) 7.81 (1 H, broad s, N*H*), 7.99 (1 H, broad s, N*H*), 8.50 (1 H, broad s, N*H*). MS-ES *m/z* 828 (M + H)$^+$, calcd for $C_{45}H_{47}F_2N_3O_{10}$ 827.87 g mol$^{-1}$.

### 6-{*N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycyl}-aminocaproyl L-ascorbic acid, 2

The protected ascorbic acid derivative **7** (200 mg, 1.0 equiv., 0.24 mmol) was dissolved in 3 mL of MeOH. 10% in weight of Pearlman's catalyst ($Pd(OH)_2$ over activated charcoal) was added to the previous solution and the resulting suspension was stirred at room temperature under $H_2$ atmosphere for 2 $\frac{1}{2}$ hours. The solution was filtered and concentrated under reduced pressure to yield the deprotected ascorbic acid derivative **2** (140 mg, yield: 90%) as a white solid (Found: C, 57.71; H, 5.25; N, 6.54. $C_{31}H_{35}F_2N_3O_{10}$ requires C, 57.49; H, 5.45; N, 6.49%). $\delta_H$ (250 MHz, $CD_3OD$) 1.22–1.63 (9 H, m, -NH-CH$_2$-(C*H*$_2$)$_3$-CH$_2$-C(O)-O- + C*H*$_3$ β Ala, $^3J$ = 7.2 Hz), 2.34–2.72 (2 H, m, -C*H*$_2$-C(O)-O-), 3.06–3.21 (2 H, m, -NH-C*H*$_2$-), 3.58 (2 H, broad s, C*H*$_2$-C(O)-NH), 4.05–4.41 (4 H, m, -O-C*H*$_2$-C*H*(OH)-CH- + C*H* α Ala), 4.71–4.73 (1 H, m, -O-CH$_2$-CH(OH)-C*H*-), 5.32 (1 H, s, C*H* α Phg), 6.78–6.94 (3 H, m, Ar*H*), 7.29–7.35 (5 H, m, Ar*H*). ES-MS *m/z* 648 (M + H)$^+$, calcd for $C_{31}H_{35}F_2N_3O_{10}$ 647.62 g mol$^{-1}$.

### 6-{*N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycyl}-aminocaproic acid, 8

The ester derivative **7** (200 mg, 1.0 equiv., 0.24 mmol) was smoothly hydrolysed by LiOH (13 mg, 1.5 equiv., 0.36 mmol) in

a iPrOH–$H_2O$ (v/v, 7 : 3) solution of $CaCl_2$ 0.8 M (10 mL). The reaction mixture was stirred for 2 hours at room temperature. After concentration under reduced pressure, the residue was diluted in $H_2O$ (5 mL) and washed once with $CH_2Cl_2$ (10 mL). The aqueous layer was acidified until pH 1 by addition of aqueous HCl 1 M and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to afford the carboxylic acid derivative **8** which was used without any further purification (68 mg, yield: 58%) (Found: C, 61.58; H, 6.05; N, 8.43. $C_{25}H_{29}F_2N_3O_5$ requires C, 61.34; H, 5.97; N, 8.58%). $\delta_H$ (250 MHz, $CD_3OD$) 1.25–1.67 (9 H, m, -NH-$CH_2$-($CH_2$)$_3$-$CH_2$-C(O)-O- + $CH_3$ β Ala), 2.21–2.26 (2 H, m, -$CH_2$-C(O)-O-), 3.05–3.14 (2 H, m, -NH-$CH_2$-), 3.55 (2 H, s, -$CH_2$-C(O)-NH-), 4.02–4.28 (1 H, m, C*H* α Ala), 5.58 (1 H, broad s, C*H* α Phg), 6.87–7.15 (3 H, m, Ar*H*), 7.25–7.55 (5 H, m, Ar*H*). MS-ES *m*/*z* 490 $(M + H)^+$, calcd for $C_{25}H_{29}F_2N_3O_5$ 489.51 g $mol^{-1}$.

### 6-{*N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycyl}-aminocaproyl L-dehydroascorbic acid, 9

A solution of **2** (80 mg, 0.12 mmol) in methanol (2 mL) was subjected to oxidation with 5% hydrogen peroxide (0.1 mL) for 5 minutes. The excess of $H_2O_2$ was destroyed by addition of a few crystals of ascorbic acid. The solution was evaporated to dryness under reduced pressure. The residue was kept dry in a desiccator under nitrogen atmosphere and reconstituted just before injection. The purity of the sample has been checked by HPLC analysis and was found at about 80% of DHAA derivative **9**.

## Biological studies

***In vitro* biological stability.** The *in vitro* biological stability of compound **2** was evaluated on mice liver and brain extracts. The tested compound **2** was dissolved in DMSO immediately prior to use at a concentration of 0.155 mol $L^{-1}$. This solution (10 μL) was incubated at 37 °C in the presence of the selected biological medium (300 μL of liver extract or brain extract). At the end of the incubation, the reaction was quenched by addition of methanol (300 μL). The crude mixture was sonicated for 5 minutes at room temperature and centrifuged at 4000 rpm for 10 minutes. The supernatant was then purified by PLC (silica $60F_{254}$ Merck gel plates 0.2 mm thick, EtOAc–methanol 1 : 1) by using pure sample of **2** as reference. The purified residue was extracted from the silica gel by ethanol (5 mL). The suspension was sonicated for 5 minutes at room temperature, centrifuged at 4000 rpm for 10 minutes to eliminate the solid silica gel. The resulting solution was concentrated under reduced pressure. The residue was dissolved in methanol (1 mL), sonicated for 5 minutes, centrifuged at 4000 rpm for 10 minutes and filtered on Celite. An aliquot of the filtrate (50 μL) was diluted with 450 μL of methanol and the absorbance of the resulting sample was measured at 267 nm on a Safas UV mc$^2$ spectrophotometer at 25 °C.

The concentration of the sample was determined by comparison to a calibration curve obtained in very similar conditions by using the same biological sample previously deactivated by addition of methanol prior to introduction of the DMSO solution of compound **2**.

***In vitro* γ-secretase cell free assay.** The ability to block specifically *in vitro* γ-secretase was performed by using solubilized γ-secretase prepared as described by Li *et al.*[28] A DNA fragment encoding amino acids 596–695 of the 695-aa isoform of APP (APP695) and the Flag sequence (DYKD-DDDK) at the C terminus was generated by PCR amplification with suitably designed oligonucleotides and the APP695 cDNA. The Met that serves as the translation start site is residue 596 of APP695 (the P1 residue with respect to the β-secretase cleavage site). C100Flag (1.7 μM) was incubated with cell membranes (0.5 mg $mL^{-1}$) in the presence of CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate). The reactions were stopped by adding RIPA.

***In vitro* cell based assay.** The ability to inhibit Aβ peptide production in a cell based assay was measured according to a well known procedure using APP transfected HEK 293 cells.[30] HEK 293 E cells were maintained in DMEM supplemented with 10% fetal bovine serum, 25 μg $mL^{-1}$ penicillin, 25 μg $mL^{-1}$ streptomycin, and 250 μg $mL^{-1}$ G 418. Cells were transfected following specifications provided by the manufacturer (Gibco-BRL). One day after transfection, the conditioned medium was replaced with selection medium (media as above containing in addition 250 μg $mL^{-1}$ hygromycin B). For immunoblotting analysis, the 24-hour serum-free conditioned medium was harvested and cellular debris was removed by centrifugation for 10 minutes. The monolayers were washed with PBS, and cell lysates were prepared by the addition of SDS-sample buffer containing 50 mM dithiothreitol. For Aβ determination by ELISA, confluent cultures were incubated in serum-free medium containing 0.2% BSA for 16 hours. To test the effects of compounds on Aβ formation, cells were plated at confluency ($10^6$ cells $cm^{-2}$) in 96-well tissue culture dishes in serum-free medium containing 0.2% BSA in the presence of the indicated concentration of either γ-secretase reference inhibitor DAPT. After 16 hours, the conditioned medium was harvested and analysed using the HA 11 Aβ-specific ELISA.

### BBB permeation evaluation[31,10]

Six groups, each of four Sprague Dawley male rats of average weight of 55–65 g were anaesthetized with urethane. A freshly prepared solution (50 mmol) of each compound in DMSO (diluted to 20% with water) was injected through the jugular vein at a dose of 20 mg $kg^{-1}$.

**Blood sample analysis.** At time intervals (0.25, 0.50, 1.00, 2.00, 3.00 and 4.00 hours) blood samples were withdrawn from the eyeball, immediately added to previously weighed centrifuge tubes containing 5 mL of 1% TFA in methanol and weighed to determine the amount of blood added. The blood samples were mixed by vortex at room temperature for about 10 minutes, centrifuged at about 4000 rpm for 5 minutes. The supernatant was withdrawn and evaporated under helium gas to dryness. The residue was reconstituted in 1 mL of mobile phase and analysed by HPLC.

**Brain sample analysis.** The animals were decapitated and the brains were taken. Each brain was weighed and immediately homogenized with 1 mL saline and diluted with 5 mL of 5% DMSO in methanol, homogenized again and centrifuged at 4000 rpm for 10 minutes. The supernatant was evaporated under reduced pressure. The residue was diluted to 0.5 mL with the mobile phase and analysed by HPLC.

**HPLC analysis method.** HPLC method with UV detection.

*Equipment.* Knauer HPLC with Marathon Plus Autosampler with switch column injector.

*Column.* Knauer HPLC column C18, 4.6 mm × 20 cm, 5 μm.

*Detector.* Diode array detector at wavelength range 245–290 nm.

*Injection volume.* 150 μL.

*Temperature.* Room temperature.

*Flow rate.* 1 mL $min^{-1}$.

*Software.* Eurochrom 2000.

*Mobile phase.* Filtered and degassed mixture of 0.1% hexane–sulfonic acid (prepared by pH adjustment of 0.1% solution of hexane–sulfonic acid sodium salt with 20% trifluoroacetic acid to pH about 4.7), acetonitrile and isopropanol (45 : 52 : 3). The peak symmetry was achieved by addition of chloroform (0.5 mL per litre of mobile phase).

***Retention time.*** **1** (about 18.5 minutes), **2** (about 7.5 minute) and **10** (about 12.7 minutes).

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