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Aust. J. Chem. **2005**, *58*, 585–594

Design of β-Secretase Inhibitors by Introduction of a Mandelyl Moiety in DAPT Analogues

Nicolas Pietrancosta,[A] *Gilles Quéléver*,[A] *Younes Laras*,[A] *Cédrik Garino*,[A] *Stéphane Burlet*,[A] *and Jean Louis Kraus*[A,B]

[A] INSERM U-623, Institut de Biologie du Développement de Marseille (CNRS–INSERM–Université de la Méditerranée), Laboratoire de Chimie Biomoléculaire, Faculté des Sciences de Luminy, case 907, 13288 Marseille Cedex 09, France.

[B] Corresponding author. Email: kraus@luminy.univ-mrs.fr

We report the synthesis of two series of compounds with 3,5-difluoromandelyl-alanyl or 3,5-difluorophenylacetyl-alanyl backbones coupled to various heterocyclic or peptidic moieties. These two series of compounds were evaluated for their inhibitory properties on β-secretase (BACE-1) enzymatic assay, a target enzyme for Alzheimer's disease (AD) pathology. We found that both diastereomers obtained from the racemic mixture **7** of the coumarin derivative bearing a mandelyl moiety were the most potent BACE-1 inhibitors studied in this work ($IC_{50} = 1 \times 10^{-6}$ M). Analysis of the obtained results led to the hypothesis that introduction of a difluoromandelyl residue in place of a difluorophenylacetyl moiety may induce β-secretase inhibitory activity.

Manuscript received: 21 April 2005.
Final version: 17 June 2005.

Introduction

A key advance in the understanding of the Alzheimer's disease (AD) ethiology was the identification of two enzymes, β- and γ-secretases, that play a critical role in the amyloid cascade.[1,2] β-Secretase, also called Asp-2 or BACE-1, is a unique member of the pepsin family of aspartyl proteases that initiates the production of the amyloidogenic Aβ peptide. This neurotoxic peptide is the principal component of the senile plaques found during postmortem analysis of AD patients. To date, several secretase inhibitors have been described. They are peptide-based analogues in which the scissile amide bond has been replaced by a non-cleavable isostere.[3] From a literature study, it was observed that among the potential motifs of potent secretase inhibitors, 3,5-difluorophenylacetyl and 3,5-difluoromandelyl moieties are of particular interest (Fig. 1).[4–6] When these structural motifs were coupled to bulky heterocyclic moieties, the resulting derivatives displayed secretase inhibitory activities, mainly on γ-secretase.

Moreover, it was observed that introduction of an hydroxy function on the methylene group of the 3,5-difluorophenylacetyl moiety, to give the corresponding 3,5-difluoromandelic analogues, substantially enhanced the γ-secretase inhibitory potency of the resulting analogues.[6,7] Nevertheless, various studies suggest caution in testing γ-secretase inhibitors on humans. The major concern surrounding the use of potent γ-secretase inhibitors for AD clinical treatments lies in their side effects at the level of neural stem cell differentiation as well as in the control of gliogenesis.[8] Consequently, on the one hand, it has been suggested that a limited reduction of amyloid precursor protein (APP) processing will suffice to limit amyloid precipitation,[9] and, on the other hand, β-secretase appears to be a promising target for the treatment of AD.

Herein we report the synthesis and β-secretase inhibitory activity of new derivatives. These new analogues, which incorporate a difluoromandelyl-alanyl backbone coupled to various heterocyclic or pseudopeptidic moieties, were assayed as BACE-1 inhibitors, and compared to their difluorophenylacetyl counterparts that lack the hydroxy group (Fig. 2). These observations are discussed in terms of stereochemistry as well as in terms of structure inhibitory relationships. It appears that numerous heterocycles seem to be suitable for secretase inhibitory activity, such as the benzodiazepine moiety, for example.[4] These heterocyclic moieties confer to the resulting molecules a planar structure with enhancement of their hydrogen donor or acceptor binding properties. For these reasons, we selected various heterocyclic entities which present these structural features while having not yet been described in the design of BACE-1 inhibitors (Fig. 2).

Results and Discussion

The syntheses of the new derivatives were achieved either through the direct coupling of the pseudopeptidic backbones to the heterocyclic moiety, or by constructing the pseudopeptidic moiety step by step by an extension of the heterocyclic core used as the starting scaffold. Some heterocyclic entities involved in this study were commercially available, such as 6-amino-3,4-benzocoumarin **5**, or were synthesized

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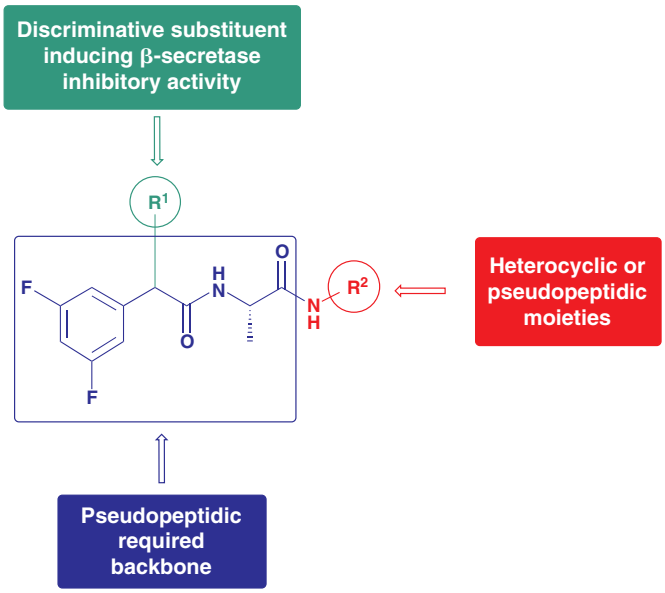


Fig. 1. General structure for secretase inhibitors.

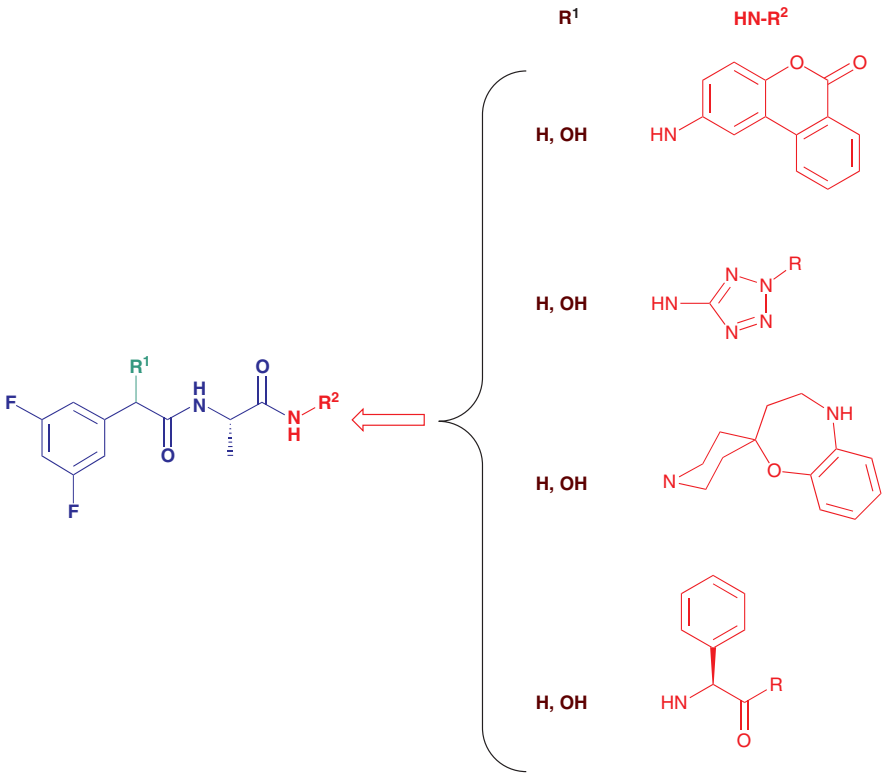
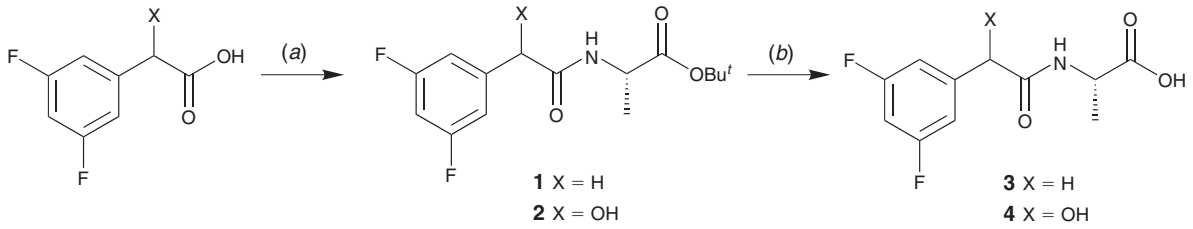


Fig. 2. General target molecule structures.

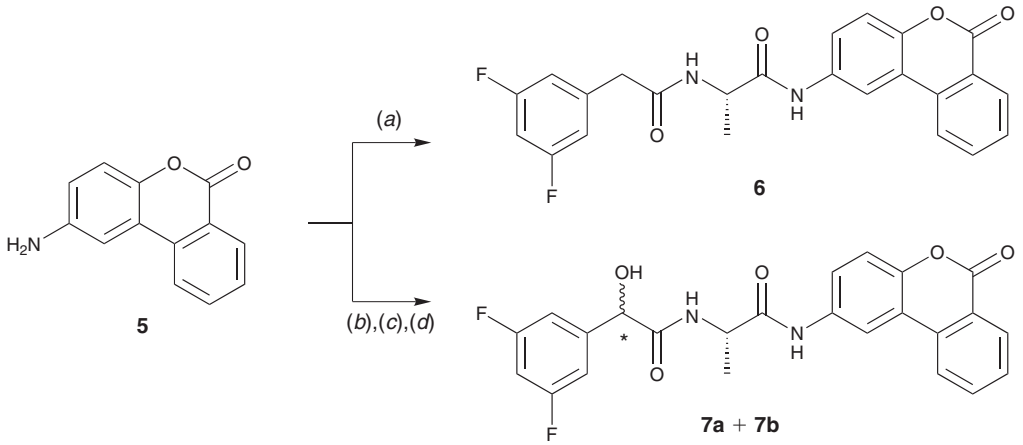
according to described procedures, such as the benzoxazepine piperidine **8**[10] or the tetrazole derivative **11**.[11]

The first strategy involved the synthesis of the phenylacetyl-alanyl and mandelyl-alanyl building blocks before their coupling to the heterocyclic moiety. These pseudopeptidic moieties were synthesized according to a classical coupling methodology as summarized in Scheme 1 to give the desired compounds **3** (X = H) and **4** (X = OH). This first strategy was used in order to synthesize compounds **6** (Scheme 2), **9**, and **10** (Scheme 3) while compounds **7a**/**7b** (Scheme 2), **12**, **13a**/**13b** (Scheme 4), **15**, **16a**/**16b**, **19**,[14] and **20** (Scheme 5) were built by successive introduction of each acylating building block, which is similar to a classical solution-phase peptide synthesis. All the compounds were unambiguously characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. In the case of compounds **7a**/**7b**, **13a**/**13b**, and **16a**/**16b**, we were able to separate both diastereomers.

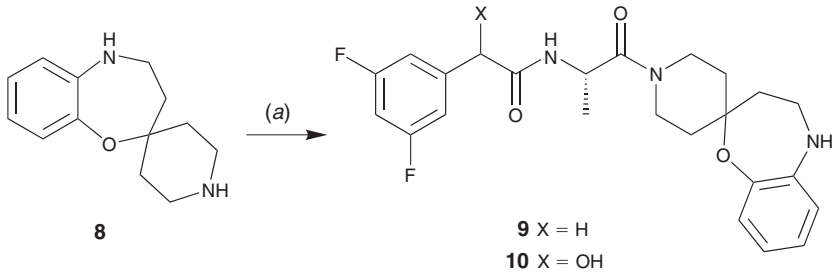
The compounds were assayed as BACE-1 inhibitors by using a fluorescence resonance energy transfer (FRET) assay,



Scheme 1. Synthesis of the pseudopeptidic moieties. Conditions and reagents: (*a*) L-alanine *tert*-butyl ester hydrochloride, BOP, DIEA, CH_2Cl_2, rt; 90% (**1**), 30% (**2**); (*b*) TFA, CH_2Cl_2, rt; 90% (**3**), quantitative (**4**).



Scheme 2. Synthesis of *N*-acylated 6-aminocoumarin derivatives. Conditions and reagents: (*a*) **3**, DCC, HOBt, DMF, rt; 48% (**6**); (*b*) BocAla, DCC, HOBt, CH_2Cl_2, rt; quantitative; (*c*) TFA, CH_2Cl_2, rt; quantitative; (*d*) 3,5-difluoromandelic acid, BOP, DIEA, CH_2Cl_2, rt; quantitative (**7a** + **7b**).



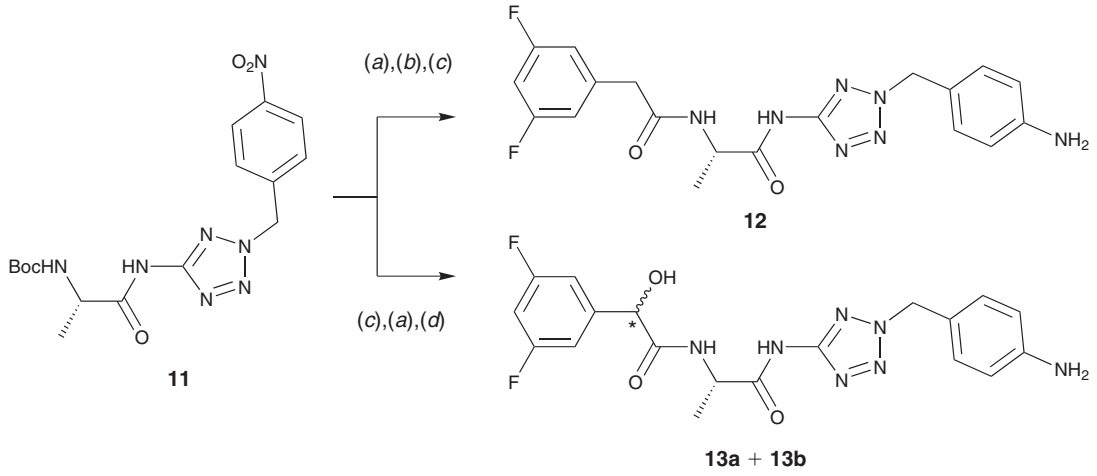
Scheme 3. Synthesis of *N*-acylated benzoxazepine piperidine derivatives. Conditions and reagents: (*a*) **3** or **4**, BOP, DIEA, CH_2Cl_2, rt; 37% (**9**), 38% (**10**).

which uses purified baculovirus-expressed (BACE-1) and a specific substrate (Rh-EVNLDAEFK-Quencher) based on the Swedish mutation of the amyloid precursor protein (APP). This peptidic substrate becomes highly fluorescent upon enzymatic cleavage.

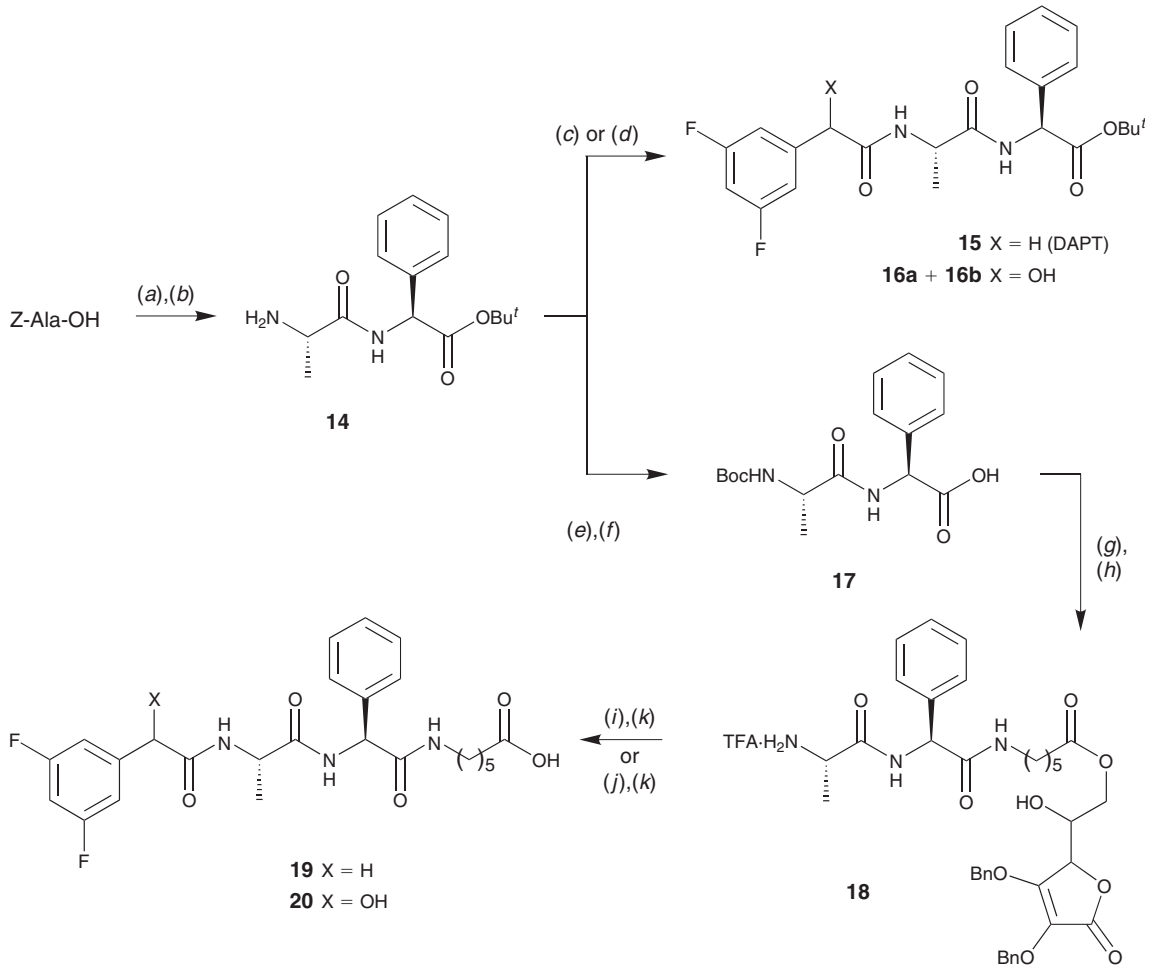
IC_{50} (50% inhibitory concentration) values were determined at least six times from kinetic curves obtained on a Wallac Victor^2 1420 spectrofluorometer (530–545 nm excitation and 570–590 nm emission wavelengths) using *Workout 1.5 Prism* software from GraphPad. A standard statine-derived inhibitor, H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-3*S*,4*S*-Stat-Val-Ala-Glu-Phe-OH, was used to calibrate inhibition of BACE-1 activity. The IC_{50} values obtained for both series of compounds are summarized in Table 1. With the exception of compound **20**, the *N*-terminal 3,5-difluoromandelyl derivatives exhibited β-secretase inhibitory activity. Moreover, the inhibitory potency of the mandelic analogue series was higher compared to that of their corresponding 3,5-difluorophenylacetyl analogues, which demonstrated poor or no β-secretase inhibitory activities. The most active compounds of the mandelic series were the two coumarin analogue diastereomers **7a** and **7b**, with IC_{50} values of about 1×10^{-6} M.

It should also be underlined that *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-L-phenylglycine *tert*-butyl ester (DAPT, **15**), which belongs to the 3,5-difluorophenylacetic series and is known as a very potent γ-secretase inhibitor ($IC_{50} = 20 \times 10^{-9}$ M),[17] was found to be a weak BACE-1 inhibitor (IC_{50} approx. 100×10^{-6} M). In contrast, its corresponding mandelyl analogue **16** was found to be 20 times more potent on BACE-1.

Moreover, the very potent γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-3-*S*-amino-1-methyl-5-phenyl-1,3-dihydrobenzo[*e*](1,4)diazepin-2-one, commonly

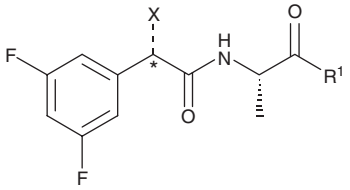


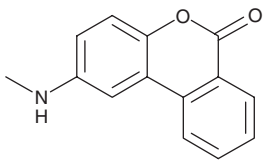
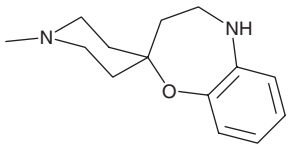
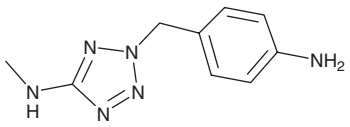
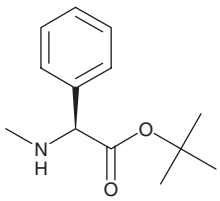
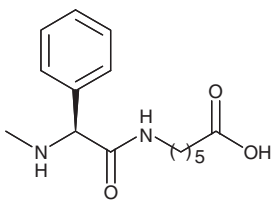
Scheme 4. Synthesis of *N*-acylated 5-aminotetrazole derivatives. Conditions and reagents: (*a*) TFA, CH_2Cl_2, rt; (*b*) 3,5-difluorophenylacetic acid, BOP, DIEA, CH_2Cl_2, rt; (*c*) Fe, NH_4Cl, H_2O/EtOH, reflux;[12] (*d*) 3,5-difluoromandelic acid, EDC, HOBt, DIEA, NMM, THF, rt;[13] 49% (**12**, for 3 steps (*a*), (*b*), and (*c*)); 4% (**13a** + **13b**, for 3 steps (*c*), (*a*), and (*d*)).



Scheme 5. Synthesis of DAPT and DAPT-like derivatives. Conditions and reagents: (*a*) phenylglycine *tert*-butyl ester hydrochloride salt, BOP, DIEA, CH_2Cl_2, rt; quantitative; (*b*) H_2, $Pd(OH)_2$/C, MeOH, rt; 96% (**14**); (*c*) 3,5-difluorophenylacetic acid, BOP, DIEA, CH_2Cl_2, rt; 88% (**15**); (*d*) 3,5-difluoromandelic acid, $Cl_3COCOCl$, activated charcoal, THF, rt[15] then DIEA, CH_2Cl_2, rt; 21% (**16a** + **16b**); (*e*) TFA, CH_2Cl_2, rt; quantitative; (*f*) Boc_2O, DIEA, CH_2Cl_2, rt; 80% (**17**); (*g*) 6-aminocaproyl 2,3-di-*O*-benzyl-L-ascorbic acid trifluoroacetic acid salt,[14] BOP, DIEA, CH_2Cl_2, rt; 60%; (*h*) TFA, CH_2Cl_2, rt; quantitative (**18**); (*i*) 3,5-difluorophenylacetic acid, BOP, DIEA, CH_2Cl_2, rt; 73%; (*j*) 3,5-difluoromandelic acid, $Cl_3COCOCl$, activated charcoal, THF, rt[15] then DIEA, CH_2Cl_2, rt; 26%; (*k*) LiOH, $CaCl_2$, Pr^iOH/H_2O, rt;[14,16] 58% (**19**),[14] 22% (**20**).

Table 1. BACE-1 inhibitory activity determined by FRET assay
Compared to the standard statine-derived reference inhibitor,
H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-(3*S*,4*S*)-Stat-Val-Ala-Glu-Phe-OH (IC_{50} 30×10^{-9} M)



Compound	X	R^1	BACE-1 (IC_{50}) [$\times 10^{-6}$ M]
6	H		80 ± 6
7a[A]	OH		1 ± 0.2
7b[A]	OH		1 ± 0.2
9	H		>100
10 (*racemic*)	OH		40 ± 3
12	H		100 ± 5
13a[A]	OH		30 ± 2.5
13b[A]	OH		25 ± 2.5
15 (DAPT)	H		50 ± 5
16a[A]	OH		5 ± 0.5
16b[A]	OH		5 ± 0.5
19	H		>100
20 (*racemic*)	OH		>100

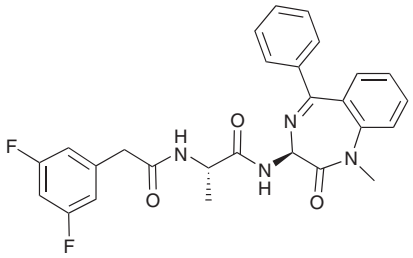
[A]Pure diastereomer separated from the racemic mixture by chromatographic techniques.

known as compound E (Fig. 3), the structure of which bears a phenylacetyl residue, was only a very weak β-secretase inhibitor ($IC_{50} = 100 \times 10^{-6}$ M).

We also found that the effect of substitution at the *C*-Ala-terminal position greatly influences the potency of the resulting derivatives. The introduction of heterocyclic moieties, such as spirobenzoxazepine **10**, or pseudopeptidic moieties, such as phenylacetyl caproylamide **20**, almost abolished BACE-1 inhibitory activity, with IC_{50} values ranging from (40 to 100) $\times 10^{-6}$ M.

The introduction of other heterocyclic moieties, such as a substituted tetrazole for compound **13**, or a pseudopeptidic moiety, such as phenylglycyl *tert*-butyl ester for compound **16**, led to moderate inhibitory activities ranging over (5–30) $\times 10^{-6}$ M.

We also studied the influence of the chirality of the mandelic moiety on BACE-1 inhibitory potency. We found that between the diastereomeric pairs of the racemic mixtures **7a**/**7b**, **13a**/**13b**, and **16a**/**16b**, no noticeable differences were observed. These similar activities for both diastereomers may



Compound E

Fig. 3. Structure of γ-secretase inhibitor compound E.

indicate that the stereochemistry of the hydroxyl group of the mandelic moiety is not determinative for the interactions within the enzymatic site of the β-secretase.

In conclusion, we synthesized new BACE-1 inhibitors bearing 3,5-difluoromandelyl-alanyl or 3,5-difluorophenylacetyl-alanyl backbones linked to various functionalized heterocyclic or aliphatic moieties. The present results clearly show that the introduction of a difluoromandelyl-alanyl moiety in a pseudopeptidic backbone coupled to various specific heterocyclic scaffolds induces potent β-secretase inhibitory activity. In contrast, the corresponding difluorophenylacetyl-alanyl residue coupled to the same heterocyclic scaffold was devoid of any β-secretase inhibitory activity.

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. A BACE-1 (β-secretase) FRET kit assay was purchased from the PanVera Corporation. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl immediately before use. Dichloromethane (CH_2Cl_2) was distilled over P_2O_5 just before 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 Brüker AC-250 spectrometer. Chemical shifts are expressed as δ 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 the 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 chromatography was carried out on silica gel (230–400 mesh, G60 Merck). Melting points were not determined because of the amorphous character of our synthesized compounds.

N-*(3,5-Difluorophenylacetyl)-L-alanine* tert-*Butyl Ester,* ***1***

3,5-Difluorophenylacetic acid (1.07 g, 1.0 equiv., 6.22 mmol) was dissolved in freshly distilled CH_2Cl_2 (30 mL) with 1.2 equiv. (3.30 g, 7.46 mmol) of BOP reagent (benzotriazol-l-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate). The solution was cooled to 0°C and then 1.0 equiv. of DIEA (*N,N*-diisopropylethylamine; 1.08 mL, 6.22 mmol) was added dropwise. The reaction mixture was stirred for 1 h at room temperature and once again cooled to 0°C. A CH_2Cl_2 solution (30 mL) of 1.0 equiv. (1.13 g, 6.22 mmol) of L-alanine *tert*-butyl ester hydrochloride and 3.0 equiv. (3.25 mL, 18.66 mmol) of DIEA was added dropwise. The solution was allowed to warm to room temperature and was stirred for 4 h. 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). It was then dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc/hexane, 1 : 2) to afford the title compound **1** (1.68 g, yield: 90%) as a white solid. (Found: C 60.1, H 6.5, N 4.9%. $C_{15}H_{19}F_2NO_3$ requires C 60.2, H 6.4, N 4.7%.) R_f 0.31 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.35 (3 H, d, CH_3 β Ala, ^3J 7.0), 1.45 (9 H, s, CH_3 *t*-Bu), 3.53 (2 H, s, –C*H*$_2$–C(O)–), 4.39–4.50 (1 H, m, CH α Ala), 6.08 (1 H, br s, NH), 6.69–6.87 (3 H, m, ArH). *m/z* (ES) 300 $(M + H)^+$, calc. for $C_{15}H_{19}F_2NO_3$ 299.31.

N-*(3,5-Difluoromandelyl)-L-alanyl* tert-*Butyl Ester,* ***2***

The title compound **2** was synthesized according to a similar procedure as described for the previous derivative **1**. This procedure involved the use of 3,5-difluoromandelic acid (0.50 g, 1.0 equiv., 2.65 mmol), L-alanine *tert*-butyl ester hydrochloride (430 mg, 0.9 equiv., 2.38 mmol), and BOP reagent (1.17 g, 1.0 equiv., 2.65 mmol) as coupling agent. Compound **2** was isolated after flash chromatography (CH_2Cl_2/MeOH, 95 : 5) as a white solid (250 mg, yield: 30%). (Found: C 57.0, H 6.0, N 4.6%. $C_{15}H_{19}F_2NO_4$ requires C 57.1, H 6.1, N 4.4%.) R_f 0.65 (CH_2Cl_2/MeOH, 95 : 5). δ_H (250 MHz, $CDCl_3$) 1.34 (3 H, d, CH_3 β Ala, ^3J 7.0), 1.45 (9 H, s, CH_3 *t*-Bu), 4.26–4.32 (1 H, m, CH α Ala), 4.96 (1 H, s, C*H*OH), 5.05 (1 H, s, CHO*H*), 6.81–7.15 (3 H, m, ArH), 7.34 (1 H, d, NH, ^3J 7.4). *m/z* (ES) 316 $(M + H)^+$, calc. for $C_{15}H_{19}F_2NO_4$ 315.31.

N-*(3,5-Difluorophenylacetyl)-L-alanine,* ***3***

The *tert*-butyl ester derivative **1** (1.68 g, 1.0 equiv., 5.62 mmol) was dissolved in CH_2Cl_2 (20 mL). Trifluoroacetic acid (4.33 mL, 10.0 equiv., 56.19 mmol) was then added and the reaction mixture was stirred overnight at room temperature. The solvent and excess TFA were removed under reduced pressure and the resulting solid was triturated in hexane. The title compound **3** was isolated as a white solid (1.22 g, yield: 90%). (Found: C 54.2, H 4.3, N 5.9%. $C_{11}H_{11}F_2NO_3$ requires C 54.3, H 4.6, N 5.8%.) δ_H (250 MHz, [D_6]DMSO) 1.28 (3 H, d, CH_3 β Ala, ^3J 7.5), 3.52 (2 H, s, –CH_2–C(O)–), 4.12–4.53 (1 H, m, CH α Ala), 6.97–7.14 (3 H, m, ArH), 8.51 (1 H, d, NH, ^3J 7.0), 12.60 (1 H, br s, COOH). *m/z* (ES) 244 $(M + H)^+$, calc. for $C_{11}H_{11}F_2NO_3$ 243.21.

N-*(3,5-Difluoromandelyl)-L-alanine,* ***4***

The title compound **4** was obtained according to a similar procedure to that described previously for the synthesis of **3**. Acidolysis of the *tert*-butyl ester derivative **2** (250 mg, 1.0 equiv., 0.79 mmol) by trifluoroacetic acid led quantitatively to the free carboxylic acid **4**, which was isolated as a yellow solid (200 mg) as a racemic mixture of two diastereomers. (Found: C 51.2, H 4.4, N 5.3%. $C_{11}H_{11}F_2NO_4$ requires C 51.0, H 4.3, N 5.4%.) δ_H (250 MHz, $CDCl_3$) 1.46 (3 H, d, CH_3 β Ala, ^3J 7.2), 4.49–4.58 (1 H, m, CH α Ala), 5.10 (1 H, s, C*H*OH), 6.76–7.01 (3 H, m, ArH), 7.25 (1 H, d, NH), 12.91 (1 H, br s, COOH). *m/z* (ES) 260 $(M + H)^+$, calc. for $C_{11}H_{11}F_2NO_4$ 259.21.

6-N-*[(3,5-Difluorophenylacetyl)-L-alanyl]-3,4-benzocoumarin,* ***6***

N-(3,5-Difluorophenylacetyl)-L-alanine **3** (140 mg, 1.2 equiv., 0.57 mmol) was dissolved in anhydrous DMF (10 mL). The solution was cooled to 0°C and DCC (1,3-dicyclohexylcarbodiimide; 195 mg, 2.0 equiv., 0.95 mmol) and HOBt (128 mg, 2.0 equiv., 0.95 mmol) were added. The resulting mixture was stirred for a few minutes. A solution of the commercially available 6-amino coumarin derivative **5** (100 mg, 1.0 equiv., 0.47 mmol) and DIEA (250 μL, 3.0 equiv., 1.42 mmol) in anhydrous DMF (5 mL) was added dropwise. The solution was allowed to warm to room temperature and stirred until completion. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (20 mL). The organic layer was washed successively with 5% aqueous citric acid (2 × 5 mL), brine (2 × 5 mL), 5% aqueous $NaHCO_3$ (2 × 2 mL), brine (2 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by precipitation to yield the desired compound **6** as a white solid (100 mg, yield: 48%). (Found: C 65.9,

H 4.4, N 6.6%. $C_{24}H_{18}F_2N_2O_4$ requires C 66.1, H 4.2, N 6.4%.) R_f 0.38 (toluene/EtOAc, 9 : 1). δ_H (250 MHz, $CDCl_3$) 1.48 (3 H, d, CH_3 β Ala, ^3J 6.9), 3.62 (2 H, s, $–CH_2–C(O)–$), 4.12 (1 H, br s, NH Ala), 4.62–4.74 (1 H, m, CH α Ala), 6.60–6.92 (3 H, m, ArH), 7.20 (1 H, d, H_2, ^3J 8.8), 7.29 (1 H, dd, H_1, ^3J 9.0, ^4J 2.2), 7.55 (1 H, td, H'_3, ^3J 8.0, ^4J 1.1), 7.74 (1 H, td, H'_4, ^3J 7.7, ^4J 1.2), 7.93 (1 H, d, H'_5, ^3J 8.2), 8.32 (1 H, dd, H'_2, ^3J 7.9, ^4J 1.0), 8.39 (1 H, d, H_5, ^4J 2.2), 9.15 (1 H, s, NH). *m/z* (ES) 437 $(M+H)^+$, calc. for $C_{24}H_{18}F_2N_2O_4$ 436.41.

6-N-*[(3,5-Difluoromandelyl)-L-alanyl]-3,4-benzocoumarin, **7a**/**7b***

6-Amino-3,4-benzocoumarin **5** (100 mg, 1.0 equiv., 0.47 mmol) was acylated by Boc-Ala (Boc = *tert*-butyloxycarbonyl) according to a similar procedure to that described previously for the synthesis of compound **6**. This procedure involved the use of DCC/HOBt as a coupling system and led to the corresponding *N*-Boc-alanyl coumarine derivative, 6-*N*-[(*N*-*tert*-butoxycarbonyl)-L-alanyl]-3,4-benzocoumarin, which was quantitatively isolated (180 mg) as a pale yellow solid. (Found: C 65.8, H 6.1, N 7.6%. $C_{21}H_{22}N_2O_5$ requires C 66.0, H 5.8, N 7.3%.) R_f 0.31 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, [D_6]DMSO) 1.30 (3 H, d, CH_3 β Ala, ^3J 7.0), 1.39 (9 H, s, CH_3 Boc), 4.11–4.17 (1 H, m, CH α Ala), 7.16 (1 H, d, NH Ala, ^3J 7.3), 7.42 (1 H, d, ArH, ^3J 9.0), 7.69–7.74 (2 H, m, ArH), 7.98 (1 H, td, ArH, ^3J 8.0, ^4J 1.4), 8.17 (1 H, d, ArH, ^3J 8.3), 8.28 (1 H, dd, ArH, ^3J 8.0, ^4J 1.3), 8.58 (1 H, d, ArH, ^4J 2.5), 10.18 (1 H, br s, NH). *m/z* (ES) 383 $(M+H)^+$, calc. for $C_{21}H_{22}N_2O_5$ 382.41. The previous *N*-Boc-alanyl coumarine derivative (137 mg, 1.0 equiv., 0.36 mmol) was deprotected by using TFA (276 μL, 10.0 equiv., 3.60 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred overnight at room temperature and the solvent and excess TFA were removed under reduced pressure. The resulting TFA salt was quantitatively isolated as a white solid (170 mg) after trituration in ether. δ_H (250 MHz, [D_6]DMSO) 1.50 (3 H, d, CH_3 β Ala, ^3J 7.0), 4.00–4.11 (1 H, m, CH α Ala), 7.47 (1 H, d, ArH, ^3J 8.8), 7.70–7.78 (2 H, m, ArH), 8.01 (1 H, td, ArH, ^3J 7.6, ^4J 1.3), 8.10–8.20 (4 H, m, 2 × ArH + NH_2), 8.51 (1 H, d, ArH, ^4J 2.3), 10.74 (1 H, br s, NH). *m/z* (ES) 283 $(M+H)^+$, calc. for $C_{16}H_{14}N_2O_3$ 282.29 and for $C_{16}H_{14}N_2O_3 \cdot CF_3COOH$ 396.32. The previous TFA salt (150 mg, 1.2 equiv., 0.36 mmol) was acylated by 3,5-difluoromandelic acid (56 mg, 1.0 equiv., 0.30 mmol) in the presence of BOP reagent as a coupling agent, according to a similar procedure to that described previously for the synthesis of **1**. The desired compound was quantitatively isolated as a mixture of diastereomers (140 mg). Both diastereomers were separated by flash chromatography (EtOAc/hexane, 2 : 1) but their respective stereochemistry was not fully attributed. Compound **7a** (70 mg) (Found: C 63.6, H 4.0, N 6.3%. $C_{24}H_{18}F_2N_2O_5$ requires C 63.7, H 4.0, N 6.2%.) R_f 0.27 (EtOAc/hexane, 2 : 1). δ_H (250 MHz, [D_6]DMSO) 1.37 (3 H, d, CH_3 β Ala, ^3J 7.3), 3.30 (1 H, br s, CHO*H*), 4.43–4.55 (1 H, m, CH α Ala), 5.09 (1 H, d, C*H*OH, ^3J 5.0), 6.59 (1 H, d, NH Ala, ^3J 5.5), 7.11–7.21 (3 H, m, ArH), 7.43 (1 H, d, ArH, ^3J 9.3), 7.66–7.76 (2 H, m, ArH), 7.96–8.04 (1 H, m, ArH), 8.17–8.30 (2 H, m, ArH), 8.56 (1 H, d, ArH, ^4J 2.5), 10.31 (1 H, br s, NH). *m/z* (ES) 453 $(M+H)^+$, calc. for $C_{24}H_{18}F_2N_2O_5$ 452.12. Compound **7b** (70 mg) (Found: C 63.8, H 4.1, N 6.3%. $C_{24}H_{18}F_2N_2O_5$ requires C 63.7, H 4.0, N 6.2%.) R_f 0.22 (EtOAc/hexane, 2 : 1). δ_H (250 MHz, [D_6]DMSO) 1.40 (3 H, d, CH_3 β Ala, ^3J 7.0), 3.30 (1 H, br s, CHO*H*), 4.40–4.51 (1 H, m, CH α Ala), 5.09 (1 H, d, C*H*OH, ^3J 5.3), 6.56 (1 H, d, NH Ala, ^3J 5.0), 7.09–7.19 (3 H, m, ArH), 7.42 (1 H, d, ArH, ^3J 9.0), 7.64–7.75 (2 H, m, ArH), 7.95–8.03 (1 H, m, ArH), 8.16–8.30 (2 H, m, ArH), 8.54 (1 H, d, ArH, ^4J 2.3), 10.31 (1 H, br s, NH). *m/z* (ES) 453 $(M+H)^+$, calc. for $C_{24}H_{18}F_2N_2O_5$ 452.12.

1′-N-*[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-4,5-dihydrospiro[1,5-benzoxazepine-2(3H),4′-piperidine], **9***

The 3,5-difluorophenylacetyl alanine acid **3** (114 mg, 1.0 equiv., 0.46 mmol) was dissolved in freshly distilled CH_2Cl_2 (5 mL) in the presence of BOP reagent (244 mg, 1.2 equiv., 0.55 mmol). The reaction mixture was cooled to 0°C and then DIEA (80 μL, 1.0 equiv., 0.46 mmol) was added dropwise. The reaction mixture was stirred for 1 h at room temperature and then cooled once again to 0°C. A CH_2Cl_2 solution of the benzoxazepine piperidine derivative **8** (129 mg, 1.0 equiv., 0.46 mmol) and DIEA (240 μL, 3.0 equiv., 1.38 mmol) 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 (20 mL). The organic layer was washed successively by using H_2O (10 mL), brine (10 mL), 5% aqueous $NaHCO_3$ (2 × 10 mL), and brine (10 mL), was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash chromatography (CH_2Cl_2/MeOH, 95 : 5) to give the title compound **9** (76 mg, yield: 37%) as a white solid. (Found: C 64.9, H 6.0, N 9.6%. $C_{24}H_{27}F_2N_3O_3$ requires C 65.0, H 6.1, N 9.5%.) R_f 0.48 (CH_2Cl_2/MeOH, 95 : 5). δ_H (250 MHz, $CDCl_3$) 1.32 (3 H, d, CH_3 β Ala, ^3J 6.6), 1.38–1.46 (2 H, m, $–(CHH–CH_2)_2–N–$), 1.88–1.92 (2 H, m, $–CH_2–CH_2–NH–$), 2.05–2.16 (2 H, m, $–(CHH–CH_2)_2–N–$), 3.05–3.19 (1 H, m, $–(CH_2–CHH)_2–N–$), 3.28–3.33 (2 H, m, $–CH_2–CH_2–NH–$), 3.51 (2 H, s, $–CH_2–C(O)–$), 3.59–3.64 (2 H, m, $–(CH_2–CHH)_2–N–$), 4.25–4.42 (1 H, br s, NH aniline), 4.85–4.90 (1 H, m, CH α Ala), 6.63–6.92 (7 H, m, ArH), 8.05 (1 H, br s, NH Ala). *m/z* (ES) 444 $(M+H)^+$, calc. for $C_{24}H_{27}F_2N_3O_3$ 443.49.

1′-N-*[*N*-(3,5-Difluoromandelyl)-L-alanyl]-4,5-dihydrospiro[1,5-benzoxazepine-2(3H),4′-piperidine], **10***

The title compound was prepared according to a procedure similar to that described for the synthesis of the previous derivative **9** by using the mandelyl derivative **4** (119 mg, 1.0 equiv., 0.46 mmol) as an acidic moiety and BOP reagent (244 mg, 1.2 equiv., 0.55 mmol). The desired compound **10** was isolated as a white solid (80 mg, yield: 38%) after flash chromatography (CH_2Cl_2/MeOH, 95 : 5). (Found: C 62.5, H 6.1, N 9.3%. $C_{24}H_{27}F_2N_3O_4$ requires C 62.7, H 5.9, N 9.2%.) R_f 0.47 (CH_2Cl_2/MeOH, 95 : 5). δ_H (250 MHz, $CDCl_3$) 1.25–1.60 (5 H, m, CH_3 β Ala + $–(CHH–CH_2)_2–N–$), 1.88–1.93 (2 H, m, $–CH_2–CH_2–NH–$), 2.04–2.12 (2 H, m, $–(CHH–CH_2)_2–N–$), 3.07–3.19 (2 H, m, $–(CH_2–CHH)_2–N–$), 3.25–3.35 (2 H, m, $–CH_2–CH_2–NH–$), 3.56–3.81 (3 H, m, $–(CH_2–CHH)_2–N–$ + OH), 4.31–4.42 (1 H, m, NH aniline), 4.83–4.89 (1 H, m, CH α Ala), 5.04 (1 H, br s, $–CH(OH)–$), 6.64–7.41 (7 H, m, ArH), 7.95 (1 H, br s, NH Ala). *m/z* (ES) 460 $(M+H)^+$, calc. for $C_{24}H_{27}F_2N_3O_4$ 459.49.

*5-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-amino-2-(4-aminobenzyl)-tetrazole, **12***

The substituted *N*-Boc protected 5-aminotetrazole derivative **11**[10] (140 mg, 1.0 equiv., 0.36 mmol) was dissolved in CH_2Cl_2 (5 mL). Trifluoroacetic acid (275 μL, 10.0 equiv., 3.60 mmol) was then added at 0°C and the reaction mixture was stirred overnight at room temperature. The solvent and excess TFA were removed under reduced pressure. The resulting trifluoroacetic acid salt was isolated as a white solid after trituration in ether (140 mg, yield: 97%). δ_H (250 MHz, CD_3OD) 1.62 (3 H, d, CH_3 β Ala, ^3J 7.0), 4.15 (1 H, br s, CH α Ala), 6.02 (2 H, s, $–CH_2–$), 7.64 (2 H, d, $CH_{m\text{-}NO_2}$, ^3J 8.8), 8.28 (2 H, d, $CH_{o\text{-}NO_2}$, ^3J 8.8). *m/z* (ES) 292 $(M+H)^+$, calc. for $C_{11}H_{13}N_7O_3$ 291.27 and for $C_{11}H_{13}N_7O_3 \cdot CF_3COOH$ 405.29. The following coupling reaction involving this trifluoroacetic acid salt was performed similarly to the procedure described for the synthesis of **1** by using BOP as a coupling agent. The tetrazole derivative was the result of a coupling reaction between 3,5-difluorophenylacetic acid (34 mg, 1.0 equiv., 0.20 mmol) and the previous TFA salt (80 mg, 1.0 equiv., 0.20 mmol). The resulting nitro derivative was isolated by flash chromatography as a yellow solid (60 mg, yield: 68%). (Found: C 51.1, H 4.1, N 21.9%. $C_{19}H_{17}F_2N_7O_4$ requires C 51.2, H 3.9, N 22.0%.) R_f 0.50 (EtOAc). δ_H (250 MHz, $CDCl_3$) 1.36 (3 H, d, CH_3 β Ala, ^3J 7.0), 3.50 (2 H, s, $–CH_2–C(O)–$), 4.90–5.01 (1 H, m, CH α Ala), 5.76 (2 H, s, $N–CH_2–$), 6.52–6.74 (3 H, m, ArH), 7.47–7.50 (3 H, m, NH Ala + $H_{m\text{-}NO_2}$, ^3J 8.8), 8.10 (2 H, d, $H_{o\text{-}NO_2}$, ^3J 8.8), 10.55 (1 H, br s, NH tetrazole). *m/z* (ES) 446 $(M+H)^+$, calc. for $C_{19}H_{17}F_2N_7O_4$ 445.38. This nitro derivative (45 mg, 1.0 equiv., 0.10 mmol) was heated to reflux in a H_2O/EtOH solution (5 mL, 3 : 5 v/v). Powdered iron (28 mg, 5.0 equiv., 0.51 mmol) and NH_4Cl (11 mg, 2.0 equiv., 0.20 mmol) were then added. After 2 h,

the solution was cooled to room temperature, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography to afford the desired anilino compound **12** as a yellow solid (31 mg, yield: 74%). (Found: C 56.1, H 4.5, N 23.4%. $C_{19}H_{19}F_2N_7O_2$ requires C 54.9, H 4.6, N 23.6%.) R_f 0.51 (EtOAc). δ_H (250 MHz, $CDCl_3$) 1.32 (3 H, d, CH_3 β Ala, ^3J 7.3), 3.44 (2 H, s, $–CH_2–C(O)–$), 3.75 (2 H, br s, $–NH_2$), 4.80 (1 H, br s, CH α Ala), 5.47 (2 H, s, $–CH_2–C_6H_4–NH_2$), 6.49–6.58 (3 H, m, ArH), 6.73 (2 H, d, $H_{o\text{-}NH_2}$, ^3J 8.5), 7.08 (2 H, d, $H_{m\text{-}NH_2}$, ^3J 8.5), 7.63 (1 H, br s, NH Ala), 10.38 (1 H, br s, NH). *m/z* (ES) 416 $(M + H)^+$, calc. for $C_{19}H_{19}F_2N_7O_2$ 415.40.

5-N-*[*N-*(3,5-Difluoromandelyl)-*L*-alanyl]-amino-2-(4-aminobenzyl)-tetrazole,* ***13a/13b***

The aromatic nitro derivative **11** (970 mg, 1.0 equiv., 2.48 mmol) was reduced to its aniline analogue by using Fe/NH_4Cl as a reducing system according to a similar procedure described for the last step of the synthesis of **12**. The desired aniline was isolated as a pale yellow solid (211 mg, yield: 24%). (Found: C 53.3, H 6.5, N 27.3%. $C_{16}H_{23}N_7O_3$ requires C 53.2, H 6.4, N 27.1%.) R_f 0.47 (EtOAc/hexane, 2 : 1). δ_H (250 MHz, $CDCl_3$) 1.40 (12 H, br s, CH_3 Boc + CH_3 β Ala), 3.86 (2 H, br s, NH_2), 4.40–4.58 (1 H, m, CH α Ala), 5.46 (1 H, br s, NH Ala), 5.54 (2 H, br s, $–N–CH_2–C_6H_4–NH_2$), 6.58 (2 H, d, $H_{o\text{-}NH_2}$, ^3J 7.3), 7.16 (2 H, d, $H_{m\text{-}NH_2}$, ^3J 7.3), 10.04 (1 H, br s, NH). *m/z* (ES) 362 $(M + H)^+$, calc. for $C_{16}H_{23}N_7O_3$ 361.40. The *N*-Boc protecting group on the alanyl moiety of the resulting aniline derivative (171 mg, 1.0 equiv., 0.47 mmol) was removed by acidolysis according to a procedure similar to that described previously for the first step of the synthesis of compound **12**. The diTFA salt was quantitatively isolated as a yellow solid after trituration in ether (231 mg). δ_H (250 MHz, [D_6]DMSO) 1.43 (3 H, d, CH_3 β Ala), 4.46 (3 H, br s, $–C_6H_4–NH_2$ + CH α Ala), 5.74 (2 H, br s, $–N–CH_2–C_6H_4–NH_2$), 6.80 (2 H, d, $H_{o\text{-}NH_2}$, ^3J 7.1), 7.21 (2 H, d, $H_{m\text{-}NH_2}$, ^3J 7.1), 8.27 (2 H, br s, NH_2 Ala), 11.60 (1 H, br s, NH). *m/z* (ES) 262 $(M + H)^+$, calc. for $C_{11}H_{15}N_7O$ 261.28 and for $C_{11}H_{15}N_7O.2\ CF_3COOH$ 489.33. The diTFA salt (220 mg, 1.0 equiv., 0.59 mmol) was acylated by 3,5-difluoromandelic acid (110 mg, 1.0 equiv., 0.59 mmol) in anhydrous THF (3 mL). The coupling system, EDC·HCl (226 mg, 2.0 equiv., 1.18 mmol), HOBt (159 mg, 2.0 equiv., 1.18 mmol), and *N*-methyl morpholine (195 μL, 3.0 equiv., 1.77 mmol), was added at 0°C and the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in H_2O (5 mL). The aqueous layer was extracted with EtOAc (2 × 5 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The two desired diastereomers were separated as yellow solids by flash chromatography (EtOAc/hexane, 3 : 1) (40 mg, yield: 16%), but their respective stereochemistry was not fully attributed. Compound **13a** (20 mg) (Found: C 53.2, H 4.3, N 22.8%. $C_{19}H_{19}F_2N_7O_3$ requires C 52.9, H 4.4, N 22.7%.) R_f 0.42 (EtOAc/hexane, 3 : 1). δ_H (250 MHz, CD_3OD) 1.40 (3 H, d, CH_3 β Ala, ^3J 7.1), 4.45–4.65 (1 H, m, CH α Ala), 5.05 (1 H, br s, C*H*OH), 5.74 (2 H, br s, $–N–CH_2–C_6H_4–NH_2$), 6.78–7.19 (3 H, m, ArH), 7.41 (2 H, d, $H_{o\text{-}NH_2}$, ^3J 8.8), 7.64 (2 H, d, $H_{m\text{-}NH_2}$, ^3J 7.3). *m/z* (ES) 432 $(M + H)^+$, calc. for $C_{19}H_{19}F_2N_7O_3$ 431.40. Compound **13b** (20 mg) (Found: C 52.9, H 4.5, N 22.6%. $C_{19}H_{19}F_2N_7O_3$ requires C 52.9, H 4.4, N 22.7%.) R_f 0.36 (EtOAc/hexane, 3 : 1). δ_H (250 MHz, CD_3OD) 1.44 (3 H, d, CH_3 β Ala, ^3J 7.1), 4.44–4.61 (1 H, m, CH α Ala), 5.05 (1 H, br s, C*H*OH), 5.72 (2 H, br s, $–N–CH_2–C_6H_4–NH_2$), 6.76–7.16 (3 H, m, ArH), 7.39 (2 H, d, $H_{o\text{-}NH_2}$, ^3J 8.8), 7.63 (2 H, d, $H_{m\text{-}NH_2}$, ^3J 7.3). *m/z* (ES) 432 $(M + H)^+$, calc. for $C_{19}H_{19}F_2N_7O_3$ 431.40.

N-*[L-Alanyl]-L-phenylglycine* tert-*Butyl Ester,* ***14***

N-Carbobenzyloxycarbonyl-L-alanine (2.00 g, 1.0 equiv., 8.96 mmol) was coupled to phenylglycine *tert*-butyl ester hydrochloride (2.18 g, 1.0 equiv., 8.96 mmol) by using BOP (4.75 g, 1.2 equiv., 10.75 mmol) as a coupling agent. The orthogonally protected dipeptide residue was purified by flash chromatography (EtOAc/hexane, 1 : 1) and quantitatively isolated (3.70 g) as a white solid. (Found: C 67.1, H 7.1, N 6.5%. $C_{23}H_{28}N_2O_5$ requires C 67.0, H 6.8, N 6.8%.) R_f 0.56 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.42 (12 H, br s, CH_3 β Ala + CH_3 *t*-Bu), 4.25–4.37 (1 H, m, CH α Ala), 5.13 (2 H, s, $–CH_2–O$), 5.33 (1 H, br s, NH), 5.43 (1 H, d, CH α Phg, ^3J 7.3), 7.03 (1 H, br s, NH), 7.28–7.36 (10 H, m, ArH). *m/z* (ES) 413 $(M + H)^+$, calc. for $C_{23}H_{28}N_2O_5$ 412.48. *N*-(*N*-Carbobenzyloxy-L-alanyl)-L-phenylglycine *tert*-butyl ester (3.70 g, 8.96 mmol) was dissolved in 10 mL of MeOH and 10 wt.-% of Pearlman's catalyst ($Pd(OH)_2$ over activated charcoal) was added to the solution. The resultant suspension was stirred overnight at room temperature under a H_2 atmosphere. The solution was filtered over celite and concentrated under reduced pressure to yield the title compound **14** (2.41 g, yield: 96%) as a white solid which was used without any further purification. (Found: C 64.5, H 8.1, N 9.8%. $C_{15}H_{22}N_2O_3$ requires C 64.7, H 8.0, N 10.1%.) R_f 0.17 (EtOAc). δ_H (250 MHz, $CDCl_3$) 1.34 (3 H, d, CH_3 β Ala, ^3J 7.0), 1.42 (9 H, s, CH_3 *t*-Bu), 3.48–3.60 (1 H, m, CH α Ala), 1.67 (2 H, br s, NH_2), 5.47 (1 H, d, CH α Phg, ^3J 7.8), 7.32–7.41 (5 H, m, ArH), 8.19 (1 H, broad d, NH Phg, ^3J 7.8). *m/z* (ES) 279 $(M + H)^+$, calc. for $C_{15}H_{22}N_2O_3$ 278.35.

N-*[*N-*(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycine* tert-*Butyl Ester (DAPT),* ***15***

DAPT **15** was isolated as a white solid (3.40 g, yield: 88%) according to a procedure similar to that described previously for the synthesis of compound **1**, by using BOP as a coupling agent. This procedure involved the use of 1.0 equiv. (1.49 g, 8.70 mmol) of the carboxylic acid entity, 3,5-difluorophenylacetic acid, and 1.0 equiv. (2.41 g, 8.70 mmol) of the previous amino moiety **14** (Found: C 63.8, H 5.9, N 6.8%. $C_{23}H_{26}F_2N_2O_4$ requires C 63.9, H 6.1, N 6.5%.) R_f 0.35 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.41 (12 H, br s, CH_3 β Ala, ^3J 7.0, + CH_3 *t*-Bu), 3.45 (2 H, s, $–CH_2–C(O)–NH–$), 4.54–4.65 (1 H, m, CH α Ala, ^3J 7.0), 5.38 (1 H, d, CH α Phg, ^3J 7.3), 6.46 (1 H, broad d, NH Ala, ^3J 7.0), 6.68–6.85 (3 H, m, ArH), 7.14 (1 H, d, NH Phg, ^3J 7.3), 7.25–7.35 (5 H, m, ArH). *m/z* (ES) 433 $(M + H)^+$, calc. for $C_{23}H_{26}F_2N_2O_4$ 432.46.

N-*[*N-*(3,5-Difluoromandelyl)-L-alanyl]-L-phenylglycine* tert-*Butyl Ester,* ***16a/16b***

3,5-Difluoromandelic acid (188 mg, 1.0 equiv., 1.0 mmol) was suspended in freshly distilled THF (2 mL). The suspension was cooled to 0°C and diphosgene (150 μL, 1.2 equiv., 1.2 mmol) was added in one batch. A small amount of activated charcoal was then added and the resulting solution was stirred overnight at room temperature. The activated charcoal was filtered off and the solvent and excess phosgene were removed under reduced pressure. The resulting 1,3-dioxolane-2,4-dione intermediate was used for the second step without any further purification. This intermediate was dissolved in freshly distilled CH_2Cl_2 (3 mL) and the resulting solution was cooled to 0°C. A solution of the free amino derivative **14** (278 mg, 1.0 equiv., 1.0 mmol) and DIEA (209 μL, 1.2 equiv., 1.2 mmol) in CH_2Cl_2 (2 mL) was then added dropwise. The reaction mixture was stirred for 24 h at room temperature. The solvent was eliminated under reduced pressure. The residue was dissolved in EtOAc (10 mL) and the organic layer was successively washed by 5% aqueous citric acid (2 × 5 mL), brine (2 × 5 mL), and 5% aqueous $NaHCO_3$ (2 × 5 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc/hexane, 1 : 1) to afford both diastereomers of the racemic mixture (96 mg, yield: 21%), but their respective stereochemistry was not fully attributed. Compound **16a** (48 mg) (Found: C 61.8, H 6.0, N 6.0%. $C_{23}H_{26}F_2N_2O_5$ requires C 61.6, H 5.8, N 6.3%.) R_f 0.47 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.36–1.40 (12 H, m, CH_3 *t*-Bu + CH_3 β Ala), 4.32 (1 H, br s, OH), 4.49–4.58 (1 H, m, CH α Ala), 4.92–5.00 (2 H, m, C*H*OH + NH), 5.34 (1 H, d, CH α Phg, ^3J 7.3), 6.69–7.00 (3 H, m, ArH), 7.22–7.40 (6 H, m, ArH + NH). *m/z* (ES) 449 $(M + H)^+$, calc. for $C_{23}H_{26}F_2N_2O_5$ 448.46. Compound **16b** (48 mg) (Found: C 61.5, H 6.0, N 6.4. $C_{23}H_{26}F_2N_2O_5$ requires C 61.6, H 5.8, N 6.3%.) R_f 0.24 (EtOAc/hexane, 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.40 (12 H, br s, CH_3 Boc + CH_3 β Ala), 4.43 (1 H, br s, OH), 4.53–4.64 (1 H, m, CH α Ala), 5.00 (1 H, br s, C*H*OH), 5.31 (1 H, d, CH α Phg, ^3J 6.8), 6.68–6.98 (3 H, m, ArH), 7.19–7.40 (7 H, m, ArH + 2 NH). *m/z* (ES) 449 $(M + H)^+$, calc. for $C_{23}H_{26}F_2N_2O_5$ 448.46.

N-*[*N-*(*tert-*Butyloxycarbonyl)-*L*-alanyl]-*L*-phenylglycine, **17***

N-[L-Alanyl]-L-phenylglycine *tert*-butyl ester **14** (1.94 g, 1.0 equiv., 6.98 mmol) was dissolved in 50 mL of CH_2Cl_2. The solution was cooled to 0°C and trifluoroacetic acid (5.40 mL, 10.0 equiv., 69.80 mmol) was added dropwise. The reaction mixture was stirred overnight at room temperature. The solvent and excess TFA were removed under reduced pressure and the residue was triturated in ether. The corresponding trifluoroacetic acid salt was isolated quantitatively as a white solid (2.30 g). It was characterized by mass spectrometry but not by NMR as it was not soluble in the conventional deuterated solvents. *m/z* (ES) 223 $(M+H)^+$, calc. for $C_{11}H_{14}N_2O_3$ 222.24 and for $C_{11}H_{14}N_2O_3{\cdot}CF_3COOH$ 336.26. The trifluoroacetic acid salt (2.30 g, 1.0 equiv., 6.98 mmol) was suspended in 50 mL of freshly distilled CH_2Cl_2. The solution was cooled to 0°C and a solution of 1.2 equiv. of Boc_2O (1.83 g, 8.38 mmol) and 3.0 equiv. of DIEA (3.60 mL, 20.94 mmol) in 20 mL of CH_2Cl_2 was added dropwise. The reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in H_2O (50 mL). The basic aqueous layer was washed with EtOAc (2 × 25 mL), acidified to 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. The title compound **17** was isolated as a white solid (1.80 g, yield: 80%). (Found: C 59.9, H 7.1, N 8.6%. $C_{16}H_{22}N_2O_5$ requires C 59.6, H 6.9, N 8.7%.) δ_H (250 MHz, $CDCl_3$) 1.32 (3 H, d, CH_3 β Ala, ^3J 6.8), 1.38 (9 H, s, CH_3 Boc), 4.22–4.40 (1 H, m, CH α Ala), 5.26 (1 H, br s, NH), 5.55 (1 H, d, CH α Phg, ^3J 7.0), 7.25–7.34 (5 H, m, ArH), 7.61 (1 H, br s, NH), 8.70 (1 H, br s, COOH). *m/z* (ES) 323 $(M+H)^+$, calc. for $C_{16}H_{22}N_2O_5$ 322.36.

*6-[*N-*(*L*-Alanyl)-*L*-phenylglycyl]-aminocaproyl 2,3-di-*O*-benzyl-*L*-ascorbic Acid Trifluoroacetic Acid Salt, **18***

6-Aminocaproyl 2,3-di-*O*-benzyl-L-ascorbic acid trifluoroacetic salt[14] (3.64 g, 1.2 equiv., 6.22 mmol) was acylated by the *N*-Boc protected dipeptide **17** (1.67 g, 1.0 equiv., 5.19 mmol) in the presence of BOP as a coupling agent according to a procedure similar to that described previously for the synthesis of **1**. The desired *N*-Boc derivative was isolated as a white solid (2.40 g, yield: 60%). (Found: C 65.0, H 6.4, N 5.6%. $C_{42}H_{51}N_3O_{11}$ requires C 65.2, H 6.6, N 5.4%.) R_f 0.25 (EtOAc/hexane, 2 : 1). δ_H (250 MHz, $CDCl_3$) 1.33–1.78 (6 H, m, –NH–CH_2–(CH_2)$_3$–CH_2–C(O)–O–), 1.34 (3 H, d, CH_3 β Ala, ^3J 7.5), 1.41 (9 H, s, CH_3 Boc), 2.30 (2 H, t, –CH_2–C(O)–O–, ^3J 7.5), 3.16–3.24 (3 H, m, –NH–CH_2– + OH), 4.04–4.37 (4 H, m, –O–CH_2–C*H*(OH)–CH– + CH α Ala), 4.66 (1 H, d, –O–CH_2–CH(OH)–C*H*–, ^3J 2.5), 5.07–5.24 (5 H, m, 2 × –O–CH_2–C_6H_5 + NH), 5.38 (1 H, d, CH α Phg, ^3J 7.5), 6.45 (1 H, br s, NH), 6.64 (1 H, br s, NH), 7.19–7.38 (15 H, m, ArH). *m/z* (ES) 774 $(M+H)^+$, calc. for $C_{42}H_{51}N_3O_{11}$ 773.87. The *N*-Boc protecting group of the previous compound (2.40 g, 1.0 equiv., 3.10 mmol) was removed under acidic conditions according to a procedure similar to that described previously for the first step of the synthesis of compound **7**. The resulting TFA salt **18** was quantitatively isolated as a white solid (2.50 g). δ_H (250 MHz, $CDCl_3$) 1.13–1.46 (9 H, m, –NH–CH_2–(CH_2)$_3$–CH_2–C(O)–O– + CH_3 β Ala), 2.20 (2 H, br s, –CH_2–C(O)–O–), 3.02–3.14 (2 H, m, –NH–CH_2–), 4.02–4.29 (4 H, m, –O–CH_2–C*H*(OH)–CH– + CH α Ala), 4.63 (1 H, d, –O–CH_2–CH(OH)–C*H*–, ^3J 2.5), 4.99 (2 H, s, –O–CH_2–C_6H_5), 5.07–5.21 (2 H, m, –O–CH_2–C_6H_5), 5.61–5.64 (2 H, m, CH α Phg + OH), 7.18–7.36 (15 H, m, ArH), 7.71 (1 H, br s, NH), 8.13 (2 H, br s, NH_2), 8.55 (1 H, br s, NH). *m/z* (ES) 674 $(M+H)^+$, calc. for $C_{37}H_{43}F_3N_3O_9$ 673.75 and $C_{37}H_{43}F_3N_3O_9{\cdot}CF_3COOH$ 787.78.

6-N-*{*N-*[*N-*(3,5-Difluorophenylacetyl)-L-alanyl]-L-phenylglycyl}-aminocaproic Acid, **19***

The title compound **19** was synthesized according to a two-step sequence. The first step was performed according to a procedure similar to that described previously for the synthesis of **1** by using BOP as a coupling agent. The corresponding 3,5-difluorophenylacetyl ascorbic acid ester derivative was isolated as a white solid (800 mg, yield: 73%) after flash chromatography (EtOAc). (Found: C 65.2, H 5.9, N 5.0%. $C_{45}H_{47}F_2N_3O_{10}$ requires C 65.3, H 5.7, N 5.1%.) R_f 0.47 (EtOAc). δ_H (250 MHz, $CDCl_3$) 1.11–1.63 (9 H, m, –NH–CH_2–(CH_2)$_3$–CH_2–C(O)–O– + CH_3 β Ala, ^3J 7.5), 2.18–2.33 (2 H, m, –CH_2–C(O)–O–), 2.93–3.30 (3 H, m, –NH–CH_2– + OH), 3.51 (2 H, br s, –CH_2–C(O)–NH), 4.06–4.36 (4 H, m, –O–CH_2–C*H*(OH)–CH– + CH α Ala), 4.71 (1 H, d, –O–CH_2–CH(OH)–C*H*–, ^3J 3.1), 5.05 (2 H, br s, –O–CH_2–C_6H_5), 5.11 (1 H, d, –O–C*H*H–C_6H_5, ^2J 10.9), 5.23 (1 H, d, –O–CH*H*–C_6H_5, ^2J 10.9), 5.74 (1 H, d, CH α Phg, ^3J 7.8), 6.60 (3 H, m, ArH), 7.18–7.44 (15 H, m, ArH), 7.81 (1 H, br s, NH), 7.99 (1 H, br s, NH), 8.50 (1 H, br s, NH). *m/z* (ES) 828 $(M+H)^+$, calc. for $C_{45}H_{47}F_2N_3O_{10}$ 827.87. The previous ester derivative (200 mg, 1.0 equiv., 0.24 mmol) was smoothly hydrolyzed by LiOH (13 mg, 1.5 equiv., 0.36 mmol) in a Pr^iOH/H_2O (7 : 3, v/v) solution of 0.8 M $CaCl_2$ (10 mL). The reaction mixture was stirred for 2 h 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 the addition of aqueous 1 M HCl 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 **19** which was used without any further purification (68 mg, yield: 58%). (Found: C 61.6, H 6.1, N 8.4%. $C_{25}H_{29}F_2N_3O_5$ requires C 61.3, H 6.0, N 8.6%.) δ_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, CH α Ala), 5.58 (1 H, m, CH α Phg), 6.87–7.15 (3 H, m, ArH), 7.25–7.55 (5 H, m, ArH). *m/z* (ES) 490 $(M+H)^+$, calc. for $C_{25}H_{29}F_2N_3O_5$ 489.51.

6-N-*{*N-*[*N-*(3,5-Difluoromandelyl)-L-alanyl]-L-phenylglycyl}-aminocaproic Acid, **20***

The title compound **20** was also synthesized according to a two-step synthetic sequence. The first step involved the use of a methodology similar to that in the procedure described previously for the synthesis of the mandelic analogue **16a/16b** of DAPT. The desired 3,5-difluoromandelyl ascorbic acid ester derivative was isolated as a racemic mixture (200 mg, yield: 26%). The diastereomers of the resulting racemic mixture were not separated (Found: C 63.9, H 5.4, N 5.1%. $C_{45}H_{47}F_2N_3O_{11}$ requires C 64.1, H 5.6, N 5.0%.) R_f 0.20 (EtOAc/hexane, 3 : 1). δ_H (250 MHz, $CDCl_3$) 1.09–1.67 (9 H, m, –NH–CH_2–(CH_2)$_3$–CH_2–C(O)–O– + CH_3 β Ala), 2.17–2.32 (3 H, m, –CH_2–C(O)–O– + O*H*), 2.96–3.27 (2 H, m, –NH–CH_2–), 3.99–4.31 (4 H, m, –O–CH_2–C*H*(OH)–CH + CH α Ala), 4.66 (1 H, d, O–CH_2–CH(OH)–C*H*, ^3J 1.7), 5.02 (2 H, s, –O–CH_2–C_6H_5), 5.06–5.22 (5 H, m, –O–CH_2–C_6H_5 + –C*H*(O*H*)– + NH), 5.57–5.61 (1 H, m, CH α Phg), 6.62–7.03 (3 H, m, ArH), 7.18–7.35 (16 H, m, ArH + NH), 8.05 (1 H, br s, NH). *m/z* (ES) 844 $(M+H)^+$, calc. for $C_{45}H_{47}F_2N_3O_{11}$ 843.87. The previous ascorbic acid derivative (151 mg, 1.0 equiv., 0.18 mmol) was smoothly hydrolyzed in basic conditions according to a procedure similar to that described previously for the synthesis of the corresponding non mandelic analogue **19**. The carboxylic acid **20** was isolated as a white solid (20 mg, yield: 22%) (Found: C 59.4, H 5.6, N 8.2%. $C_{25}H_{29}F_2N_3O_6$ requires C 59.4, H 5.8, N 8.3%.) δ_H (250 MHz, CD_3OD) 1.18–1.90 (9 H, m, –NH–CH_2–(CH_2)$_3$–CH_2–C(O)–O– + CH_3 β Ala), 2.16–2.24 (2 H, m, –CH_2–C(O)–O–), 3.08–3.24 (2 H, m, –NH–CH_2–), 3.99–4.23 (1 H, m, CH α Ala), 5.35 (1 H, br s, C*H*(OH)–C(O)–NH–), 5.46 (1 H, m, CH α Phg), 6.78–7.10 (3 H, m, ArH), 7.22–7.45 (5 H, m, ArH). *m/z* (ES) 506 $(M+H)^+$, calc. for $C_{25}H_{29}F_2N_3O_6$ 505.51.

BACE-1 Enzymatic Assay

These experiments were preformed using a BACE-1 (γ-secretase) FRET Kit assay, from PanVera Corp. (Madison, WI), according to the described protocol and using a multiwell spectrofluorometer instrument capable of 530–545 nm excitation and 570–590 nm emission wavelengths (Victor^2 1420, Wallac). The procedure was as follows: the substrate and enzyme were diluted according to the described protocol into the

provided assay buffer (50×10^{-3} M Tris, pH 7.5, 10% glycerol). Each inhibitor was diluted into DMSO at the desired concentration. The substrate (Rh-EVNLDAEFK-Quencher; 10 μL of the main solution) and each inhibitor (1 μL of the corresponding solution) were introduced in a 96-well flat bottom black polystyrene plate (Corning). The resulting mixtures were gently mixed and 10 μL of the enzyme solution was then added to each well to start the reaction. The reaction mixtures were incubated at 25°C for 90 min and the fluorescence was monitored at 530–545 nm (excitation wavelength) and 570–590 nm (emission wavelength). The kinetic assays were performed in duplicate for each inhibitor, using BACE-1 inhibitor (H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-3*S*,4*S*-Stat-Val-Ala-Glu-Phe-OH, $IC_{50} = 30 \times 10^{-9}$ M; Calbiochem) as reference (negative test, no cleavage), the provided BACE-1 Product Standard (Rh-EVNL) as a positive test (100% cleavage), and a control test using only the enzyme and the substrate under the same conditions to allow a 15% cleavage of the substrate after 90 min.

Acknowledgments

INSERM and Conseil Régional Provence-Alpes-Côte d'Azur are greatly acknowledged for financial support (fellowships for N.P. and C.G.). We are grateful to Dr Philippe Pierre and members of his research team (CIML, Inserm-CNRS, Université de la Méditerranée, France) for their assistance when we performed the spectrofluorimetric measurements for the BACE-1 (β-secretase) FRET assays. The authors thank Prof. Keith Dudley (INSERM U-623) for manuscript preparation.

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