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 active biological molecules to specific sites or organs based on delivery systems which represent a systematic way of targeting engineered cells secreting a drug or a polymeric matrix containing the hematoencephalic barrier.

Supported by the design of prodrugs. These prodrugs involve the conjugation of a non-transportable drug to a lipophilic system; such as: liposomes exploiting receptor mediated transcytosis or chemical drug delivery systems uses the biochemical properties of ascorbic acid (AA), where it can then be protected brain tissues in a wide variety of CNS pathologies and eventually it may protect against cognitive impairment and reduce the risk for development of Alzheimer’s disease (AD). Selective 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, neuronal dysfunction and possibly inflammation and oxidative damage, with neurodegeneration and dementia as final outcome. 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.

Numerous very potent inhibitors of γ-secretase have been described so far. N-(3,5-Difluorophenylacetyl)-(S)-alanyl-(S)-phenylglycine tert-butyler 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. Nevertheless, various studies suggest caution in the testing of γ-secretase inhibitors in humans and unwanted
side-effects could be observed during long-term treatment in humans. 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 \( \gamma \)-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 \( \gamma \)-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 \( \alpha \)-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 \( \alpha \)-amino acid spacer. It was first \( \alpha \)-\( N \)-protected before esterification of the terminal carboxylic acid function. This esterification reaction was performed using 2,3-di-O-benzyl-L-ascorbic acid as primary alcohol entity. This alcohol was itself synthesized according to a well-documented three-step synthetic sequence. The \( N \)-Boc protecting group was removed in acidic conditions and the resulting TF A salt was quantitatively isolated. This TF A 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 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

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

**Scheme 2** Conditions and reagents: (i) Boc\(_2\)O, 1 M NaOH, dioxane–H\(_2\)O (v/v, 2 : 1), quantitative; (ii) 2,3-di-O-benzyl-L-ascorbic acid 5, BOP, DIEA, CH\(_2\)Cl\(_2\), rt; 57%; (iii) TFA, CH\(_2\)Cl\(_2\), rt; quantitative.
analogue of the target molecule 2. This analogue 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 via a hydrolysable ester bond. Prior to any in vivo study, it was of interest to investigate the stability of the synthesised 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 ($\lambda_{max} = 266 \text{ nm}$, $\varepsilon \approx 10\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ (H$_2$O)) while the non ascorbic carboxylic analogue 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 compound disappeared, indicating that compound 2 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 tract 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 nipeotic acid increased the latency of induced convulsions in mice. However, ascorbic acid derivative of nipeotic acid increased the latency of induced convulsions in mice.

**Cell free Aβ peptide production inhibitory activities**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aβ production</th>
<th>log $P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (DAPT)</td>
<td>50% (10 nM)$^a$</td>
<td>3.07</td>
</tr>
<tr>
<td>2</td>
<td>36% (1 µM)$^d$</td>
<td>0.63</td>
</tr>
<tr>
<td>8</td>
<td>42% (1 µM)$^d$</td>
<td>2.19</td>
</tr>
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$^a$ Potency determination for the ability of compounds to reduce total Aβ production from HEK293 cells. $^b$ Potency determination to reduce Aβ$_{40}$ production. $^c$ Potency determination to reduce Aβ$_{42}$ 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 H2O2 into its DHAA analog DHAA, 2. It can be observed that compound 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{\text{concentration of drug in brain (ng/g of brain)}}{\text{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. 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. 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.

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. 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. 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 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 Service Central d’Analyses du CNRS (Vesoul, 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 (60F254, 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 mc2 spectrophotometer (Safas, Monaco).

N-[N-(3,5-Difluorophenylacetyl)-t-]alanyl-l-t-phenylglycine tert-butyl ester (DAPT), 1

N-Carbobenzyloxy-carbonyl-t-alanine (2.00 g, 1.0 equiv., 8.96 mmol) was dissolved in freshly distilled CH2Cl2 (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 CH2Cl2 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 reaction mixture was cooled to 0 °C (1.56 mL, 8.96 mmol) was added dropwise. The reaction mixture was stirred overnight at room temperature under H2 (2.40 mL, 17.40 mmol) of DIEA was added dropwise. The reaction mixture was allowed to warm and stirred overnight at room temperature and then once again cooled to 0 °C. 1.09–1.55 (15 H, m, C–H CH2–), 2.20 (2 H, t, –CH2–N), 4.40 (1 H, broad s, N=CH), 6.68–6.85 (3 H, m, ArH), 7.14 (1 H, d, NH Phg, J = 7.3 Hz), 7.25–7.35 (5 H, m, ArH). MS-ES m/z 433 (M + H)+, calc for C23H23F2N2O4 432.46 g mol−1.

N-[N-(3,5-Difluorophenylacetyl)-t-]alanyl-l-phenylglycine tert-butyl ester (DAPT), 1

The tert-butyl ester DAPT (1.0 equiv., 3.40 g, 7.66 mmol) was dissolved in CH2Cl2 (40 mL). Trifluoroacetic acid (5.9 mL, 50.0 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 EtOAc. 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. C19H21NO4 requires C, 60.64; H, 4.82; N, 7.44%). δH (250 MHz, DMSO-d6) 1.26 (3 H, d, CH β Ala, J = 7.0 Hz), 3.51 (2 H, s, -CH2–C(O)–NH), 4.11–4.47 (1 H, m, CH a Al), 5.30 (1 H, d, CH a Phg, J = 7.5 Hz), 6.97–7.50 (8 H, m, ArH), 8.38 (1 H, d, NH Phg, J = 7.5 Hz), 8.63 (1 H, d, NH Ala, J = 7.0 Hz), 12.68 (1 H, broad s, NH). MS-ES m/z 377 (M + H)+, calc for C25H20F2N2O6 376.35 g mol−1.

6-N-(tert-Butyloxy carbonyl) aminoacaproic acid, 4

6-Aminocaproic acid (2.62 g, 1.0 equiv., 20.0 mmol) was dissolved in a dioxane-H2O (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 Boc2O (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 combined organic layers were dried over anhydrous MgSO4, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 1:1) to give 4 as a white solid (2.50 g, yield: 60%). δH (250 MHz, DMSO-d6) 1.19–1.59 (15 H, m, C–H CH2–), 2.20 (2 H, t, –CH2–NH), 4.20 (1 H, broad s, NH). MS-ES m/z 372 (M + H)+, calc for C25H20N2O6 371.39 g mol−1.

2,3-Di-O-benzyl-l-ascorbic acid, 5

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 mL, 0.05 equiv., 0.29 mmol) was then added and the 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. C10H12O6 requires C, 50.00; H, 5.59%). Rf 0.45 (EtOAc–hexane 4:1). δH (250 MHz, DMSO-d6) 1.79–1.93 (3 H, m, CH3–), 2.76–2.94 (4 H, t, –CH2–CH2–), 7.09–7.41 (5 H, m, ArH). MS-ES m/z 393 (M + H)+, calc for C14H14O6 392.08 g mol−1.
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 EtO (30 mL). The organic layer was washed with brine (30 mL) and the aqueous layer was extracted twice with EtO (2 × 30 mL). The combined organic layers were dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography (EtOAc–hexane 1 : 4 then 1 : 1) to afford the desired compound.

6-N-(tert-Butyloxy carbonyl)-aminocaproic acid derivative 4 (0.80 g, 1.0 equiv., 3.46 mmol) was dissolved in freshly distilled CH₂Cl₂ (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 (200 µL) was added. The reaction mixture was stirred for 1 hour at room temperature and then once again cooled to 0 °C. A CH₂Cl₂ solution (30 mL) of 1.1 equiv. (1.36 g, 3.81 mmol) of alcohol 5 and 2 equiv. (3.12 mL, 17.92 mmol) of DIEA was added dropwise. The solution was stirred 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), 5% aqueous NaHCO₃ (3 × 50 mL) and brine (50 mL), was dried over anhydrous MgSO₄, 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, 2.55. C₁₇H₂₂NO₄ requires C, 65.36; H, 6.90; N, 2.46%). Rf 0.50 (EtOAc–hexane 1 : 1). δ₁₂ (250 MHz, CDCl₃) 1.26–1.71 (15 H, m, CH₃, C₆H₅), 1.95–2.16 (2 H, t, -CH₂-C₆H₅), 3.35 (2 H, d, -O(CH₂)₂-C₆H₅), 4.05 (1 H, dd, -O-CH₂-), 7.10–7.27 (15 H, m, ArH). MS-ES m/z 357 (M + H)⁺, calcd for C₁₇H₂₂NO₄ 356.37 g mol⁻¹.

6-Aminocaproic 2,3-di-O-benzyl-1-ascorbic acid triluroacetic acid salt, 6

6-N-(tert-Butyloxy carbonyl)-aminocaproic acid 4 (0.80 g, 1.0 equiv., 3.46 mmol) was dissolved in freshly distilled CH₂Cl₂ (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 (200 µL) was added. The reaction mixture was stirred for 1 hour at room temperature and then once again cooled to 0 °C. A CH₂Cl₂ solution (30 mL) of 1.1 equiv. (1.36 g, 3.81 mmol) of alcohol 5 and 2 equiv. (3.12 mL, 17.92 mmol) of DIEA was added dropwise. The solution was stirred 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), 5% aqueous NaHCO₃ (3 × 50 mL) and brine (50 mL), was dried over anhydrous MgSO₄, 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, 1.0 equiv., 2.0 mmol) was dissolved in CH₂Cl₂ (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 EtO and the title compound 6 was quantitatively isolated as a white solid (1.15 g, 0.20 mmol). δ₁₂ (250 MHz, CDCl₃) 1.24–1.78 (15 H, m, CH₂-C₆H₅), 1.95–2.16 (2 H, t, -CH₂-C₆H₅), 3.35 (2 H, d, -O(CH₂)₂-C₆H₅), 4.05 (1 H, dd, -O-CH₂-), 7.10–7.27 (15 H, m, ArH). MS-ES m/z 740 (M + H)⁺, calcd for C₁₇H₂₂NO₄ 743.35 g mol⁻¹ and for C₁₇H₂₂O₇, CF₃COOH 583.55 g mol⁻¹.

6-[(N-([3,5-Difluorophenylacetyl]-l-alanyl)-l-phenylglyclyl]-aminocaproic 2,3-di-O-benzyl-1-ascorbic acid, 7

The title compound was synthesized according to a similar procedure that described previously for the synthesis of DAPT by using BOP as coupling agent. Compound 7 was isolated as a white solid (0.90 g, yield: 73%) after flash chromatography (EtOAc) (Found: C, 65.29; H, 5.72; N, 5.08%). Rf 0.47 (EtOAc). δ₁₂ (250 MHz, CDCl₃) 1.11–1.63 (9 H, m, -NH-CH₂-(CH₂)₆-N=C(O)-OH), 1.80–2.23 (2 H, m, -CH₂-C₆H₅), 3.93–3.30 (3 H, m, -NH-CH₂- + OH) 3.51 (2 H, broad s, -CH₂-C₆H₅)-N=N), 4.06–4.36 (4 H, m, -O-CH₂-(CH₂)₂-C₆H₅-C(O)-CH₂-Na). 7.41 (1 H, d, -O-CH₂-(CH₂)₆C₆H₅-C(O)-NH). 3.70–3.51 (7 H, m, ArH). MS-ES m/z 828 (M + H)⁺, calcd for C₁₇H₂₀F₂N₂O₁₁ 827.87 g mol⁻¹.

6-[(N-([3,5-Difluorophenylacetyl]-l-alanyl)-l-phenylglyclyl]-aminocaproic 1-ascorbic acid, 2

The protected ascorbic acid derivative 2 (200 mg, 1.0 equiv., 0.24 mmol) was dissolved in 3 mL of MeOH. 10% weight in percentage 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 H₂ atmosphere for 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. C₁₇H₁₇F₂N₂O₁₁ requires C, 57.49; H, 5.45; N, 6.49%). δ₁₂ (250 MHz, CDCl₃) 1.22–1.63 (9 H, m, -NH-CH₂-(CH₂)₆-N=C(O)-OH), 1.79–2.34 (2 H, m, -CH₂-C₆H₅), 3.06–3.21 (2 H, m, -NH-CH₂-), 3.38 (2 H, broad s, -CH₂-C₆H₅)-N=N), 4.05–4.41 (4 H, m, -O-CH₂-(CH₂)₂-C₆H₅-C(O)-CH₂-Na). 7.41–4.73 (1 H, m, CH₂-C₆H₅-C(O)-NH). 6.68–6.49 (3 H, m, ArH). MS-ES m/z 618 (M + H)⁺, calcd for C₁₇H₁₇F₂N₂O₁₁ 617.42 g mol⁻¹.
a pH 7.4 solution of CaCl₂ (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 H₂O (5 mL) and washed once with CH₂Cl₂ (10 mL). The aqueous layer was acidified with pH 1 by addition of aqueous HCl 1 M and extracted with EtOAc (3× 20 mL). The combined organic layers were dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure to afford the carboxylic acid derivative B which was used without any further purification (68 mg, yield: 58%) (Found: C 61.3; H 5.97; N 8.58%). δH (250 MHz, CDCl₃) 1.25-1.67 (9 H, m, -NH-CH₂(-CH₃)-CH₂-C(O)-O- + CH₂β, Ala), 2.21-2.62 (2 H, m, -CH₂-C(O)-O-), 3.05-3.14 (2 H, m, -NH-CH₂-), 3.55 (2 H, s, -CH₂-C(O)-NH-), 4.02-4.28 (1 H, m, CHα Ala), 5.58 (1 H, broad s, CHα Phg), 6.87-7.15 (3 H, m, ArH), 7.25-7.55 (5 H, m, ArH). MS-ES m/z 490 (M + H)+, calc for C₂₂H₂₅F₂N₃O₅ 489.51 g mol⁻¹.

6-[(N-[N-(3,5-Difluorophenacyl)-1-alamyl]-L-phenylglycyl]aminocaproyl 1-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 H₂O₂ 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 90% 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. The tested compound 2 was dissolved over anhydrous MgSO₄, filtered and concentrated under reduced pressure to afford the carboxylic acid derivative B which was used without any further purification (68 mg, yield: 58%) (Found: C 61.3; H 5.97; N 8.58%). βH (250 MHz, CDCl₃) 1.25-1.67 (9 H, m, -NH-CH₂(-CH₃)-CH₂-C(O)-O- + CH₂β, Ala), 2.21-2.62 (2 H, m, -CH₂-C(O)-O-), 3.05-3.14 (2 H, m, -NH-CH₂-), 3.55 (2 H, s, -CH₂-C(O)-NH-), 4.02-4.28 (1 H, m, CHα Ala), 5.58 (1 H, broad s, CHα Phg), 6.87-7.15 (3 H, m, ArH), 7.25-7.55 (5 H, m, ArH). MS-ES m/z 490 (M + H)+, calc for C₂₂H₂₅F₂N₃O₅ 489.51 g mol⁻¹.

BBB permeation evaluation³,⁶

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.


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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