**Enzymatic and solid-phase synthesis of new donepezil-based
L- and D-glutamic acid derivatives and their pharmacological
evaluation in models related to Alzheimer's disease and
cerebral ischemia**

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Abstract

Previously, we have described *N*-Bz-L-Glu[*NH*-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-*n*Hex (IQM9.21, **L-1**) as an interesting multifunctional neuroprotective compound for the potential treatment of neurodegenerative diseases. Here, we describe new derivatives and different synthetic routes, such as chemoenzymatic and solid-phase synthesis, aiming to improve the previously described route in solution. The lipase-catalysed amidation of L- and D-Glu diesters with *N*-benzyl-4-(2-aminoethyl)piperidine has been studied, using *Candida antarctica* and *Mucor miehei* lipases. In all cases, the α -amidated compound was obtained as the main product, pointing out that regioselectivity was independent of the reacting Glu enantiomer and the nature of the lipase. An efficient solid-phase route has been used to produce new donepezil-based L- and D-Glu derivatives, resulting in good yield. At micromolar concentrations, the new compounds inhibited human cholinesterases and protected neurons against toxic insults related to Alzheimer's disease and cerebral ischemia. The CNS-permeable compounds *N*-Cbz-L-Glu(OEt)-[*NH*-2-(1-benzylpiperidin-4-yl)ethyl] (**L-3**) and **L-1** blocked the voltage-dependent calcium channels and **L-3** protected rat hippocampal slices against oxygen-glucose deprivation, becoming promising anti-Alzheimer and anti-stroke lead compounds.

Keywords: Donepezil-based L- and D-Glu derivatives; human cholinesterases; neuroprotection; mitochondrial oxidative stress; Alzheimer's disease; stroke

Highlights

- Enzymatic and solid-phase synthesis of new donepezil-based L- and D-Glu derivatives
- Micro- and sub-micromolar inhibition of human cholinesterases
- Neuroprotection in cellular models of Alzheimer's disease and stroke
- Blockade of voltage-dependent calcium channels in a human neuroblastoma cell line

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; Alloc, allyloxycarbonyl group; β A, beta-amyloid peptide; BuChE, butyrylcholinesterase; CAL-B, *Candida antarctica* lipase B; Cbz, benzyloxycarbonyl group; CNS, central nervous system; DMAP, 4-(dimethylamino)pyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fmoc, fluorenylmethylenoxy group; HOBt, 1-hydroxybenzotriazole; HPLC-MS, high performance liquid chromatography joined to mass spectrometry; MML, *Mucor miehei* lipase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBP-NH₂, *N*-benzyl-4-(2-aminoethyl)piperidine; NOX, NADPH oxidase; OGD/R, oxygen and glucose deprivation followed by re-oxygenation; ORAC, oxygen radical absorbance capacity; PAMPA-BBB, parallel artificial membrane permeation assay for the blood-brain barrier; PSL, *Pseudomonas sp.* lipase; R/O, rotenone plus oligomycin A; SH-SY5Y, human neuroblastoma cell line; TFA, trifluoroacetic acid; VDCC, voltage-dependent calcium channels.

1. Introduction

As a consequence of the increasing life expectancy, Alzheimer's disease (AD) is currently a serious familiar, social, and economic problem [1]. AD is a complex neurodegenerative pathology occurring in the central nervous system (CNS), characterized by deposits of aberrant proteins namely β -amyloid (β A) and τ -protein, loss of synapses, and neuronal cell death, especially of cholinergic neurons. Nowadays, mitochondrial dysfunction and the subsequent oxidative stress are thought to be primary events in the complex and still non-completely known AD aetiology [2]. Over the last decades, several rational research strategies have been envisaged [3-5], but the current therapeutic options for the treatment of AD are limited to three acetylcholinesterase (AChE) inhibitors, namely donepezil, rivastigmine, and galantamine, and the *N*-methyl-D-aspartate receptor antagonist memantine. Rivastigmine and galantamine are used for the treatment of mild-to-moderate AD, and memantine was approved for the management of moderate-to-severe AD [6,7]. Donepezil (Figure 1) is today the most used AChE inhibitor to treat mild, moderate, and severe AD [8].

Several studies have shown that in healthy brains AChE hydrolyses the majority of acetylcholine (ACh), while butyrylcholinesterase (BuChE) plays a secondary role. However, as AD progresses, cortical AChE activity decreases while that of BuChE significantly increases [9]. Therefore, both enzymes are involved in the regulation of ACh and are good therapeutic targets in the search for new drugs to increase cholinergic levels [10,11]. In this regard, recent clinical trials have demonstrated that patients treated with rivastigmine, a non-selective inhibitor of AChE and BuChE, showed minor cortical atrophic changes and attenuated loss of brain volumes [12,13].

Although many aspects of AD pathology remain unclear, increasing evidence supports a pivotal role for mitochondrial dysfunctions, such as oxidative stress or activation of unfolded protein response genes, in the pathogenesis and progression of AD [14,15]. Post-mortem studies showed significantly increased levels of peroxidation of lipids, proteins, and nucleic acids in vulnerable regions of the brain of patients with severe AD [16]. Moreover, enhanced oxidative damage in cellular structures was observed as an event preceding the appearance of other pathological hallmarks of AD, namely amyloid plaques and neurofibrillary tangles [17]. These observations suggest that drugs able to protect neurons from oxidative stress could be useful for either the prevention or the treatment of AD [18].

On the other hand, several age- and AD-related alterations in specific calcium-regulating systems have been observed in brain cells, such as elevated intracellular calcium concentration, enhanced influx through voltage-dependent calcium channels (VDCC) and impaired ability of mitochondria to regulate homeostasis of this cation [19,20].

The complexity of AD and other neurodegenerative diseases has encouraged the active research for multifunctional molecules with two or more complementary biological activities, because they should be more effective than a ligand oriented to a single target [21-26]. Following this approach, in the last years we have reported several families of multifunctional compounds combining neurogenic, protective, antioxidant, cholinergic, and β A-reducing properties [27-34].

Among the above families, the donepezil-based hybrids carrying an L-glutamic acid (L-Glu) scaffold displayed interesting and complementary pharmacological properties. They efficiently inhibit human AChE and BuChE, protect neurons against damage

caused by mitochondrial free radicals, and are predicted to penetrate into the CNS by passive diffusion [34]. *N*-Bz-L-Glu[*NH*-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-*n*Hex (IQM-9.21, L-1, Figure 1) was selected for further pharmacological assays, displaying VDCC blockade [35], infarct volume reduction in a photothrombotic stroke-model in mice [36] and neuroprotection against sodium and calcium overload in motor neuron-like NSC-34 cells, as a model of amyotrophic lateral sclerosis [37].

Figure 1. Structures of donepezil, IQM-9.21 (L-1), and new L- and D-Glu derivatives

In our previous work, the synthesis of the L-Glu series was carried out following a classical 5-steps route in solution, involving protection-deprotection reactions. Although the products were obtained in acceptable overall yield (15-55%), some intermediates showed purification problems, requiring several chromatographic separations prior to their use [34]. With the aim of obtaining new L- and D-Glu *N*-benzylpiperidine derivatives related to L-1 and simplifying the previously described route in solution, in this work two different synthetic methodologies have been studied, namely enzymatic catalysis and solid-phase synthesis. The new derivatives have been evaluated as inhibitors of human cholinesterases, and as neuroprotective agents against

mitochondrial oxidative stress using two different models: the mixture of rotenone and oligomycin A, and the oxygen-glucose deprivation. We also studied the *in vitro* brain penetration of the new compounds and the effects of *N*-Cbz-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-ethyl (L-3) in VDCC and in a tissue model of cerebral ischemia, compared to the reference compound L-1.

2. Results and discussion

2.1. Chemoenzymatic synthesis

We explored the effectiveness of several hydrolases such as *Candida antarctica* lipase B (CAL-B), *Pseudomonas sp.* lipase (PSL), and *Mucor miehei* lipase (MML) in the amidation of L- and D-Glu diesters with *N*-benzyl-4-(2-aminoethyl)piperidine (NBP-NH₂). Reactions were performed at analytical scale in anhydrous diisopropylether using NBP-NH₂ (50 mM), Glu derivative (20 mM), enzyme (50 mg/mL), molecular sieves 4 Å (50 mg/mL), and acetanilide (3 mg/mL) as internal standard. Samples were stirred at 45 °C and the reaction progression was monitored by HPLC-MS.

Initially, we tested the amidation of *N*-Bz-D-Glu(OHex)-OHex with NBP-NH₂ in the presence of CAL-B, PSL or MML. After 72 h, no reaction was detected with PSL or MML, and only in the case of CAL-B low conversion was observed by HPLC-MS, remaining more than 90% of the starting material. When the above reaction was repeated using double concentration of both CAL-B and molecular sieves (100 mg/mL) no significant improvement in conversion was observed.

As the length of the *n*-hexyl chain could hinder the interaction of the D-Glu derivative with the active site of CAL-B, we envisaged the use of the diethylester of *N*-

Bz-D-Glu (**D-2**) as substrate for the enzymatic reactions. The treatment of *N*-Bz-D-Glu(*OE*t)-*OE*t with NBP-NH₂ in the presence of CAL-B during 24 h displayed better conversion (~ 30%), which did not improve substantially after 72 h. For this reason, we decided to replace the benzoyl fragment by the benzyloxycarbonyl (Cbz) group, which could be better substrate for lipase reactions according to our previous results [38].

Reaction of *N*-Cbz-D-Glu(*OE*t)-*OE*t (**D-2**) with NBP-NH₂ in the presence of CAL-B during 48 h yielded 88% of a monoamidated product, as detected by HPLC-MS. On the basis of this positive result, we performed a small-scale comparative study on **D-2** and its enantiomer **L-2** with CAL-B, MML and PSL (see Experimental Part for further details). By using PSL no reaction was detected, but in the case of CAL-B and MML we observed the formation of a monoamidated derivative as the main product of the enzymatic transformation. In the case of **L-2** no starting material was observed, whereas about 10% of starting material remained unchanged for **D-2**.

Then, the enzymatic reactions of **L-2** and **D-2** with CAL-B and MML were repeated, but in a preparative scale (without internal standard) to isolate and identify the products. Reactions using **D-2** as starting material were accomplished during 92 h, while in the case of **L-2** the enzymatic transformations were run for 24 h. Once the reactions were completed, enzyme and molecular sieves were removed by filtration and products were purified by column chromatography on silica gel, obtaining pure **L-3** and **D-3** derivatives in 32-46% yield (Table 1). In all cases, the α -monoamidated derivative was obtained, as deduced from two-dimensional correlation experiments in NMR (see supplementary information for further details).

Table 1. Summary results of the lipase-catalysed preparative reactions

Substrate	Lipase	Time (h)	Product	Yield (%) ^a
L-2	CAL-B	24	L-3	46
L-2	MML	24	L-3	41
D-2	CAL-B	92	D-3	32
D-2	MML	92	D-3	36

^aYield of isolated pure product

The fact of obtaining the α -monoamide as the main product of the enzymatic reactions could be rationalized on the basis of our previous studies. In a previous paper about the amidation of D-Glu diesters with *n*-pentylamine, we described the lower reaction rate of these substrates and the minor regioselectivity compared to their L-enantiomers, obtaining a mixture of α - and γ -pentylamides [38]. It may be expected that the bulkier nucleophile NBP-NH₂ instead of *n*-pentylamine, reduced even more the reaction rate of the γ -ester and, consequently, α -monoamide was the main product formed.

Comparing with the previously described 5-steps route in solution, enzymatic synthesis with CAL-B or MML provided similar overall yield (32-46%) and the advantage of involving only one synthetic step. Moreover, and as a result of the high enzyme regioselectivity, this methodology is suitable to obtain L- or D-Glu derivatives with a donepezil-derived fragment in the α -position, without the use of any protecting group.

2.2. Solid-phase synthesis

To obtain the L- and D-Glu derivatives with an *N*-benzylpiperidine fragment in the γ -position, a solid-phase synthesis was developed, using Wang resin (Scheme 1). The starting materials were *N*-Fmoc-L-Glu(*O*All)-OH or *N*-Fmoc-D-Glu(*O*All)-OH, which allow an orthogonal synthetic strategy. The fluorenylmethylenoxy group (Fmoc) can be easily removed under basic conditions, and the allyloxycarbonyl group (Alloc) can be deprotected under neutral conditions.

Thus, Wang resin was coupled to *N*-Fmoc-L-Glu(*O*All)-OH or *N*-Fmoc-D-Glu(*O*All)-OH, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-(dimethylamino)pyridine (DMAP), yielding intermediates *N*-Fmoc-L-Glu(*O*All)-*O*-resin (**L-4**) and *N*-Fmoc-D-Glu(*O*All)-*O*-resin (**D-4**), respectively. Afterwards, the Alloc protecting group was removed using tetrakis(triphenylphosphine)palladium(0) [Pd(PPh₃)₄] in the presence of phenylsilane (PhSiH₃) in dry dichloromethane under nitrogen atmosphere, and the resulting γ -free acids **L-5** and **D-5** were coupled with NBP-NH₂ in the presence of EDC, 1-hydroxybenzotriazole (HOBt), triethylamine, and DMAP. These reactions yielded intermediates *N*-Fmoc-L-Glu[*NH*-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-resin (**L-6**) and *N*-Fmoc-D-Glu[*NH*-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-resin (**D-6**), respectively. The next step was the Fmoc group cleavage with piperidine, providing intermediate resins **L-7** and **D-7**. Their unprotected amino group was reacted with different aromatic carboxylic acids, using the previous conditions (EDC, HOBt, Et₃N and DMAP) to give intermediates **L-(8-10)** and **D-8**. Finally, the cleavage of such resins with trifluoroacetic acid (TFA) afforded the carboxylic acids **L-(11-13)** and **D-11**, which were then purified by flash column chromatography and isolated in 23-76% overall yield (6 steps). The corresponding ethyl esters [**L-(14-17)** and **D-14**] or *n*-hexyl esters [**D-1** and **L-16**] were obtained by reaction with EtOH or *n*-HexOH and thionyl

chloride (SOCl_2) in moderate overall yield (18-46%, in 7 steps). Although this solid-phase synthesis involved 2 more steps than the previously described route in solution and overall yield were similar, it presented as advantages that intermediates' purification was not necessary and that desired final products were obtained rapidly in a high purity grade (above 80% by HPLC, after cleavage from the resin).

Scheme 1. Solid-phase synthesis of L- and D-Glu derivatives with an *N*-benzylpiperidine in the γ -position.

2.3. Inhibition of human cholinesterases

Firstly, compounds L-(**2,3,11-17**) and D-(**1-3,11,14**) were evaluated as inhibitors of human recombinant AChE (hAChE) and human plasmatic BuChE (hBuChE) using donepezil as reference and following the Ellman method [39]. Results are shown in table 2, in which we also included the previously described results of L-**1** for comparative purposes [34]. In general, the new donepezil-based L- and D-Glu derivatives L-(**3,11-17**) and D-(**1,3,11,14**) were good inhibitors of both hChEs with IC_{50} values in the low- and sub-micromolar range, whereas diethyl precursors L-**2** and D-**2** did not inhibit any enzyme.

From table 2 some structure – hChE's inhibition relationships can be derived, regarding the position of the *N*-benzylpiperidine moiety at the amino acid framework (α - or γ -), the influence of the amino acid chirality (L- or D-), and the nature of the carboxyl group not attached to the *N*-benzylpiperidine fragment (free acids, ethyl- or hexyl esters).

Compounds L- and D-**3**, containing an *N*-benzylpiperidine fragment in the α -carboxyl group, inhibited 10-fold better hBuChE than hAChE. However, the rest of L- or D-Glu derivatives **11-17**, bearing the *N*-benzylpiperidine group in the γ -position, displayed a clear preference for hAChE. These differences may be due to the fact that the *N*-benzylpiperidine moiety, responsible for hAChE-CAS binding, has more difficulties to reach the narrow catalytic gorge when is located in the α -position of the amino acid (L- and D-**3**) due to the higher steric hindrance of this part of the molecule. As the catalytic gorge of hBuChE is significantly bigger than that of hAChE [40], L- and D-**3** possibly have less difficulty to interact with hBuChE.

Comparing pairs of enantiomers L- or D-(**1,3,11,14**), chirality seemed to have little influence on the hAChE inhibition, with the exception of L-**11** that is about 2 orders of magnitude more active than D-**11**. Regarding hBuChE inhibition, D-Glu derivatives were better than their L-Glu counterparts, in some cases with a difference of one order of magnitude. Finally, ethyl- (**14**, **15**, and **17**) and hexyl ester derivatives (**1** and **16**) were better inhibitors of both hAChE and hBuChE than their corresponding free acid precursors (**11-13**). Moreover, all L-Glu or D-Glu acids **11-13** showed hBuChE IC₅₀ values above 10 µM, thus displaying a moderate selectivity towards hAChE, for which the IC₅₀ values were between 0.76 and 20.0 µM.

Table 2. Inhibitory activities of new compounds in hAChE and hBuChE [IC_{50} (μM)]; neuroprotection (%) elicited by compounds at 0.3 μM in the human neuroblastoma cell line SH-SY5Y, subjected to (i) the combination of rotenone (30 μM) and oligomycin A (10 μM) (R/O) or (ii) the oxygen-glucose deprivation followed by re-oxygenation (OGD/R); permeability values of compounds in the PAMPA-BBB assay (P_e , 10^{-6} cm s $^{-1}$) with their predictive penetration in the CNS^a.

Compd.	IC_{50} (μM)		Neuroprotection (%) vs.		PAMPA-BBB	
	hAChE	hBuChE	R/O	OGD/R	P_e (10^{-6} cm s $^{-1}$) ^c	Prediction
L-1	0.53 ± 0.02	1.75 ± 0.6	$44.1 \pm 1.5^{***}$	$53.4 \pm 1.7^{***}$	18.7 ± 1.6	cns+
D-1	0.89 ± 0.09	0.36 ± 0.04	$13.0 \pm 3.3^{**}$	$32.2 \pm 8.1^{**}$	13.9 ± 0.6	cns+
L-2	> 10	> 10	$37.3 \pm 5.6^{***}$	$51.6 \pm 8.5^{***}$	n.d.	n.d.
D-2	> 10	> 10	$34.7 \pm 4.9^{***}$	$47.1 \pm 9.8^{***}$	n.d.	n.d.
L-3	4.99 ± 0.16	0.40 ± 0.02	$35.2 \pm 4.7^{***}$	$42.7 \pm 6.8^{***}$	21.0 ± 0.2	cns+
D-3	3.00 ± 0.09	0.17 ± 0.01	$31.6 \pm 4.4^{***}$	$47.0 \pm 8.4^{***}$	16.7 ± 0.4	cns+
L-11	0.76 ± 0.11	> 10	$46.5 \pm 4.6^{***}$	$50.6 \pm 7.4^{***}$	3.5 ± 0.1	cns+/-
D-11	20.0 ± 0.8	82 ± 3	$22.6 \pm 2.1^{***}$	$38.5 \pm 8.6^*$	4.6 ± 0.1	cns+/-
L-12	6.4 ± 0.7	71 ± 2	$34.6 \pm 4.5^{***}$	$48.4 \pm 9.5^{***}$	1.8 ± 0.1	cns-
L-13	15.2 ± 0.5	97 ± 3	$35.0 \pm 2.3^{***}$	$45.0 \pm 7.0^{***}$	2.3 ± 0.1	cns-
L-14	0.85 ± 0.04	1.3 ± 0.2	$38.6 \pm 3.9^{***}$	$50.9 \pm 9.3^{***}$	5.2 ± 0.1	cns+/-
D-14	0.5 ± 0.2	0.39 ± 0.03	$15.1 \pm 2.7^{**}$	$39.8 \pm 6.9^*$	6.6 ± 0.2	cns+/-
L-15	1.9 ± 0.5	0.30 ± 0.04	$39.5 \pm 4.7^{***}$	$42.4 \pm 7.1^{***}$	4.5 ± 0.2	cns+/-
L-16	3.04 ± 0.12	1.85 ± 0.07	$22.1 \pm 3.9^{**}$	$31.8 \pm 7.1^{**}$	9.6 ± 0.3	cns+
L-17	0.89 ± 0.08	3.8 ± 0.9	$25.3 \pm 4.4^{**}$	$37.7 \pm 6.7^{**}$	8.3 ± 0.2	cns+
Donepezil	0.010 ± 0.002	2.50 ± 0.07	n.d.	n.d.	n.d.	
Melatonin	n.d.	n.d.	$33.4 \pm 4.0^{***}$	$31.4 \pm 3.2^{**}$	n.d.	

^aResults are the mean $\pm$ SEM of triplicates of at least four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.d.: not determined.

2.4. Cell viability and neuroprotection studies in a human neuroblastoma cell line.

Evaluation of antioxidant properties

To explore the therapeutic potential of L- and D-Glu *N*-benzylpiperidine derivatives (**1-3**, **11-17**), we studied cell viability and neuroprotective effects against oxidative stress in the human neuroblastoma cell line SH-SY5Y. Two toxic models were used: (i) the mixture of rotenone (30 μ M) and oligomycin A (10 μ M) (R/O) and (ii) oxygen and glucose deprivation (4 h) followed by re-oxygenation (20 h) (OGD/R).

Rotenone and oligomycin A block mitochondrial electron transport chain complexes I and V, respectively [41,42]. A cocktail of these toxics generates high concentration of mitochondrial free radicals, decreases ATP synthesis and induces apoptosis, causing cell death. Thus, this mixture is described as a good experimental model of mitochondrial oxidative stress [27,31,43].

Oxygen and glucose deprivation is an acute model of lesion produced by free radical generation during the re-oxygenation period [44]. This stimulus mimics what can occur in cerebral ischemia and AD, in which the hippocampus is profoundly affected and patients show symptoms of short-term memory loss [45].

In both models, compounds were tested at 0.3 μ M and cell survival was quantified by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay. MTT reduction in basal conditions was considered as 100% viability of cells without any treatment. Melatonin and L-**1** were also tested for comparative purposes.

First, possible cytotoxic effects of L- and D-Glu derivatives **1-3** and **11-17** were studied by exposing the cells to compounds at 0.3 μ M for 24 h. In all cases, cell survival was close to basal value, showing that the compounds had low toxicity (data not shown).

Compounds L-(**1-3,11-17**) and D-(**1-3,11,14**) displayed good levels of protection in both models (Table 2). Neuroprotection values ranged from 13% to 46% using R/O as toxic insult, and from 32% to 52% in the OGD/R protocol. In both models, L-Glu derivatives displayed better protection than their D-enantiomers, especially L-(**1,11,14**), in which the *N*-benzylpiperidine group was attached to the γ -position. We can highlight compound L-**11** with the best neuroprotection values in both models.

Then, we evaluated the radical scavenging properties of L- and D-Glu *N*-benzylpiperidine derivatives (**1,3,11-17**) using the oxygen radical absorbance capacity (ORAC) assay and trolox as internal standard [27,28]. Tested compounds were inactive at 10 μ M, with the exception of the series bearing an indole-3-carbonyl group in the side chain L-(**12,15,16**) that showed radical captures around 70-73% of the trolox value. As donepezil-based L- and D-Glu compounds showed little or null ability to capture oxygen radicals, their good neuroprotective properties towards oxidative stress as evidenced in the two cellular models used here could be mostly due to the activation of endogenous antioxidant pathways [46].

2.5. *In vitro* blood-brain barrier permeation assay

To explore whether L- and D-Glu derivatives would be able to penetrate into the brain, we used a parallel artificial membrane permeation assay for the blood-brain barrier (PAMPA-BBB) [47]. The *in vitro* permeabilities (P_e) of compounds L-(**1,3,11-17**) and D-(**1,3,11,14**) through a lipid extract of porcine brain were determined (see Table 2 and Supporting Information).

Predicted brain penetration appeared to be influenced mainly by the amino acid side chain and the nature of the carboxyl fragment (free acids, ethyl- or hexyl esters).

Regarding the side chain, the permeability order was found to be: carboxybenzyl > phenyl > 6-methoxy-2-naphthyl > indol-3-yl. In each of the above-mentioned families, hexyl esters were the most CNS-permeable compounds (L-**1**, D-**1**, L-**16**), all of them with positive prediction (cns+), followed by ethyl derivatives. Cbz ethyl esters (L-**3** and D-**3**) and the 6-methoxy-2-naphthyl ethyl derivative L-**17** could cross the BBB (cns+), whereas phenyl (L-**14** and D-**14**) and indol-3-yl L-**15** ethyl esters were predicted to have more difficulties to reach cerebral tissues (cns+/-). Finally, free acids were the worst CNS-permeable by passive diffusion, displaying negative (L-**12** and L-**13**) or uncertain predictions (L-**11** and D-**11**).

Donepezil-based compounds L-**1** and L-**3**, bearing the *N*-benzylpiperidine group in the γ - or in the α -position respectively, were chosen for additional pharmacological assays, on the basis of their balanced biological profile and their high predicted CNS-permeability that could facilitate their further drug development.

2.6. Study of the VDCC blocking effects in human neuroblastoma cells

Emerging findings suggest that disruptions in calcium homeostasis are implicated in neuronal degeneration [48]. In previous work, we found that donepezil-based L-**1** was a blocker of L-type VDCC that reduced K⁺-evoked [Ca²⁺]_c transients with an IC₅₀ value of 5.31 μ M in bovine chromaffin cells. At 10 μ M, L-**1** decreased both the amplitude and the area of the Ca²⁺ transient evoked by high K⁺ by 40% and 80%, respectively [35]. Therefore, we thought of interest to study the potential VDCC blocking effect of L-Glu derivatives L-**1** and L-**3**, but in the same cell model used to test their neuroprotective effect, the neuroblastoma SH-SY5Y line. For these experiments, cells were loaded with the Ca²⁺ sensitive fluorescent dye Fluo-4, thereafter, incubated for 15 min with

increasing concentrations of compounds. Then, cells were stimulated with a concentrated solution of KCl to obtain a final K^+ concentration of 70 mM. Changes in fluorescence as a consequence of the increase in the $[Ca^{2+}]_c$ elicited by high K^+ were measured in a fluorescence plate reader FluoStar Optima (BMG). A control with nifedipine (10 μ M), an L-type VDCC blocker, was included in every individual experiment. Concentration-response curves of compounds L-1 and L-3 were elaborated with concentrations ranging from 0.3 to 100 μ M (Figure 2).

Donepezil-based compound L-1 blocked the increase in $[Ca^{2+}]_c$ induced by high K^+ in a statistically significant manner at concentrations from 0.3 μ M to 100 μ M; blockade ranged from 21% to 62% ($IC_{50} = 14.1 \mu$ M). Compound L-3 showed its maximum blockade (63%) at 100 μ M, with an IC_{50} of 90.1 μ M. By comparing their IC_{50} values, compound L-1 was over 6-fold better to block VDCCs than compound L-3. Looking into their structures, the change in the *N*-protecting group to Cbz moiety and the change in the position of the *N*-benzylpiperidine fragment from γ - to α -position produced a significant reduction of its calcium channel blocker potency.

Figure 2. Inhibition by compounds L-1 and L-3 of the increment of $[\text{Ca}^{2+}]_i$ evoked by K^+ stimulation of neuronal SH-SY5Y cells. Panels A and B show the % of $[\text{Ca}^{2+}]_i$ increase without any compound (basal) and in presence of increasing concentrations of L-1 and L-3 respectively. Nifedipine (10 μM) was used as positive control. Panel C shows concentration-response curves, measured as the maximum amplitude from baseline to the top of the plateau, fitted to non-linear curves for L-1 (square, continuous line) and L-3 (triangle, dotted line) and calculated IC_{50} values.

2.7. Neuroprotection study in a tissue model of cerebral ischemia

The potential neuroprotective effect of compound L-3 was also studied on an *in vitro* model of cerebral ischemia, the rat hippocampal slices subjected to a 15 min OGD period followed by a 2 h re-oxygenation period (OGD/R) [49,50] (see protocol on Figure 3). The functional impairment of mitochondria, promoted by the absence of the

oxygen supply, is increased when re-oxygenation triggers a massive production of reactive oxygen species raised by the OGD-induced overwork of the NADPH oxidase (NOX) enzyme. Cell viability, normalized to 100% of control slices, was reduced by 40% after OGD/R (black column in Figure 3). This damage was mitigated by 55% in the presence of 3 μ M of compound L-1 [36]. Compound L-3 showed a concentration-dependent effect with 48 ± 2 % protection at the concentration of 1 μ M and maximal protection of 69 ± 2 % at the concentration of 3 μ M, being more efficient than the reference compound L-1 at the same concentration.

Figure 3. Compound L-3 increases cell viability of rat hippocampal slices subjected to OGD/R. The experimental protocol is illustrated on the top part of the figure. The graph corresponds to the mean $\pm$ SEM of 4 independent experiments in triplicate. ### $p < 0.001$ respect control slices (not exposed to OGD/R) and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ respect to slices subjected to OGD/R. Data of compound L-1 are taken from Ref. [36]

3. Conclusions

In summary, we have developed two synthetic routes to obtain multifunctional L- and D-Glu derivatives, which simplify the previously described synthesis in solution [34]. The chemoenzymatic synthesis allowed the regioselective introduction of an *N*-benzylpiperidine moiety in the α -carbonyl group of Glu diethyl ester derivatives. Regioselectivity was independent of the reacting Glu enantiomer and of the nature of the lipase assayed. In all cases, enzymatic reactions were clean, which facilitates the isolation and purification of products. Best conversions were obtained in the L-series and using CAL-B. The solid-phase synthesis enabled the preparation of L- and D-Glu derivatives bearing the *N*-benzylpiperidine fragment in the γ -position. The final products were obtained in high overall yield.

The synthesised compounds showed a multifunctional profile with good inhibition of human ChEs in the micro- and sub-micromolar range and neuroprotective properties against the oxidative stress originated by the mixture of rotenone and oligomycin A or by the oxygen and glucose deprivation. In the human neuroblastoma cell line SH-SY5Y, compounds L-**1** and L-**3** blocked the increase in $[Ca^{2+}]_c$ induced by high K^+ , with IC_{50} values in the micromolar range. Finally, in an *in vitro* model of cerebral ischemia using the rat hippocampal slices subjected to an OGD/R protocol, compound L-**3** at 3 μ M showed an interesting protection of 69%. Their multitarget profile is complemented by the positive data to be able to penetrate into the CNS by passive diffusion. Thus, we can conclude that these donepezil-based Glu derivatives show a balanced multifunctional profile of interest in the search for new drugs for the treatment of AD and cerebral ischemia.

4. Experimental section

4.1. Chemistry. General methods

Reagents were purchased from common commercial suppliers (Sigma-Aldrich, Acros, Bachem, Merck, ABCR or TCI) and were used without further purification. Thin-layer chromatography (TLC) was performed on silica-gel-coated aluminium foils (silica gel 60/kieselguhr F254, Merck) and compounds were detected with UV light ($\lambda = 254$ nm) or with ninhydrin. Chromatographic separations were performed either using an Isolera One (Biotage) purification system, or using flash-column chromatography on silica gel (Biotage SNAP FSK0-1107 cartridges and Kiesegel 60 Merck of 230-400 mesh, respectively). Melting points were determined in a Mettler Toledo MP70 apparatus. Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter at room temperature (ca. 293 K). Nuclear magnetic resonance spectra were recorded using a Varian 500, Varian Inova-400 and Varian Mercury-400. Chemical shifts are expressed in parts per million (δ , ppm) using the residual solvent proton as internal standard and J values are reported in hertz. Spin multiplicities are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). IR spectra were recorded with a Perkin Elmer Spectrum One FT-IR spectrometer. The electrospray ionization-high resolution mass spectra (ESI-HRMS) were performed by using a liquid chromatography apparatus HP-1200 (Agilent Technologies) with a Q-TOF 6520 hybrid mass spectrometer. HPLC-MS analyses were performed on Alliance Waters 2695 equipment, with a UV detector photodiode array Waters 2996 with MS detector Micromass ZQ (Waters), using a SunFire C₁₈, 3.5 μ m, 4.6 mm x 50 mm column, and a gradient of CH₃CN/H₂O/HCOOH (2% aq sol) from 15:80:5 to 95:0:5 v/v/v for 5 minutes at 1.0 mL/min, if not otherwise indicated. HPLC analyses were used to confirm the purity of all compounds ($\geq 95\%$).

4.2. General procedure for the lipase-catalysed reactions

From commercially available *N*-Cbz-L-Glu and D-Glu and following described methods [38,51], intermediates *N*-Cbz-L-Glu(OEt)-OEt (**L-2**) and *N*-Cbz-D-Glu(OEt)-OEt (**D-2**) were obtained in 79% and 13% yield, respectively. To a solution of **L-2** or **D-2** (20 mM) and NBP-NH₂ (50 mM) in dry diisopropylether (*i*Pr₂O), CAL-B (*Candida antarctica* lipase B) or MML (*Mucor miehei* lipase) (50 mg/mL) and molecular sieves 4 Å (50 mg/mL) were added. The reaction mixture was agitated in an orbital shaker at 45 °C. The reaction was followed by HPLC-MS. When the reaction was completed, the enzyme and molecular sieves were filtered off and washed with CH₂Cl₂. The solvent was evaporated under reduced pressure and the crude was purified by flash column chromatography using CH₂Cl₂/MeOH 9:1 as eluent.

4.2.1. *N*-Carboxybenzyl-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-ethyl (**L-3**)

Following the general procedure, from **L-2** (0.60 g, 1.78 mmol), NBP-NH₂ (0.98 mL, 4.45 mmol), 4.5 g of molecular sieves and 4.5 g of enzyme in 90 mL of dry *i*Pr₂O, compound **L-3** was obtained in 22 hours as a white solid of mp 105-106 °C (bibl. 107-108 °C [52]) and $[\alpha]_D^{20}$: -3.5 (*c* = 1, CHCl₃). Yield of **L-3** using CAL-B as enzyme: 0.42 g (0.83 mmol, 46%), yield of **L-3** using MML: 0.37 g (0.73 mmol, 41%). ¹H NMR (500 MHz, CDCl₃): δ 7.31 (m, 10H, 2Ph), 6.27 (m, 1H, NHCOCα), 5.67 (br d, 1H, NHCα), 5.08 (s, 2H, H1'), 4.17 (m, 1H, Hα), 4.12 (q, *J* = 7.2 Hz, 2H, H1''), 3.54 (s, 2H, H8), 3.26 (m, 2H, H1), 2.91 (br d, *J* = 11.5 Hz, 2H, H5eq,6eq), 2.49 (dt, *J* = 17.0, 7.0 Hz, 1H, Hγ₁), 2.36 (dt, *J* = 17.0, 7.0 Hz, 1H, Hγ₂), 2.11 (m, 1H, Hβ₁), 1.99 (m, 2H, H5ax,6ax), 1.93 (m, 1H, Hβ₂), 1.66 (br d, *J* = 10.8 Hz, 2H, H4eq,7eq), 1.43 (m, 2H, H2), 1.35 (m,

2H, H4_{ax}, 7_{ax}), 1.31 (m, 1H, H3), 1.24 (t, $J = 7.2$ Hz, 3H, H2''). ¹³C NMR (125 MHz, CDCl₃): δ 173.7 (COC γ), 171.1 (COC α), 156.4 (CONHC α), 137.6, 136.3, 129.6 (2C), 128.7 (2C), 128.4, 128.3 (2C), 128.1 (2C) and 127.4 (2 Ph); 67.2 (C1'), 63.3 (C8), 61.0 (C1''), 54.4 (C α), 53.6 (C5,6), 37.4 (C1), 36.1 (C2), 33.4 (C3), 31.8 (C4,7), 30.6 (C γ), 28.3 (C β), 14.3 (C2''). IR (neat) $\bar{\nu}$ 3436, 3305, 3066, 3033, 2928, 2799, 1726, 1690, 1650, 1540, 1447, 1379, 1280, 1243, 1196, 1017, 740, 699 cm⁻¹. HRMS (ESI⁺): m/z calcd for C₂₉H₄₀N₃O₅ [M+H]⁺ 510.2962, found 510.2967. HPLC-MS: t_R = 2.92 min (96%).

4.2.2. *N*-Carboxybenzyl-*D*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-ethyl (*D*-**3**)

Following the general procedure, from *D*-**2** (0.30 g, 0.88 mmol), NBP-NH₂ (0.49 mL, 2.20 mmol), 2.2 g of molecular sieves and 2.2 g of enzyme in 44 mL of dry *i*Pr₂O, compound *D*-**3** was obtained in 92 hours as a white solid of mp 100-102 °C and $[\alpha]_D^{20}$: +2.8 ($c = 1$, CHCl₃). Yield using CAL-B as enzyme: 0.14 g (0.28 mmol, 32%), yield with MML: 0.17 g (0.32 mmol, 36%). ¹H NMR (500 MHz, CDCl₃): δ 7.31 (m, 10H, 2Ph), 6.24 (m, 1H, NHCOC α), 5.66 (br d, 1H, NHC α), 5.09 (s, 2H, H1'), 4.17 (m, 1H, H α), 4.12 (q, $J = 7.2$ Hz, 2H, H1''), 3.49 (s, 2H, H8), 3.26 (m, 2H, H1), 2.87 (bd, $J = 11.0$ Hz, 2H, H5_{eq}, 6_{eq}), 2.49 (dt, $J = 17.0, 7.0$ Hz, 1H, H γ_1), 2.36 (dt, $J = 17.0, 7.0$ Hz, 1H, H γ_2), 2.10 (m, 1H, H β_1), 1.93 (m, 3H, H β_2 , 5_{ax}, 6_{ax}), 1.64 (br d, $J = 8.9$ Hz, 2H, H4_{eq}, 7_{eq}), 1.43 (m, 2H, H2), 1.28 (m, 3H, H3, 4_{ax}, 7_{ax}), 1.24 (t, $J = 7.2$ Hz, 3H, H2''). ¹³C NMR (125 MHz, CDCl₃): δ 173.7 (COC γ), 171.0 (COC α), 156.4 (CONHC α), 138.2, 136.3, 129.4 (2C), 128.7 (2C), 128.4, 128.3 (2C), 128.2 (2C) and 127.2 (2Ph); 67.2 (C1'), 63.5 (C8), 61.0 (C1''), 54.4 (C α), 53.8 (C5,6), 37.4 (C1), 36.2 (C2), 33.6 (C3), 32.1 (C4,7), 30.6 (C γ), 28.3 (C β), 14.3 (C2''). IR (KBr) $\bar{\nu}$ 3436, 3305,

3066, 2926, 2798, 1726, 1690, 1649, 1542, 1447, 1379, 1280, 1243, 1197, 1175, 1077, 1017, 740, 699 cm^{-1} . HRMS (ESI⁺): m/z calcd for $\text{C}_{29}\text{H}_{40}\text{N}_3\text{O}_5$ $[\text{M}+\text{H}]^+$ 510.2962, found 510.2966. HPLC-MS: t_R = 2.80 min (97%).

4.3. General procedure for the solid-phase synthesis of amino acid derivatives

All solid-phase reactions were performed in an orbital shaker at room temperature. Wang resin was suspended in DMF, shaken for 5 min, and the solvent was filtered off. This process was repeated twice for resin swelling. After every step, the following general washing procedure was carried out: twice with (MeOH, THF, pentane, CH_2Cl_2), once with (MeOH, DMF, pentane, THF), and twice with (pentane, CH_2Cl_2 , pentane), using 10-15 mL of each solvent and shaking for 2 min. After every step, we considered to get a quantitative yield to calculate the amount of reagents for the next reaction.

4.3.1. Anchor of *N*-Fmoc-L-Glu(OAll)-OH or *N*-Fmoc-D-Glu(OAll)-OH to Wang resin

To a solution of *N*-Fmoc-L-Glu(OAll)-OH or *N*-Fmoc-D-Glu(OAll)-OH (491 mg, 1.2 mmol) in 20 mL of CH_2Cl_2 :DMF (9:1, v/v), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC HCl, 230 mg, 1.2 mmol) was added, and the mixture was stirred for 10 min. Then, this solution was added to a suspension of the swollen Wang resin (909 mg, 1.1 mmol/g, 1.0 mmol) in DMF (20 mL) and the mixture was stirred for an additional 5 min period. Afterward, 4-dimethylaminopyridine (DMAP, 12 mg, 0.1 mmol) was added and the mixture was shaken for 16 h. Then, the solvent was filtered off and the corresponding resin, L-4 or D-4, was washed following the general procedure and then dried under vacuum.

4.3.1.1. *N-Fmoc-L-Glu(OAll)-O-resin (L-4)*. IR (KBr) $\bar{\nu}$ 3421, 3059, 3025, 2921, 2840, 1732, 1612, 1512, 1493, 1451, 1380, 1299, 1220, 1172, 1030, 822, 758, 740, 698, 539 cm^{-1} .

4.3.1.2. *N-Fmoc-D-Glu(OAll)-O-resin (D-4)*. IR (KBr) $\bar{\nu}$ 3420, 3060, 3025, 2920, 2840, 1735, 1615, 1514, 1496, 1453, 1382, 1298, 1224, 1174, 1033, 824, 760, 742, 670, 540 cm^{-1} .

4.3.2. Alloc deprotection

Under nitrogen atmosphere, to a suspension of resin L-4 or D-4 (1 mmol) in dry CH_2Cl_2 a solution of tetrakis(triphenylphosphine)palladium(0) ($\text{Pd(PPh}_3)_4$, 116 mg, 0.1 mmol) and phenylsilane (PhSiH_3 , 541 mg, 5 mmol) was added, and the reaction mixture was shaken for 45 min. Then, solvent was filtered off and the resin was washed 3 times with CH_2Cl_2 . The above process was repeated a second time, using the same quantity of every reagent for other 45 min. Then, the solvent was filtered off and the resulting resin, L-5 or D-5, was washed following the general procedure and dried under vacuum.

4.3.2.1. *N-Fmoc-L-Glu(OH)-O-resin (L-5)*. IR (KBr) $\bar{\nu}$ 3430, 3055, 3025, 2920, 2846, 1725, 1647, 1613, 1512, 1493, 1451, 1381, 1220, 1172, 1029, 822, 758, 741, 698, 538 cm^{-1} .

4.3.2.2. *N-Fmoc-D-Glu(OH)-O-resin (D-5)*. IR (KBr) $\bar{\nu}$ 3432, 3058, 3026, 2920, 2848, 1726, 1650, 1615, 1513, 1497, 1452, 1384, 1223, 1172, 1030, 823, 760, 742, 699, 537 cm^{-1} .

4.3.3. Amine coupling

Resin L-**5** or D-**5** (1 mmol) was suspended in 20 mL of DMF/CH₂Cl₂ (2:1, v/v) and then, EDC HCl (383 mg, 2 mmol), 1-hydroxybenzotriazole (HOBt H₂O, 306 mg, 2 mmol), NBP-NH₂ (437 mg, 2 mmol), triethylamine (348 μ L, 2.5 mmol) and DMAP (12 mg, 0.1 mmol) were successively added. The reaction mixture was shaken for 16 h and then the solvent was filtered off. Resin L- or D-**6** was washed following the general procedure and dried under vacuum.

4.3.3.1. *N-Fmoc-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (L-6)*. IR (KBr) $\bar{\nu}$ 3422, 3059, 3025, 2919, 2841, 1734, 1646, 1613, 1512, 1493, 1451, 1368, 1301, 1220, 1170, 1016, 821, 758, 740, 697, 539 cm⁻¹.

4.3.3.2. *N-Fmoc-D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (D-6)*. IR (KBr) $\bar{\nu}$ 3424, 3060, 3025, 2920, 2840, 1736, 1650, 1615, 1513, 1494, 1450, 1370, 1300, 1220, 1170, 1018, 820, 760, 745, 695, 540 cm⁻¹.

4.3.4. Fmoc deprotection

Resin L-**6** or D-**6** (1 mmol) was suspended in DMF (16 mL) and a piperidine solution 20 % in DMF (4 mL) was added. The mixture was shaken for 20 min, the solvent was filtered off, and the resin was washed twice with DMF. The same process was performed a second time with the same amount of piperidine for other 20 min. Then, the solvent was filtered off and the corresponding resin L-**7** or D-**7** was washed following the general procedure and dried under vacuum.

4.3.4.1. *L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (L-7)*. IR (KBr) $\bar{\nu}$ 3421, 3059, 3025, 2920, 2850, 1735, 1648, 1612, 1512, 1493, 1452, 1369, 1301, 1223, 1171, 1016, 822, 758, 698, 540 cm^{-1} .

4.3.4.2. *D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (D-7)*. IR (KBr) $\bar{\nu}$ 3420, 3060, 3030, 2924, 2855, 1740, 1650, 1615, 1515, 1496, 1458, 1370, 1300, 1225, 1174, 1016, 822, 760, 700, 542 cm^{-1} .

4.3.5. Introduction of the aromatic substituents – acid coupling

To a solution of the corresponding carboxylic acid (1.2 mmol) in 20 mL of CH_2Cl_2 :DMF (1:1, v/v), EDC HCl (230 mg, 1.2 mmol) and HOBT (1.2 mmol) were added and stirred for 10 min. This solution was added to a suspension of resin L-7 or D-7 (1 mmol) in 20 mL of DMF/ CH_2Cl_2 (1:1, v/v) and then, Et_3N (223 μL , 1.6 mmol) and DMAP (12 mg, 0.1 mmol) were added and the mixture was stirred for 16 h. Then the solvent was filtered off and resins L-(8-10) and D-8 were washed following the general procedure and then dried under vacuum.

4.3.5.1. *N-Benzoyl-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (L-8)*. IR (KBr) $\bar{\nu}$ 3422, 3059, 3025, 2919, 2846, 1736, 1643, 1610, 1512, 1492, 1451, 1375, 1220, 1170, 1109, 1027, 820, 757, 697, 536 cm^{-1} .

4.3.5.2. *N-Benzoyl-D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-resin (D-8)*. IR (KBr) $\bar{\nu}$ 3422, 3059, 3025, 2921, 2850, 1740, 1653, 1613, 1513, 1493, 1452, 1376, 1221, 1172, 1110, 1027, 823, 758, 698, 539 cm^{-1} .

4.3.5.3. *N*-(Indole-3-carbonyl)-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-resin (*L*-**9**). IR (KBr) $\bar{\nu}$ 3421, 3059, 3025, 2919, 2846, 1738, 1636, 1512, 1493, 1452, 1371, 1300, 1171, 1016, 821, 747, 698, 538 cm^{-1} .

4.3.5.4. *N*-(6-Methoxy-2-naphthoyl)-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-resin (*L*-**10**). IR (KBr) $\bar{\nu}$ 3429, 3055, 3025, 2921, 2856, 1736, 1629, 1511, 1452, 1370, 1214, 1169, 1121, 1028, 820, 757, 698, 538 cm^{-1} .

4.3.6. Resin cleavage – Synthesis of *N*-(arylcarbonyl)-*L*-(or *D*)-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-OH

Trifluoroacetic acid (TFA, 2 mL) was added to a suspension of each resin *L*-(**8-10**) or *D*-**8** in dry CH_2Cl_2 (8 mL) and the mixture was stirred for 20 min. Resin was filtered off and washed with CH_2Cl_2 (3 x 20 mL). The organic phases were combined and evaporated under reduced pressure, using sequential additions of EtOAc (3 x 20 mL) and Et_2O (3 x 20 mL) to remove the excess of TFA.

4.3.6.1. *N*-Benzoyl-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-OH (*L*-**11**)

Following the general procedure, from 2.1 g of Wang resin, compound *L*-**11** was obtained as a white solid (0.80 g, 1.76 mmol, 76% over six steps) of mp 70-72 °C and $[\alpha]_{\text{D}}^{20}$: -8.4 ($c = 1$, CH_3OH). ^1H NMR (400 MHz, CD_3OD): δ 7.89 (m, 2H, H Ar), 7.56 (m, 1H, H Ar), 7.48 (m, 7H, H Ar), 4.57 (m, 1H, H α), 4.26 (s, 2H, H8), 3.42 (m, 2H, H5eq, 6eq), 3.20 (m, 2H, H1), 2.97 (m, 2H, H5ax, 6ax), 2.39 (m, 2H, H γ), 2.32 (m, 1H, H β_1), 2.11 (m, 1H, H β_2), 1.95 (m, 2H, H4eq, 7eq), 1.60 (m, 1H, H3), 1.43 (m, 2H, H2), 1.37 (m, 2H, H4ax, 7ax). ^{13}C NMR (100 MHz, CD_3OD): δ 175.0 ($\text{COC}\alpha$), 174.8

($\underline{\text{COC}}\gamma$), 170.3 ($\underline{\text{CONHC}}\alpha$), 135.2 (C Ar), 133.0 (C Ar), 132.3 (2C Ar), 131.2 (2C Ar), 130.4 (C Ar), 130.3 (2C Ar), 129.6 (2C Ar), 128.5 (C Ar), 61.8 (C8), 53.8 (C α), 53.7 (C5,6), 37.2 (C1), 36.2 (C2), 33.3 (C γ), 32.0 (C3), 30.3 (C4,7), 28.0 (C β). IR (KBr) $\bar{\nu}$ 3401, 3060, 2931, 1661, 1537, 1454, 1201, 1134, 720, 702 cm^{-1} . HRMS (ESI⁺): m/z calcd for $\text{C}_{26}\text{H}_{34}\text{N}_3\text{O}_4$ [M+H]⁺ 452.2544, found 452.2556. HPLC-MS: t_R = 2.06 min (98%).

4.3.6.2. *N-Benzoyl-D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-OH (D-11)*

Following the general procedure, from 1.8 g of Wang resin, a residue was obtained and purified by flash column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (increasing polarity from 9:1 to 4:1). Compound D-11 was obtained as a white solid (0.21 g, 0.47 mmol, 23% over six steps) of mp 75-77 °C and $[\alpha]_{\text{D}}^{20}$: +7.6 (c = 1, CH_3OH). ¹H NMR (400 MHz, CD_3OD): δ 7.85 (m, 2H, H Ar), 7.48 (m, 3H, H Ar), 7.36 (m, 5H, H Ar), 4.45 (m, 1H, H α), 3.88 (s, 2H, H8), 3.14 (m, 4H, H1, 5eq, 6eq), 2.49 (m, 2H, H5ax, 6ax), 2.30 (m, 2H, H γ), 2.21 (m, 1H, H β_1), 2.06 (m, 1H, H β_2), 1.78 (m, 2H, 4eq, 7eq), 1.38 (m, 5H, H2, 3, 4ax, 7ax). ¹³C NMR (100 MHz, CD_3OD): δ 178.2 ($\underline{\text{COC}}\alpha$), 175.6 ($\underline{\text{COC}}\gamma$), 169.2 ($\underline{\text{CONHC}}\alpha$), 135.7 (C Ar), 134.1 (C Ar), 132.7 (2C Ar), 131.7 (2C Ar), 129.9 (C Ar), 129.8 (2C Ar), 129.6 (2C Ar), 128.3 (C Ar), 62.7 (C8), 56.4 (C α), 53.9 (C5,6), 37.6 (C1), 36.3 (C2), 33.9 (C γ), 33.1 (C3), 31.2 (C4,7), 30.3 (C β). IR (KBr) $\bar{\nu}$ 3401, 3063, 2929, 1643, 1602, 1578, 1544, 1482, 1397, 743, 699 cm^{-1} . HRMS (ESI⁺): m/z calcd for $\text{C}_{26}\text{H}_{34}\text{N}_3\text{O}_4$ [M+H]⁺ 452.2544, found 452.2527. HPLC-MS: t_R = 2.08 min (98%).

4.3.6.3. *N*-[Indole-3-carbonyl]-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-OH (**L-12**)

Following the general procedure, starting with 1.4 g of Wang resin, a residue was obtained and purified by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 9:1 to 4:1). Compound **L-12** was obtained as a yellow solid (0.27 g, 0.54 mmol, 36% over six steps) of mp 87-89 °C and $[\alpha]_D^{20}$: -4.2 ($c = 1$, CH₃OH). ¹H NMR (400 MHz, CD₃OD): δ 8.10 (m, 1H, H Ar), 7.99 (s, 1H, H Ar), 7.41 (m, 6H, H Ar), 7.15 (m, 2H, H Ar), 4.57 (m, 1H, H α), 4.10 (s, 2H, H δ), 3.12 (m, 4H, H ϵ , 5eq, 6eq), 2.81 (m, 2H, H5ax, 6ax), 2.38 (m, 2H, H γ), 2.29 (m, 1H, H β_1), 2.10 (m, 1H, H β_2), 1.81 (m, 2H, H4eq, 7eq), 1.52 (m, 1H, H3), 1.32 (m, 4H, H2, 4ax, 7ax). ¹³C NMR (100 MHz, CD₃OD): δ 176.4 ($\underline{\text{COC}}\alpha$), 175.1 ($\underline{\text{COC}}\gamma$), 168.1 ($\underline{\text{CONHC}}\alpha$), 138.1 (2C Ar), 132.3 (C Ar), 131.1 (C Ar), 130.5 (C Ar), 130.3 (2C Ar), 129.7 (C Ar), 127.0 (C Ar), 123.6 (C Ar), 122.2 (C Ar), 121.7 (C Ar), 113.0 (C Ar), 111.6 (C Ar), 61.6 (C8), 53.8 (C α), 53.5 (C5,6), 37.2 (C1), 35.9 (C2), 33.5 (C γ), 31.8 (C3), 30.1 (C4,7), 28.8 (C β). IR (KBr) $\bar{\nu}$ 3414, 3068, 2931, 1677, 1640, 1537, 1437, 1204, 1134, 751, 722 cm⁻¹. HRMS (ESI⁺): m/z calcd for C₂₈H₃₅N₄O₄ [M+H]⁺ 491.2653, found 491.2675. HPLC-MS: t_R = 2.47 min (96%).

4.3.6.4. *N*-[6-Methoxy-2-naphthoyl]-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-OH (**L-13**)

Following the general procedure, starting with 1.4 g of Wang resin, a residue was obtained and purified by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 9:1 to 8:2) Compound **L-13** was obtained as a white solid (0.20 g, 0.38 mmol, 25% over six steps) of mp 75-78 °C and $[\alpha]_D^{20}$: -3.7 ($c = 1$, CH₃OH). ¹H NMR (400 MHz, CD₃OD): δ 8.38 (m, 1H, H Ar), 7.88 (m, 3H, H Ar), 7.45

(m, 5H, H Ar), 7.30 (m, 1H, H Ar), 7.21 (m, 1H, H Ar), 4.60 (m, 1H, H α), 4.20 (s, 2H, H8), 3.93 (s, 3H, H11''), 3.20 (m, 4H, H1, 5eq, 6eq), 2.89 (m, 2H, H5ax, 6ax), 2.42 (m, 2H, H γ), 2.34 (m, 1H, H β_1), 2.16 (m, 1H, H β_2), 1.88 (m, 2H, 4eq, 7eq), 1.57 (m, 1H, H3), 1.35 (m, 4H, H2, 4ax, 7ax). ^{13}C NMR (100 MHz, CD $_3$ OD): δ 186.5 (COC α), 175.0 (COC γ), 170.2 (CONHC α), 160.9 (C6'), 138.1 (2C Ar), 132.2 (C Ar), 131.6 (C Ar), 131.2 (C Ar), 130.3 (3C Ar), 130.1 (C Ar), 129.3 (C Ar), 128.9 (C Ar), 128.2 (C Ar), 125.6 (C Ar), 120.9 (C Ar), 106.7 (C Ar), 61.6 (C8), 55.9 (C11''), 54.3 (C α), 53.6 (C5,6), 37.3 (C1), 36.1 (C2), 33.5 (C γ), 31.9 (C3), 30.2 (C4,7), 28.3 (C β). IR (KBr) $\bar{\nu}$ 3423, 3065, 2942, 1681, 1535, 1439, 1209, 1135, 801, 723 cm $^{-1}$. HRMS (ESI $^+$): m/z calcd for C $_{31}$ H $_{38}$ N $_3$ O $_5$ [M+H] $^+$ 532.2806, found 532.2823. HPLC-MS: t_R = 2.72 min (97%).

4.3.7. General procedure for the synthesis of esters

The corresponding acid L-(**11-13**) or D-**11** (1 mmol) was dissolved in EtOH or *n*-hexanol (20 mL) at 0 °C and then, thionyl chloride (SOCl $_2$, 1.2 mmol) was added dropwise. The reaction mixture was stirred for 16 h at room temperature and then the solvent was evaporated at reduced pressure. The crude was purified by flash column chromatography as indicated in each case.

4.3.7.1. *N*-Benzoyl-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-ethyl (L-**14**)

Following the general procedure, the reaction of acid L-**11** (0.20 g, 0.44 mmol), EtOH (10 mL), and SOCl $_2$ (39.0 μ L, 0.53 mmol), followed by flash column chromatography using CH $_2$ Cl $_2$ /MeOH (increasing polarity from 98:2 to 90:10), afforded

compound L-**14** as a white solid (0.13 g, 0.27 mmol, 61%) of mp 117-118 °C and $[\alpha]_D^{20}$: -8.1 ($c = 1$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 7.87 (m, 2H, H Ar), 7.80 (m, 1H, NHC α), 7.45 (m, 8H, H Ar), 6.78 (br s, 1H, NHCOC γ), 4.62 (m, 1H, H α), 4.17 (q, $J = 7.2$ Hz, 2H, H1''), 3.95 (s, 2H, H8), 3.24 (m, 4H, H1, 5eq, 6eq), 2.49 (m, 2H, 5ax, 6ax), 2.38 (m, 2H, H γ), 2.24 (m, 1H, H β_1), 2.13 (m, 1H, H β_2), 1.77 (m, 4H, H2, 4eq, 7eq), 1.48 (m, 3H, H3, 4ax, 7ax), 1.27 (t, $J = 7.2$ Hz, 3H, H2''). ¹³C NMR (100 MHz, CDCl₃): δ 172.8 (COC γ), 172.1 (COC α), 167.5 (CONHC α), 133.6 (C Ar), 132.0 (C Ar), 131.2 (2C Ar), 129.7 (2C Ar), 129.2 (C Ar), 128.7 (4C Ar), 127.4 (C Ar), 61.7 (C1''), 61.3 (C8), 53.1 (C α), 52.5 (C5,6), 36.8 (C1), 35.2 (C2), 32.8 (C γ), 31.7 (C3), 29.2 (C4,7), 27.8 (C β), 14.3 (C2''). IR (KBr) $\bar{\nu}$ 3436, 3315, 3067, 3027, 2929, 2800, 1733, 1646, 1548, 1453, 1342, 1301, 1177, 1028, 730, 697 cm⁻¹. HRMS (ESI⁺): m/z calcd for C₂₈H₃₈N₃O₄ [M+H]⁺ 480.2857, found 480.2837. HPLC-MS: $t_R = 2.71$ min (100%).

4.3.7.2. *N-Benzoyl-D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-ethyl (D-14)*

Following the general procedure, the reaction of acid D-**11** (0.15 g, 0.33 mmol), EtOH (10 mL), and SOCl₂ (29 μ L, 0.40 mmol), followed by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 97:3 to 90:10), afforded compound D-**14** as a white solid (0.14 g, 0.29 mmol, 89%) of mp 90-92 °C and $[\alpha]_D^{20}$: +5.4 ($c = 1$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 7.86 (m, 2H, H Ar), 7.80 (m, 1H, NHC α), 7.44 (m, 8H, H Ar), 6.82 (br s, 1H, NHCOC γ), 4.60 (m, 1H, H α), 4.17 (q, $J = 7.2$ Hz, 2H, H1''), 3.95 (s, 2H, H8), 3.23 (m, 4H, H1, 5eq, 6eq), 2.50 (m, 2H, 5ax, 6ax), 2.37 (m, 2H, H γ), 2.23 (m, 1H, H β_1), 2.11 (m, 1H, H β_2), 1.77 (m, 4H, H2, 4eq, 7eq), 1.44 (m, 3H, H3, 4ax, 7ax), 1.25 (t, $J = 7.2$ Hz, 3H, H2''). ¹³C NMR (100 MHz, CDCl₃): δ 172.8 (COC γ), 172.1 (COC α), 167.5 (CONHC α), 133.5 (C Ar), 132.0 (C Ar),

131.3 (2C Ar), 129.8 (2C Ar), 129.2 (C Ar), 128.7 (4C Ar), 127.4 (C Ar), 61.7 (C1''), 61.1 (C8), 53.1 (C α), 52.5 (C5,6), 36.8 (C1), 35.2 (C2), 32.8 (C γ), 31.7 (C3), 29.0 (C4,7), 27.8 (C β), 14.3 (C2''). IR (KBr) $\bar{\nu}$ 3430, 3062, 2931, 1737, 1651, 1537, 1455, 1208, 1028, 701 cm^{-1} . HRMS (ESI⁺): m/z calcd for C₂₈H₃₈N₃O₄ [M+H]⁺ 480.2857, found 480.2875. HPLC-MS: t_R = 2.62 min (100%).

4.3.7.3. *N-Benzoyl-D-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-n-hexyl (D-1)*

Following the general procedure, the reaction of acid D-**11** (0.10 g, 0.22 mmol), *n*-HexOH (4 mL), and SOCl₂ (19.4 μL , 0.27 mmol), followed by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 97:3 to 90:10), afforded compound D-**1** as a white solid (0.10 g, 0.19 mmol, 86%) of mp 68-70 °C and $[\alpha]_D^{20}$: +10.2 (c = 1, CHCl₃). ¹H NMR (500 MHz, CDCl₃): δ 7.87 (m, 2H, H Ar), 7.75 (m, 1H, NHC α), 7.45 (m, 8H, H Ar), 6.78 (br s, 1H, NHCOC γ), 4.64 (m, 1H, H α), 4.12 (m, 2H, H1''), 4.03 (s, 2H, H8), 3.24 (m, 4H, H1, 5eq, 6eq), 2.54 (m, 2H, H5ax, 6ax), 2.38 (m, 2H, H γ), 2.23 (m, 1H, H β_1), 2.13 (m, 1H, H β_2), 1.80 (m, 2H, H2, 4eq, 7eq), 1.62 (m, 2H, H2''), 1.46 (m, 3H, H3, 4ax, 7ax), 1.29 (m, 6H, H3'', 4'', 5''), 0.86 (t, J = 7.3 Hz, 3H, H6''). ¹³C NMR (125 MHz, CDCl₃): δ 172.9 (COC γ), 172.1 (COC α), 167.5 (CONHC α), 133.5 (C Ar), 132.1 (C Ar), 131.4 (2C Ar), 130.1 (2C Ar), 129.3 (C Ar), 128.7 (4C Ar), 127.4 (C Ar), 65.9 (C1''), 61.1 (C8), 53.1 (C α), 52.5 (C5,6), 36.7 (C1), 35.2 (C2), 32.8 (C γ), 31.5 (C3), 28.9 (C4,7), 28.6 (C2''), 27.9 (C β), 25.6 (C3''), 22.6 (C4'', 5''), 14.1 (C6''). IR (KBr) $\bar{\nu}$ 3430, 3065, 2931, 1738, 1647, 1535, 1455, 1202, 749, 701 cm^{-1} . HRMS (ESI⁺): m/z calcd for C₃₂H₄₆N₃O₄ [M+H]⁺ 536.3483, found 536.3478. HPLC-MS: t_R = 3.38 min (95%).

4.3.7.4. *N*-[Indole-3-carbonyl]-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-ethyl (*L*-**15**)

Following the general procedure, the reaction of acid **L-12** (0.10 g, 0.20 mmol), EtOH (5 mL), and SOCl₂ (17.9 μ L, 0.24 mmol), followed by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 95:5 to 90:10), afforded compound **L-15** as a yellow solid (0.053 g, 0.10 mmol, 50%) of mp 87-90 °C and $[\alpha]_D^{20}$: -9.1 ($c = 1$, CH₃OH). ¹H NMR (400 MHz, CD₃OD): δ 8.11 (m, 1H, H Ar), 8.00 (s, 1H, H Ar), 7.40 (m, 6H, H Ar), 7.16 (m, 2H, H Ar), 4.57 (m, 1H, H α), 4.20 (q, $J = 7.2$ Hz, 2H, H1''), 4.0 (m, 2H, H8), 3.16 (m, 4H, H1, 5eq, 6eq), 2.71 (m, 2H, 5ax, 6ax), 2.40 (m, 2H, H γ), 2.29 (m, 1H, H β_1), 2.08 (m, 1H, H β_2), 1.80 (m, 2H, H4eq, 7eq), 1.49 (m, 1H, H3), 1.35 (m, 4H, H2, 4ax, 7ax), 1.27 (t, $J = 7.2$ Hz, 3H, H2''). ¹³C NMR (100 MHz, CD₃OD): δ 174.8 ($\underline{\text{COC}}\gamma$), 174.0 ($\underline{\text{COC}}\alpha$), 168.4 ($\underline{\text{CONHC}}\alpha$), 138.1 (2C Ar), 132.1 (C Ar), 130.9 (C Ar), 130.2 (C Ar), 129.7 (2C Ar), 127.2 (C Ar), 123.7 (C Ar), 122.2 (C Ar), 121.9 (C Ar), 113.0 (C Ar), 111.3 (C Ar), 62.4 (C1''), 61.7 (C α), 53.4 (C5,6), 37.2 (C1), 36.0 (C2), 33.3 (C γ), 31.9 (C3), 30.2 (C4,7), 28.0 (C β), 14.5 (C2''). IR (KBr) $\bar{\nu}$ 3401, 3264, 3088, 2930, 1735, 1632, 1538, 1455, 1199, 1029, 750, 701 cm⁻¹. HRMS (ESI⁺): m/z calcd for C₃₀H₃₉N₄O₄ [M+H]⁺ 519.2966, found 519.2987. HPLC-MS: $t_R = 2.72$ min (96%).

4.3.7.5. *N*-[Indole-3-carbonyl]-*L*-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-*O*-*n*-hexyl (*L*-**16**)

Following the general procedure, the reaction of acid **L-12** (0.11 g, 0.22 mmol), *n*-HexOH (4 mL), and SOCl₂ (19.6 μ L, 0.27 mmol), followed by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 98:2 to 90:10), afforded

compound L-**16** as a yellow solid (0.086 g, 0.15 mmol, 70%) of mp 85-87 °C and $[\alpha]_{\text{D}}^{20}$: -8.0 ($c = 1$, CH₃OH). ¹H NMR (500 MHz, CD₃OD): δ 8.01 (m, 1H, H Ar), 7.92 (s, 1H, H Ar), 7.32 (m, 6H, H Ar), 7.07 (m, 2H, H Ar), 4.49 (m, 1H, H α), 4.05 (m, 2H, H1''), 3.94 (m, 2H, H8), 3.08 (m, 4H, H1, 5eq, 6eq), 2.62 (m, 2H, 5ax, 6ax), 2.32 (m, 2H, H γ), 2.21 (m, 1H, H β_1), 2.01 (m, 1H, H β_2), 1.70 (m, 2H, H4eq, 7eq), 1.54 (m, 2H, H2''), 1.40 (m, 1H, H3), 1.21 (m, 10H, H2, 4ax, 7ax, 3'', 4'', 5''), 0.73 (t, $J = 7.2$ Hz, 3H, H6''). ¹³C NMR (125 MHz, CD₃OD): δ 174.7 ($\underline{\text{COC}}\gamma$), 174.1 ($\underline{\text{COC}}\alpha$), 168.4 ($\underline{\text{CONHC}}\alpha$), 138.1 (2C Ar), 132.1 (2C Ar), 130.9 (C Ar), 130.2 (C Ar), 129.6 (2C Ar), 127.3 (C Ar), 123.7 (C Ar), 122.2 (C Ar), 121.9 (C Ar), 113.0 (C Ar), 111.4 (C Ar), 66.4 (C1''), 61.7 (C8), 53.6 (C5,6), 53.4 (C α), 37.3 (C1), 35.9 (C2), 33.4 (C γ), 32.6 (C3''), 31.9 (C3), 30.1 (C4,7), 29.7 (C2''), 27.9 (C β), 26.7 (C4''), 23.6 (C5''), 14.3 (C6''). IR (KBr) $\bar{\nu}$ 3412, 3266, 3086, 2930, 1737, 1635, 1538, 1455, 1196, 1135, 750, 701 cm⁻¹. HRMS (ESI⁺): m/z calcd for C₃₄H₄₇N₄O₄ [M+H]⁺ 575.3592, found m/z 575.3585. HPLC-MS: $t_{\text{R}} = 3.28$ min (95%).

4.3.7.6. *N*-[6-Methoxy-2-naphthoyl]-L-Glu[NH-2-(1-benzylpiperidin-4-yl)ethyl]-O-ethyl (L-**17**)

Following the general procedure, the reaction of acid L-**13** (0.082 g, 0.15 mmol), EtOH (5 mL), and SOCl₂ (13.1 μ L, 0.18 mmol), followed by flash column chromatography using CH₂Cl₂/MeOH (increasing polarity from 95:5 to 90:10), afforded compound L-**17** as a white solid (0.063 g, 0.11 mmol, 75%) of mp 68-71 °C and $[\alpha]_{\text{D}}^{20}$: -8.3 ($c = 1$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 8.34 (m, 1H, H Ar), 7.80 (m, 4H, 3H Ar, NHC α), 7.47 (m, 2H, H Ar), 7.35 (m, 3H, H Ar), 7.13 (m, 2H, H Ar), 6.85 (m, 1H, NHCOC γ), 4.66 (m, 1H, H α), 4.19 (q, $J = 7.2$ Hz, 2H, H1''), 3.91 (s, 5H, H11''),

H8), 3.21 (m, 4H, H1, 5eq, 6eq), 2.40 (m, 4H, H γ , 5ax, 6ax), 2.26 (m, 1H, H β_1), 2.15 (m, 1H, H β_2), 1.75 (m, 4H, H2, 4eq, 7eq), 1.40 (m, 3H, H3, 4ax, 7ax), 1.26 (t, $J = 7.2$ Hz, 3H, H2''). ^{13}C NMR (100 MHz, CDCl_3): δ 172.9 ($\text{COC-}\gamma$), 172.2 ($\text{COC-}\alpha$), 167.6 ($\text{CONHC-}\alpha$), 159.4 (C6'), 136.6 (2C Ar), 131.4 (C Ar), 130.7 (C Ar), 130.0 (C Ar), 129.3 (3C Ar), 128.5 (C Ar), 128.1 (C Ar), 128.0 (C Ar), 127.3 (C Ar), 124.4 (C Ar), 120.0 (C Ar), 105.8 (C Ar), 61.8 (C1''), 61.0 (C8), 55.6 (C11''), 53.1 (C α), 52.5 (C5,6), 36.8 (C1), 35.2 (C2), 33.0 (C γ), 31.6 (C3), 28.9 (C4,7), 27.9 (C β), 14.4 (C2''). IR (KBr) $\bar{\nu}$ 3413, 3060, 2933, 1737, 1644, 1630, 1535, 1502, 1456, 1267, 1214, 1028, 860, 749, 701 cm^{-1} . HRMS (ESI^+): m/z calcd for $\text{C}_{33}\text{H}_{42}\text{N}_3\text{O}_5$ $[\text{M}+\text{H}]^+$ 560.3119, found 560.3121. HPLC-MS: $t_R = 2.97$ min (99%).

4.4. Biochemical studies

4.4.1. Inhibition of human AChE and BuChE

The method of Ellman et al. was followed [39]. The assay solution consisted of 0.1 M phosphate buffer pH 8, 400 μ M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 0.05 U/mL hAChE (human recombinant acetylcholinesterase, Sigma Chemical Co.) or 0.024 U/mL hBuChE (butyrylcholinesterase from human serum, Sigma Chemical Co.), and 800 μ M acetylthiocholine iodide, or 500 μ M butyrylthiocholine as the substrate of the enzymatic reaction, respectively. Compounds were added to the assay solution and pre-incubated with the enzyme for 5 minutes at 30 °C. After that period, the substrate was added. The absorbance changes at 412 nm were recorded for 5 minutes with a UV/Vis microplate spectrophotometer, Multiskan Spectrum, Thermo-Electron Co. The reaction rates were compared and the percent inhibition due to the presence of tested compound was calculated. The IC₅₀ is defined as the concentration of each compound that reduces at 50% the enzymatic activity without any inhibitor, which is expressed as the mean $\pm$ SEM of three independent experiments.

4.4.2. Neuroprotection Studies

4.4.2.1. Culture of SH-SY5Y cells

SH-SY5Y cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) (Sigma-Aldrich) and Eagle's minimum essential medium (EMEM) supplemented with 15 non-essential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated foetal bovine serum (FBS), 100 units/mL penicillin, and 100 μ g/mL streptomycin (reagents from Invitrogen). Cultures were seeded into flasks containing supplemented medium

and maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air (v/v). For assays, SH-SY5Y cells were sub-cultured in 48-well plates at a seeding density of 1 x 10⁵ cells per well. Cells were treated with the drugs before confluence in F-12/EMEM with 1% FBS. All the cells used in this study were used passages below 18.

4.4.2.2. Rotenone/oligomycin-A toxicity

Concentrated solutions of drugs were prepared in DMSO. For the control group, 0.1% DMSO was incubated having the same DMSO concentration of the tested compound's group. Compounds, at the concentration of 0.3 µM, were administered 24 h before adding the cytotoxic stimuli (rotenone/oligomycin A); then, cells were co-incubated for another 24 h with the drug in the presence of stimuli. All toxic stimuli were incubated in serum-free medium.

4.4.2.3. In vitro OGD/R model of ischemia

Briefly, after 24 h of pre-treatment with compounds of interest, the culture medium was changed to the glucose-free PBS 0.1 M with 2-deoxyglucose containing either melatonin (0.1 µM, positive control) or target compounds at 0.3 µM before placing them into an anaerobic chamber that was flushed with 5% CO₂ and 95% N₂ (v/v). The same anaerobic gas mixture was bubbled previously through the glucose-free PBS with 2-deoxyglucose. The cell cultures within the anaerobic chamber were kept in a humidified incubator at 37 °C for 4 h. To terminate the OGD, the PBS medium was changed to normal medium containing the same concentrations of tested compound alone before returning to the normoxic incubating conditions. In the sham-OGD groups, the cell cultures were subjected to the same experimental procedures plus vehicle without exposure to OGD.

4.4.2.4. *Quantification of Viability by MTT in SH-SY5Y cells*

Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich), as described previously [53]. To evaluate viability after the treatment with the compounds, MTT was added to all wells (5 mg/mL) and allowed to incubate in the dark at 37 °C for 2 h. The tetrazolium ring of MTT is cleaved by active reductases producing a precipitated formazan derivative. The formazan produced was dissolved by adding 300 μ L DMSO, resulting in a coloured compound whose optical density was measured in an ELISA reader at 540 nm in 100 μ L of the resulting solution for each well transferred to a 96-well plate. All MTT assays were performed in triplicate.

4.4.3. *In vitro blood–brain barrier permeation assay*

Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA), in a similar manner as described previously [47]. Commercial drugs, phosphate buffer saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma-Aldrich, Across and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μ m) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μ m) and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 180 μ L of PBS:ethanol (70:30) and the filter surface of the donor microplate was impregnated with 4 μ L of porcine brain lipid (PBL) in dodecane (20 mg mL⁻¹). Compounds were dissolved in PBS:ethanol (70:30) at 1 mg mL⁻¹, filtered

through a Millex filter, and then added to the donor wells (180 μ L). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 4 h at 25 °C. After incubation, the donor plate is carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample was analysed at five wavelengths, in four wells and in three independent runs, and the results are given as the mean $\pm$ standard deviation. In each experiment, 15 quality control standards of known BBB permeability were included to validate the analysis set.

4.4.4. Measurement of cytosolic Ca^{2+} transients in SH-SY5Y neuroblastoma cells

SH-SY5Y cells were plated in black, bottom transparent 96-well plates and grown at confluence. Cells were loaded with 4 μ M Fluo-4/AM for 45 min at 37 °C in MEM. Then, cells were washed twice with Krebs-HEPES solution containing the following composition (in mM): 140 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 11 D-glucose, at pH 7.4 and kept at room temperature for 15 min before the beginning of the experiment. Compounds were incubated 15 min before injecting potassium (70 mM) to enhance VDCC opening and $[Ca^{2+}]_c$ increase. Fluorescence measurements were carried out for 14 seconds after the injection of the agonists in a microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 and 520 nm, respectively. At the end of the experiment, 50 μ L of triton 5% was added to each well to calculate maximum fluorescence (F_{max}) and then 50 μ L of $MnCl_2$ 1 M to obtain the minimum fluorescence (F_{min}). Drug-evoked responses were expressed as percentage of the fluorescence values at each time point (F) minus minimum fluorescence values

(F_0) divided by $F_{max}-F_{min}$ as: $F_{520}=(F-F_0) / (F_{max}-F_{min})\%$ The maximum value of F_{520} , obtained for each experiment, was considered as the Peak F_{520} value.

4.4.5. Preparation of rat hippocampal slices and induction of oxygen and glucose deprivation followed by re-oxygenation (OGD/R)

All experiments were performed using adult male Sprague-Dawley rats (275-325 g) from a colony of our animal quarters. The experiments were performed after approval of the protocol by the UAM Institutional Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Following a described protocol [49], rats were quickly decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.), forebrains were removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4), containing (in mM): NaCl 120, KCl 2, CaCl₂ 0.5, NaHCO₃ 26, MgSO₄ 10, KH₂PO₄ 1.18, glucose 11 and sucrose 200. The chamber solutions were pre-bubbled with either 95% O₂/5% CO₂ or 95% N₂/5% CO₂ gas mixtures, for at least 45 min before slice immersion, to ensure O₂ saturation or removal. The hippocampi were quickly dissected, and slices (350 μ m thick) were rapidly prepared using a McIlwain Tissue Chopper. Then, the slices were transferred to vials of sucrose-free dissection buffer, bubbled with 95% O₂/5% CO₂ in a water bath at room temperature for 60 min to recover from slicing trauma before starting the experiments (equilibration period). The slices corresponding to the control group were incubated 15 min in a Krebs solution with the following composition (in mM): NaCl 120, KCl 2, CaCl₂ 2, NaHCO₃ 26, MgSO₄ 1.19, KH₂PO₄ 1.18 and glucose 11; this solution was equilibrated with 95% O₂/5% CO₂. Oxygen and glucose

deprivation was induced by incubating the slices for a 15 min period in a glucose-free Krebs solution, equilibrated with a 95% N₂/5% CO₂ gas mixture; glucose was replaced by 2-deoxyglucose. After this OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose (re-oxygenation period). Experiments were performed at 37 °C. A control and OGD group were included in all experiments and four drug concentrations were tested in each experiment. When treated, OGD slices received compound L-3 and remained there during the OGD and re-oxygenation period.

Hippocampal cell viability was determined through the ability of the cells to reduce MTT [53]. Hippocampal slices were collected immediately after the re-oxygenation period and were incubated with MTT (0.5 mg/mL) in Krebs bicarbonate solution for 30 min at 37 °C. The tetrazolium ring of MTT is cleaved by active dehydrogenases to produce a precipitated formazan derivative. The formazan produced was solubilized by adding 200 µL DMSO, resulting in a coloured compound whose optical density was measured in an ELISA reader at 540 nm.

Data are represented as means ± standard error of the mean. Comparisons between experimental and control groups were performed by One-way ANOVA followed by Newman-Keuls post-hoc test. Differences were considered to be statistically significant when $p < 0.05$. All statistical procedures were carried out using GraphPad Prism software version.

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List of captions

Figure 1. Structures of donepezil, IQM-9.21 (L-1), and new L- and D-Glu derivatives

Figure 2. Inhibition by compounds L-1 and L-3 of the increment of $[Ca^{2+}]_c$ evoked by K^+ stimulation of neuronal SH-SY5Y cells. Panels A and B show the % of $[Ca^{2+}]_c$ increase without any compound (basal) and in presence of increasing concentrations of L-1 and L-3 respectively. Nifedipine (10 μ M) was used as positive control. Panel C shows concentration-response curves, measured as the maximum amplitude from baseline to the top of the plateau, fitted to non-linear curves for L-1 (square, continuous line) and L-3 (triangle, dotted line) and calculated IC_{50} values.

Figure 3. Compound L-3 increases cell viability of rat hippocampal slices subjected to OGD/R. The experimental protocol is illustrated on the top part of the figure. The graph corresponds to the mean $\pm$ SEM of 4 independent experiments in triplicate. ^{###} $p < 0.001$ respect control slices (not exposed to OGD/R) and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ respect to slices subjected to OGD/R. Data of compound L-1 are taken from Ref. [36]

Scheme 1. Solid-phase synthesis of L- and D-Glu derivatives with an *N*-benzylpiperidine in the γ -position

Table 1. Summary results of the lipase-catalysed preparative reactions

Table 2. Inhibitory activities of new compounds in hAChE and hBuChE [IC_{50} (μ M)]; neuroprotection (%) elicited by compounds at 0.3 μ M in the human neuroblastoma cell

line SH-SY5Y, subjected to (i) the combination of rotenone (30 μM) and oligomycin A (10 μM) (R/O) or (ii) the oxygen-glucose deprivation followed by re-oxygenation (OGD/R); permeability values of compounds in the PAMPA-BBB assay (P_e , $10^{-6} \text{ cm s}^{-1}$) with their predictive penetration in the CNS^a