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Synthesis and cholinesterase inhibition of cativic acid derivatives

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder associated with memory impairment and cognitive deficit. Most of the drugs currently available for the treatment of AD are acetylcholinesterase (AChE) inhibitors. In a preliminary study, significant AChE inhibition was observed for the ethanolic extract of *Grindelia ventanensis* (IC_{50} = 0.79 mg/mL). This result prompted us to isolate the active constituent, a normal labdane diterpenoid identified as 17-hydroxycativic acid (**1**), through a bioassay guided fractionation. Taking into account that **1** showed moderate inhibition of AChE (IC_{50} = 21.1 μM), selectivity over butyrylcholinesterase (BChE) (IC_{50} = 171.1 μM) and that it was easily obtained from the plant extract in a very good yield (0.15% w/w), we decided to prepare semisynthetic derivatives of this natural diterpenoid through simple structural modifications. A set of twenty new cativic acid derivatives (**3**–**6**) was prepared from **1** through transformations on the carboxylic group at C-15, introducing a C2–C6 linker and a tertiary amine group. They were tested for their inhibitory activity against AChE and BChE and some structure–activity relationships were outlined. The most active derivative was compound **3c**, with an IC_{50} value of 3.2 μM for AChE. Enzyme kinetic studies and docking modeling revealed that this inhibitor targeted both the catalytic active site and the peripheral anionic site of this enzyme. Furthermore, **3c** showed significant inhibition of AChE activity in SH-SY5Y human neuroblastoma cells, and was non-cytotoxic.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with progressive memory loss, a decline in language skills, and other cognitive impairments. AD is characterized by neuronal loss and atrophy in crucial memory structures of the brain and causes functional deterioration of neurotransmitter systems, particularly a deficiency of acetylcholine (ACh) in the basal forebrain, which contributes to the cognitive deficits.[1,2] Cholinesterases (ChEs) play a vital role in the regulation of cholinergic transmission. The inhibition of ChEs increases the ACh level in the brain and has thus been implicated in the treatment of AD. Currently approved AD medications mainly comprise acetylcholinesterase (AChE) inhibitors that offer symptomatic treatment, but are unable to prevent disease progression and alter the outcome of the disease.[1–4] Most AChE inhibitors interact with the catalytic site of the enzyme (CAS) located at the bottom of the gorge, where the hydrolysis of ACh takes place.[5] The entrance of this gorge contains the peripheral anionic site (PAS), which can be also targeted, either separately or simultaneously by potential inhibitors.[2–7] The interest in dual binding site AChE inhibitors is due to the so-called 'non-cholinergic action of AChE'.[2] Interaction of amyloid-β (Aβ) at the PAS of AChE greatly accelerates the aggregation of this toxic peptide and catalyzes some conformational changes in Aβ fibrils to form the β-sheets with increased aggregating potential.[2,3,6,7] Thus, inhibitors targeting the PAS of the enzyme will decrease the aggregation rate of Aβ, keeping it in solution, therefore facilitating its clearance.

In the healthy brain, another enzyme, namely butyrylcholinesterase (BChE), is involved in the metabolic degradation of ACh. BChE activity increases as AD progresses. Therefore, the concurrent inhibition of both AChE and BChE should provide additional benefits in the treatment of AD.[2,4,8]

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The potential use of natural products in the field of AD has been successfully demonstrated in several review articles reporting AChE inhibitors obtained from plants, fungus and marine organisms, as well as their future-promising synthetic analogs.[9–11] In the course of our ongoing study of Argentinean plants with potential interest for AD therapy, we observed significant in vitro AChE inhibition in a preliminary assay for the ethanolic extract of *Grindelia ventanensis* Bartola & Tortosa (Asteraceae). The genus *Grindelia* (Asteraceae) is represented in South America by 26 species, 23 of them endemic.[12] Plants from this genus are known to be sources of bioactive compounds, mostly diterpenoids of the labdane type and manoyl oxide derivatives, mono- and sesquiterpenes, polyacetylenes, flavonoids, and saponins.[13–24] Anti-inflammatory, expectorant, antispasmodic, and antimicrobial activities, as well as antifeedant effects towards insects, have been reported for extracts or secondary metabolites obtained from *Grindelia* plants.[17,22–27] *G. ventanensis* is an endemic species growing wildly in Sierra de la Ventana, southwest of Buenos Aires province, Argentina. No phytochemical analysis or bioactivity study of *G. ventanensis* has been conducted so far. A bioassay-guided approach was applied to identify the active secondary metabolites present in the extract that had displayed in vitro AChE inhibition.

Herein, we describe the isolation and structure elucidation of a labdane diterpenoid, identified as 17-hydroxycativic acid (**1**), obtained from the active fractions of the nonpolar sub-extract of *G. ventanensis*. In addition, we describe the preparation of twenty cativic acid derivatives (**3**–**6**) obtained from the naturally occurring diterpenoid **1**, and their evaluation as AChE and BChE inhibitors. The structure–activity relationships are discussed based on their inhibitory activities. The most active derivative (**3c**) was chosen to study the kinetics of AChE inhibition and the key binding interactions between this compound and AChE through docking modeling. Furthermore, in order to evaluate the effects of **3c** on the AChE activity in live cells and its potential cytotoxicity, we used SH-SY5Y human neuroblastoma cells.

2. Results and discussion

2.1. Compound 1

2.1.1. Extraction, isolation and biological activity of compound 1

A bioactivity guided approach was taken to identify AChE inhibitory agents in the ethanolic extract of *G. ventanensis* (IC_{50} = 0.79 mg/mL). The active extract was partitioned with CH_2Cl_2 to obtain an active sub-extract, which was submitted to chromatographic separation. Compound **1** was spontaneously crystallized, pure and in excellent yield, from the active fractions. After that, anti-AChE activity of these fractions declined noticeably, which discouraged us to pursue their study and led us to focus on **1**. This compound elicited good AChE inhibition (IC_{50} = 21.1 μM) and selectivity over BChE (IC_{50} = 171.1 μM). To understand the interaction between **1** and AChE we used graphical analysis of steady state inhibition data following the procedure given below (see Section 2.4). The Lineweaver–Burk plots showed a pattern of lines characteristic of a mixed type inhibition, which suggested that **1** was able to bind both the CAS and PAS of AChE.[28]

2.1.2. Structural characterization of compound 1

^{1}H NMR data of **1** were similar to those previously reported for a labdane diterpenoid known as 17-hydroxycativic acid, isolated as a methyl ester from *Haplopappus pectinatus* Phil.[29] Neither ^{13}C NMR data nor the absolute configuration were described in that paper. We were able to unambiguously assign all the ^{1}H and ^{13}C signals with the aid of COSY, HSQC, and HMBC experiments (see Section 4).

From the stereochemical point of view, labdanes occur in nature as two antipodal groups of bicyclic molecules known as the normal and the *ent* series. An X-ray diffraction study was carried out with compound **1** in order to elucidate its stereochemistry. Figure 1 shows an ellipsoid plot of **1**. In addition, Supplementary material Table 1 presents crystal data and refinement details, Table S1 presents the geometric parameters and Table S2 provides the most relevant H-bonding interactions. This data allowed us to confirm the 13-(*S*) stereochemistry for the normal labdane **1**.

2.2. Chemistry

In order to enhance the activity of **1** and anticipating the development of an effective dual binding site inhibitor, we decided to connect the diterpenoid scaffold with tertiary amine groups through carbon spacers of different lengths. This strategy has been successfully applied to the synthesis of flavonoid and coumarin derivatives.[30–32] At physiological pH, the protonated amine group could interact with the CAS of the enzyme, while the diterpenoid scaffold could interact with the PAS of AChE.

The preparation of the derivatives of compound **1** was accomplished using the procedures shown in Scheme 1. Compound **1** was reacted with five commercially available α,ω-dibromoalkanes

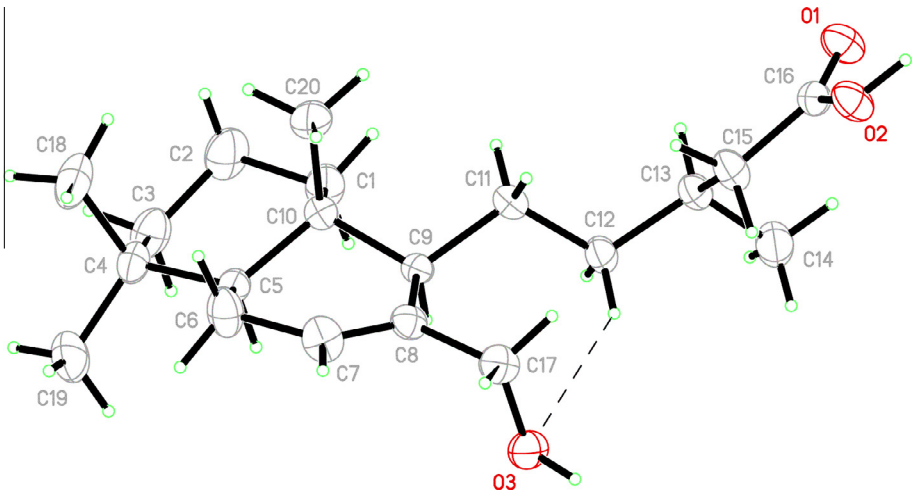
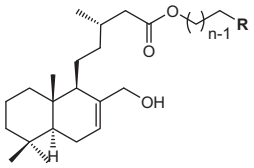


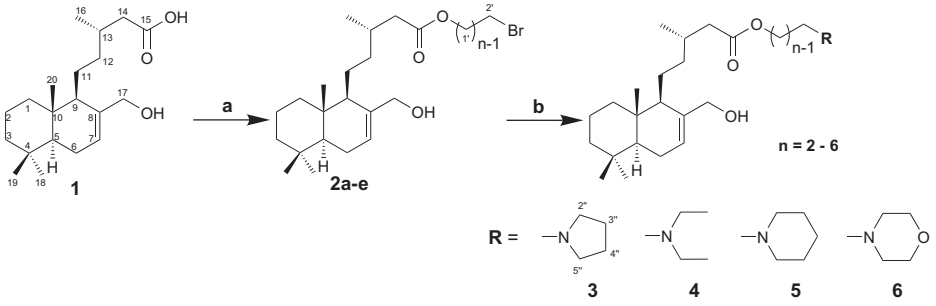
Figure 1. Molecular view of **1**, with displacement ellipsoids drawn at a 30% level. In broken lines, the intramolecular C–H···O bond. The intramolecular H-bond C12–H12b···O3 [C12–H12b: 0.97 Å, H12b...O3 2.57 Å, C12...O3 3.397(3) Å, C12–H12b...O3 143°_] is shown in broken lines.

Table 1
Inhibition of ChE activity for compounds **1**, **3**–**6**



Compds	–R	*n*	AChE		BChE		Selectivity index[a]
			IC_{50} (μM)	log IC_{50} ± SD	IC_{50} (μM)	log IC_{50} ± SD	
1			21.1		171.1		8.1
3a		2	>50.0		17.0	1.233 ± 0.101	<0.3
3b		3	33.8	1.530 ± 0.186	43.8	1.642 ± 0.145	1.3
3c		4	3.2	0.507 ± 0.079	10.3	1.013 ± 0.038	3.2
3d		5	5.5	0.744 ± 0.151	22.1	1.343 ± 0.156	4.1
3e		6	13.5	1.129 ± 0.225	10.6	1.026 ± 0.208	0.8
4a		2	>50.0		39.9	1.601 ± 0.134	<0.8
4b		3	11.8	1.074 ± 0.058	18.1	1.258 ± 0.070	1.5
4c		4	9.4	0.976 ± 0.062	23.6	1.373 ± 0.371	1.8
4d		5	13.8	1.140 ± 0.146	27.2	1.435 ± 0.186	2.0
4e		6	17.8	1.251 ± 0.079	15.2	1.183 ± 0.223	0.9
5a		2	>50.0		>50.0		–
5b		3	6.2	0.792 ± 0.055	34.7	1.539 ± 0.218	5.6
5c		4	15.0	1.176 ± 0.169	37.1	1.570 ± 0.413	2.5
5d		5	14.7	1.166 ± 0.149	24.8	1.394 ± 0.394	1.7
5e		6	14.5	1.161 ± 0.162	10.5	1.021 ± 0.247	0.7
6a		2	>50.0		>50.0		–
6b		3	>50.0		>50.0		–
6c		4	>50.0		>50.0		–
6d		5	>50.0		>50.0		–
6e		6	>50.0		>50.0		–
Tacrine	–	–	0.029	−1.53 ± 0.05	0.004	−2.35 ± 0.07	–

[a] Selectivity index = IC_{50} (BChE)/IC_{50} (AChE).



Scheme 1. Synthesis of derivatives **2**–**6**. (a) $Br(CH_2)_nBr$, K_2CO_3, DMF; (b) NHR, K_2CO_3, DMF.

in the presence of anhydrous K_2CO_3 to provide the intermediates **2a**–**e**. Finally, the reaction of **2a**–**e** with four secondary amines rendered compounds **3**–**6**. After chromatographic purification, the identity of all the derivatives was confirmed by 1D and 2D NMR spectroscopy and HRMS.

2.3. In vitro inhibition studies on AChE and BChE

The AChE and BChE inhibitory activity of compounds **3**–**6** was evaluated and compared to that of the natural diterpenoid **1**. AChE and BChE activities were measured in vitro by the spectrophotometric method developed by Ellman with slight modifications, with tacrine as the reference inhibitor.[33] The IC_{50} values for ChE inhibition and their selectivity index (SI) for the inhibition of AChE over BChE are summarized in Table 1.

The results show that most of the tested compounds were better AChE and BChE inhibitors than natural diterpenoid **1**. Compound **3c**, with a four carbon spacer and a pyrrolidine moiety showed the most potent and selective inhibition for AChE with an IC_{50} value of 3.2 μM. Also, compound **3c** exhibited the strongest inhibition to BChE with an IC_{50} value of 10.3 μM. The optimal chain lengths for AChE inhibition were a four-carbon spacer for compounds with a pyrrolidine or diethylamine group (**3c** and **4c**) and a three-carbon spacer for compounds with a piperidine group (**5b**); for BChE inhibition, the optimal chain lengths were a six-carbon spacer for compounds with a pyperidine or diethylamine group (**5e** and **4e**) and a four- and six-carbon spacer for compounds with a pyrrolidine group (**3c** and **3e**).

Compounds with a morpholine moiety (**6a**–**e**) exhibited the weakest inhibition activities for both AChE and BChE. These results can be attributed to the electron-withdrawing effect of the oxygen atom that might reduce the electronic density of the tertiary amine, thereby affecting protonation, which could diminish the interaction between the ammonium and the CAS of AChE. A similar effect was observed by Li et al. for flavonoids linked to cyclic amines possessing an additional nitrogen or oxygen atom in the terminal group.[30]

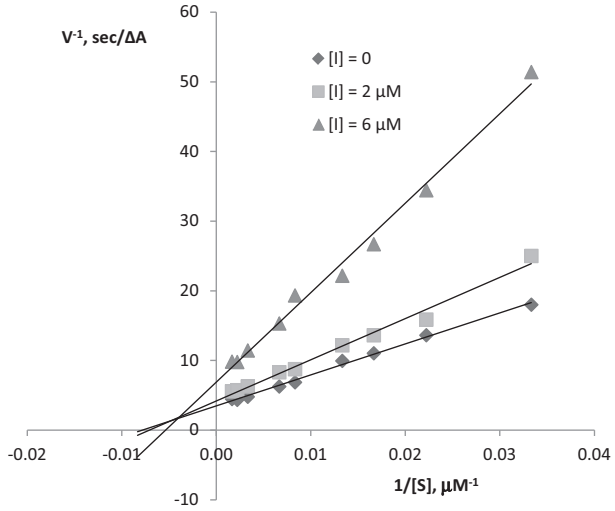


Figure 2. Lineweaver–Burk plots of the inhibition of AChE by compound **3c** with acetylthiocholine (*S*) as substrate.

Since compound **3c** was the most effective AChE inhibitor of the series, it was selected for the kinetic study of enzyme inhibition and for the molecular docking study.

2.4. Kinetic characterization of AChE inhibition

Enzyme activity was evaluated at different fixed substrate concentrations and increasing inhibitor concentrations. The data were used to elucidate the enzyme inhibition mechanism. The results are illustrated in the form of Lineweaver–Burk plots (Fig. 2). The double-reciprocal plots showed an increasing slope (decreased V_{max}) and an increasing intercept (higher K_m) on the *y*-axis at a higher concentration of **3c**, indicating mixed-type inhibition. Thus, the enzyme kinetic study suggests that the inhibitor binds to both the CAS and PAS sites of AChE. The inhibition constant K_i was equal to 4.6 ± 0.6 μM.

2.5. Molecular modeling study

Molecular docking studies were performed in order to obtain more information about the binding mode and the interactions between the enzyme and compound **3c**, and gain a structural insight into the mechanism of inhibition. The docking studies were achieved with AChE. The best results of the molecular modeling study were the conformations of cluster N°2 (−12.4 Kcal/mol) and cluster N°14 (−10.4 Kcal/mol), the largest ones because solutions that are found many times in reiterated docking experiments typically correspond to compounds with better free energy of binding.[34,35]

The conformation adopted in cluster N°2 is shown in Figure 3. Compound **3c** is fully buried into the gorge of the enzyme. It occupies the entire CAS, mid-gorge and PAS, explaining the mixed-type inhibition mechanism of AChE. It penetrates the gorge through the side chain leaving rings *A* and *B* at the peripheral site. The protonated nitrogen of the pyrrolidine resulted in a π-cation interaction with TRP84—placed at the catalytic site-with a distance of 2.6 Å (Fig. S3, Supplementary data). The docking simulation also showed that the affinity of **3c** for the complex enzyme–substrate is favored by hydrogen bonding interactions, which involve the oxygen of the ester group of **3c** and the hydrogen of the hydroxyl group of SER122. The distance between them is 2.12 Å. Also, the oxygen at C-17 comes close to TYR70 (1.88 Å) resulting in another hydrogen bond interaction (Fig. S3, Supplementary data). The main hydrophobic interactions between the hydrocarbon skeleton of the inhibitor and the protein were observed with the residues TRP279, PHE288, PHE331 and TYR334, and the residues ASP72, PHE330 and GLY441 interact with the side chain of the inhibitor (Fig. S4, Supplementary data).

The conformation adopted in the cluster N°14 is shown in Figure 3. Rings *A* and *B* of **3c** are located at the PAS of the enzyme and penetrates the gorge through ring *A* due to its aliphatic character in comparison with that of the side chain. This is in accordance with the many aromatic residues located at the peripheral site. The main hydrophobic interactions between the hydrocarbon skeleton of the inhibitor **3c** and the protein were observed with the residues: TRP279, LEU282, ILE287, PHE290 and LEU358 (Fig. S5, Supplementary data). Binding is also assisted by a hydrogen bond between the oxygen of the ester group of **3c** and the amidic hydrogen of SER286 (2.03 Å) (Fig. S6, Supplementary data). Also, an electrostatic contribution is observed between the protonated nitrogen of the pyrrolidine and the carboxylate of the ASP285 (1.73 Å) (Fig. S6, Supplementary data).

The docking studies revealed that the inhibitor can adopt two possible conformations explaining the mixed-type inhibition mechanism of action. Also, the molecular modeling allowed us to establish the orientation of the inhibitor **3c** relative to AChE as well as its conformation when bound each other. This study permitted to identify all the interactions inside the gorge as the stabilizing factors in the enzyme–substrate–inhibitor complex. Further molecular dynamics studies of this complex as starting point are necessary to check the complex inhibitor–enzyme stability, to determinate if the enzyme undergoes structural rearrangements and verify the distances and an angles observed in the interactions are within a suitable range.

2.6. Biological assays

In order to evaluate the effects of **3c** on the AChE activity in live cells, we used SH-SY5Y human neuroblastoma cells. For this purpose, we incubated SH-SY5Y cells for 1 h in the presence of **3c** (1–5 μM in PBS) and determined the AChE activity by Ellman's

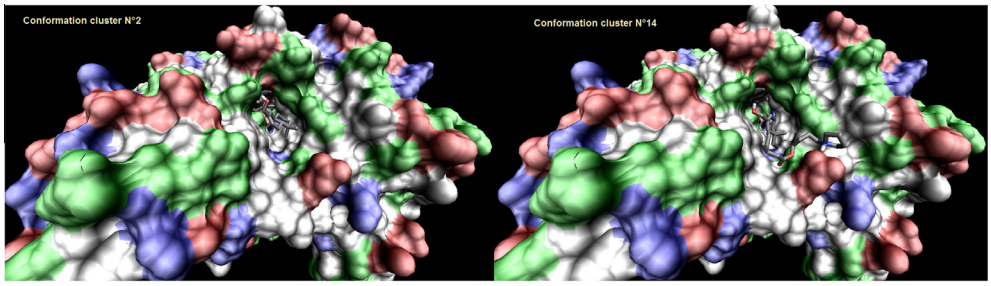


Figure 3. Docking results for compound **3c**: conformation adopted in cluster N° 2 (left) and cluster N°14 (right). Blue: basic residues, red: acid residues, green: polar residues, white: non-polar residues.

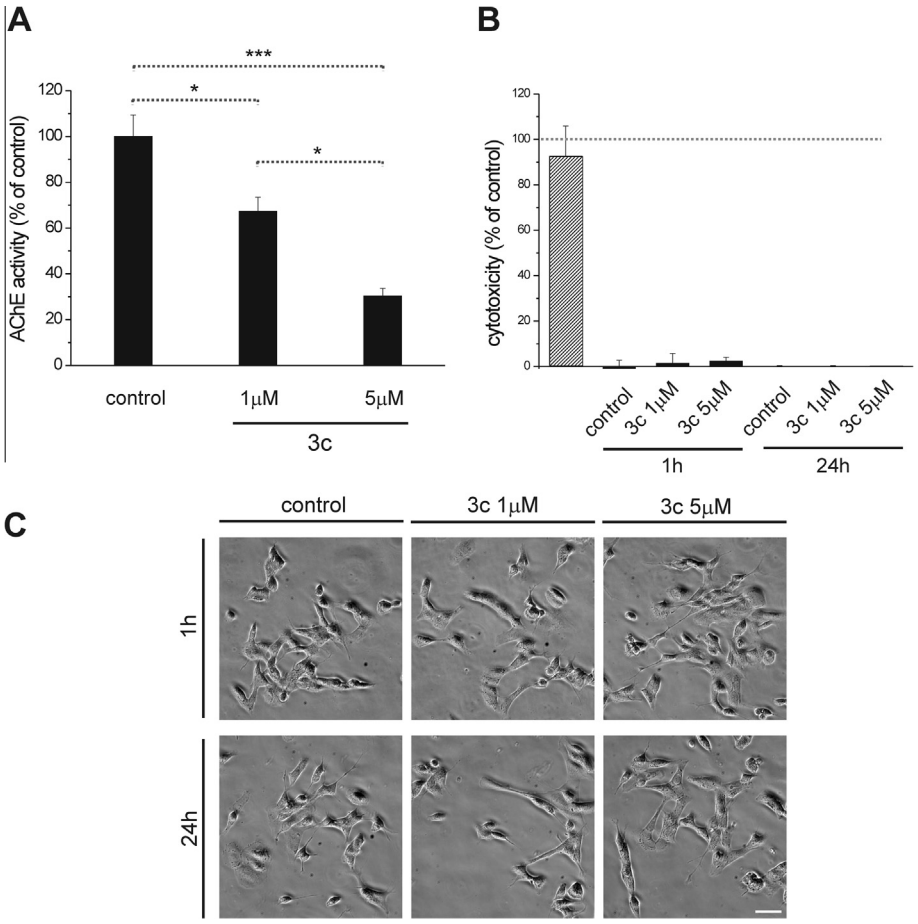


Figure 4. (A) AChE activity of SH-SY5Y cells treated for 1 h with vehicle (control) or 1–5 μM **3c**. AChE activity was determined according to Ref. 33 in intact cells. The activities of the enzyme are expressed as % of control (AChE activity) ± SEM of three independent experiments. One-way ANOVA followed by Turkey's test: control versus 1 μM **3c**, $p = 0.0194$; control versus 5 μM **3c**, $p = 0.0008$; 1 μM **3c** versus 5 μM **3c**, $p = 0.0364$. (*) and (***) denote p values <0.05 and <0.001, respectively. (B) Cell viability of SH-SY5Y cells treated for 1–24 h with vehicle (control) or 1–5 μM **3c**. Cell viability was determined by LDH assay and represented respect of 100% LDH release (bar with diagonal lines). (C) Phase contrast images of SH-SY5Y cells treated for 1 h or 24 h with vehicle (control) or 1–5 μM **3c**. Bar: 40 μm.

method.[33] Figure 4A shows the significant inhibitory effect of **3c** after 1 h of treatment at both tested concentrations (AChE activity: control, 100 ± 9.4; 1 μM **3c**, 67.3 ± 6.1; 5 μM **3c**, 30.4 ± 3.3; *n*: 3).

We analyzed the cytotoxic effect of **3c** on SH-SY5Y cells. LDH is a soluble enzyme localized in the cellular cytoplasm, and its release into the cell culture medium is an accepted marker of cell death. Treatment of SH-SY5Y cells with 1–5 μM **3c** did not affect cell viability within the time range used in the experiments (Fig. 4B) [LDH release (respect 100% LDH release): control 1 h, −0.78 ± 3.50; 1 μM **3c** 1 h, 1.34 ± 4.42; 5 μM **3c** 1 h, 2.33 ± 1.68; control 24 h, −0.02 ± 0.35; 1 μM **3c** 24 h, −0.02 ± 0.37; 5 μM **3c** 24 h, 0.11 ± 0.05; *n*: 3]. Additionally, the treatment of SH-SY5Y cells with **3c** for 1 h or 24 h did not affect the cell morphology (Fig. 4C)

3. Conclusion

In conclusion, the active metabolite responsible for AChE inhibition observed in the ethanolic extract of *G. ventanensis* was isolated and fully characterized as 17-hydroxy-(5*S*,9*R*,10*S*,13*S*)-labd-7-en-15-oic acid (**1**), a normal labdane diterpenoid. This compound, with a unique structure, proved to be a selective mixed-type AChE inhibitor, which can be easily obtained in good yield by crystallization from the plant extract. These characteristics allowed us to design and prepare twenty new derivatives (**3**–**6**), in two steps, through five intermediates (**2a**–**e**), from the natural diterpenoid **1**. All the derivatives were tested for in vitro anticholinesterase activity against AChE and BChE and most of them were better inhibitors than **1** for both enzymes. Compound **3c**, with a pyrrolydine linked to the diterpenoid by a four carbon chain, was found to be the most effective AChE ($IC_{50} = 3.2$ μM) and BChE ($IC_{50} = 10.3$ μM) inhibitor. Enzyme kinetic studies and molecular modeling indicated that **3c** targeted both the CAS and the PAS of AChE. In addition, compound **3c** elicited significant inhibition of AChE activity in SH-SY5Y human neuroblastoma cells, with no cytotoxicity. Overall, compound **3c**, synthesized from a natural diterpenoid, has potential to serve as a dual binding site AChE inhibitor for the treatment of AD and might provide a useful template for the development of new anti-AD agents.

4. Experimental section

4.1. General

Melting points (mp) were determined using a Reichert melting points apparatus. Optical rotations were determined using a Polar IBZ Messtechnik polarimeter. NMR spectra were recorded with a Bruker ARX300 spectrometer operated at 300 and 150 MHz for ^{1}H and ^{13}C, respectively, in $CDCl_3$. Chemical shifts are given in ppm (δ) with TMS as an internal standard. High resolution mass spectra (HRMS) were obtained on a QTOF mass spectrometer (micrOTOF-Q11 Series, Bruker) equipped with an electrospray ionization (ESI) interface. UV spectra were recorded on a JASCO

V-630BIO spectrophotometer. Column chromatography (CC) was performed with Silica gel 60 (70–230 mesh, Merck) and column flash chromatography with Silica gel 60 (200–425 mesh, Merck). Analytical thin layer chromatography (TLC) was performed on Silica gel 60 F_{254} sheets (0.2 mm thickness, Merck) and the spots were detected with *p*-anisaldehyde-acetic acid spray reagent.

All chemicals and solvents were analytical grade and solvents were purified by general methods before being used. AChE from electric eel (type VI-S), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), tacrine and eserine were purchased from Sigma. BChE (horse serum) was purchased from MP Biomedicals.

All derivatives were rigorously characterized by NMR spectroscopy and mass spectrometry. Compounds **2**–**6** are described here for the first time and bidimensional NMR spectra (COSY, HMBC, HSQC) were used for the unequivocal assignments. Selected NMR spectra are included in the Supplementary data.

4.2. Plant material

Aerial parts of *G. ventanensis* were collected in Sierras Grandes, Sierra de la Ventana, Buenos Aires Province, Argentina (November 2012). A voucher specimen was identified by Dra Maria Gabriela Murray and deposited in the Herbarium of the Universidad Nacional del Sur (BBB) in Bahía Blanca, Argentina, under the number *Murray*, *M.G.* 546.

4.3. Extraction and isolation of compound 1

Fresh aerial parts from *G. ventanensis* (731.8 g) were extracted with EtOH (4.5 L) at room temperature for two weeks, filtered and evaporated to dryness to yield 61.3 g of the active extract (EE, IC_{50} = 0.79 mg/mL). EE was suspended in H_2O/MeOH (9:1) (0.5 L) and then partitioned with CH_2Cl_2 to obtain EE_1 sub-extract (30.0 g). After evaporation to dryness, EE_1 was subjected to CC (silica gel 60, 70–230 mesh, 600 g), eluting with mixtures of hexane/EtOAc of increasing polarity and fractions $EE_{1.A}$–$EE_{1.M}$ (1 L each) were obtained. Fractions $EE_{1.H}$ (1.8 g) and $EE_{1.I}$ (1.7 g), eluted with hexane/EtOAc 60:40, and fractions $EE_{1.J}$ (0.7 g) and $EE_{1.K}$ (0.5 g), eluted with hexane/EtOAc 50:50, showed the highest AChE inhibition (33–67% at 0.4 mg/mL). From these fractions, spontaneously crystallized compound **1** (1.1 g, 0.15% w/w), which was unambiguously identified by ^{1}H- and ^{13}C NMR, and X-ray diffraction.

4.3.1. Compound 1

4.3.1.1. 17-Hydroxy-(5*S*,9*R*,10*S*,13*S*)-labd-7-en-15-oic acid (1). Colorless crystal; mp 135–137 °C; α = 14.5 ($CHCl_3$, *c* 0.025). ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ 5.62 (br s, 1H, H-7), 3.67 (m, 2H, H-17), 2.21 (dd, *J* = 5.4 Hz, 15.0 Hz, 1H, H-14a), 1.97 (m, 1H, H-6a), 1.93 (os, H-14b), 1.84 (os, H-6b), 1.79 (os, H-13), 1.79 (os, H-1a), 1.64 (os, H-9), 1.52 (m, 1H, H-12a), 1.40 (os, H-2), 1.39 (os, H-11a), 1.36 (os, H-3a), 1.13 (os, H-5), 1.11 (os, H-3b), 1.09 (os, H-12b), 1.09 (os, H-11b), 0.91 (os, H-1b), 0.87 (d, *J* = 6.5 Hz, 3H, H-16), 0.84 (s, 3H, H-19), 0.82 (s, 3H, H-18), 0.68 (s, 3H, H-20); ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ 174.4 (s, C-15), 139.5 (s, C-8), 121.7 (d, C-7), 63.4 (t, C-17), 52.6 (d, C-9), 49.9 (d, C-5), 42.1 (t, C-3), 41.3 (t, C-14), 38.8 (t, C-1), 38.5 (t, C-12), 36.6 (s, C-10), 33.2 (c, C-18), 32.8 (s, C-4), 30.8 (d, C-13), 23.8 (t, C-11), 23.2 (t, C-6), 21.9 (c, C-19), 20.0 (c, C-16), 18.6 (t, C-2), 13.7 (c, C-20).

4.3.2. X-ray determination of compound 1

Crystallographic measurements for compound **1** were carried out with an Oxford Diffraction Gemini CCD S Ultra diffractometer equipped with graphite-monochromated Cu–Ka radiation. The unit cell determination and data integration were carried out using the CrysAlis package of Oxford Diffraction.[36] The structure was solved by direct methods using SHELXS97[37] and refined by full-matrix least-squares on F^2 with SHELXL97.[37] The analysis of the final results was made using PLATON,[38] and the figures were prepared with XP in the SHELXTL package.[37] Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as Supplementary publication no. CCDC 988054. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.4. General procedures for the syntheses of compounds 2a–e

To a solution of compound **1** (170 mg, 0.5 mmol) and anhydrous K_2CO_3 (138 mg, 1 mmol) in dry DMF (10 mL), 0.5 mmol of α,ω-dibromoalkane was added. After stirring at room temperature until the disappearance of the starting material was confirmed by TLC (13–17 h), the solvent was removed under vacuum. The oily residue was poured into dichloromethane (15 mL) and extracted with water (3 × 5 mL), the organic layer was dried over Na_2SO_4. Then, the crude was purified by flash CC with hexane/EtOAc (85:15) to afford the desired ester.

4.4.1. Compound 2a

Compound **1** was treated with 1,2-dibromoethane (172 μL) according to general procedure to give compound **2a** as a yellow oil (62% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.38 (t, *J* = 6.3 Hz, 2H, H-1′), 4.12 (d, *J* = 12.0 Hz, 1H, H-17a), 3.96 (d, *J* = 12.0 Hz, 1H, H-17b), 3.51, (t, *J* = 6.3 Hz, 2H, H-2′), 2.37 (dd, *J* = 6.3 Hz, 15.0 Hz, 1H, H-14a), 2.20 (dd, *J* = 7.5 Hz, 15.0 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.94 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.77 (os, H-9), 1.64 (os, H-12a), 1.52 (os, H-11a), 1.47 (os, H-2), 1.41 (os, H-3a), 1.20 (os, H-5), 1.19 (os, H-11b), 1.18 (os, H-12b), 1.15 (os, H-3b), 0.98 (d, *J* = 6.6 Hz, 3H, H-16), 0.97 (os, H-1b), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.1 (s, C-15), 139.4 (s, C-8), 125.8 (d, C-7), 66.1 (t, C-17), 63.8 (t, C-1′), 52.6 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.4 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.4 (d, C-13), 28.9 (t, C-2′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20).

4.4.2. Compound 2b

Compound **1** was treated with 1,3-dibromopropane (203 μL) according to general procedure to give compound **2b** as a yellow oil (70% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.21 (t, *J* = 6.3 Hz, 2H, H-1′), 4.12 (d, *J* = 11.8 Hz, 1H, H-17a), 3.96 (d, *J* = 11.8 Hz, 1H, H-17b), 3.46 (t, *J* = 6.6 Hz, 2H, H-3′), 2.34 (dd, *J* = 6.3 Hz, 15.0 Hz, 1H, H-14a), 2.18 (os, H-2′), 2.16 (os, H-14b), 2.05 (m, 1H, H-6a), 1.93 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.78 (br s, 1H, H-9), 1.63 (os, H-12a), 1.51 (os, H-11a), 1.47 (os, H-2), 1.41 (os, H-3a), 1.20 (os, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.1 (s, C-15), 139.4 (s, C-8), 125.8 (d, C-7), 66.1 (t, C-17), 62.1 (t, C-1′), 52.6 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.4 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.9 (t, C-2′), 31.5 (d, C-13), 29.6 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20).

4.4.3. Compound 2c

Compound **1** was treated with 1,4-dibromobutane (245 μL) according to general procedure to give compound **2c** as a yellow oil (82% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H,

H-7), 4.11 (os, H-17a), 4.10 (os, H-1′), 3.95 (d, *J* = 12.0 Hz, 1H, H-17b), 3.42 (t, *J* = 6.6 Hz, 2H, H-4′), 2.32 (dd, *J* = 6.3 Hz, 14.7 Hz, 1H, H-14a), 2.14 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.93 (os, H-2′), 1.92 (os, H-13), 1.91 (os, H-6b), 1.85 (os, H-1a), 1.78 (os, H-3′), 1.77 (os, H-9), 1.62 (os, H-12a), 1.50 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.19 (os, H-5), 1.17 (os, H-12b), 1.16 (os, H-11b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.95 (d, *J* = 6.6 Hz, 3H, H-16), 0.87 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.4 (s, C-8), 125.7 (d, C-7), 66.0 (t, C-17), 63.4 (t, C-1′), 52.6 (d, C-9), 50.0 (d, C-5), 42.4 (t, C-3), 41.5 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.2 (s, C-4), 33.1 (t, C-4′), 31.4 (d, C-13), 29.5 (t, C-2′), 27.5 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20).

4.4.4. Compound 2d

Compound **1** was treated with 1,5-dibromopentane (280 μL) according to general procedure to give compound **2d** as a yellow oil (55% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H, H-7), 4.11 (os, H-17a), 4.07 (os, H-1′), 3.95 (d, *J* = 12.3 Hz, 1H, H-17b), 3.40 (t, *J* = 6.9 Hz, 2H, H-5′), 2.31 (dd, *J* = 6.3 Hz, 14.7 Hz, 1H, H-14a), 2.13 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.03 (m, 1H, H-6a), 1.93 (os, H-13), 1.91 (os, H-6b), 1.86 (os, H-4′), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.65 (os, H-2′), 1.62 (os, H-12a), 1.50 (os, H-3′), 1.47 (os, H-11a), 1.46 (os, H-2), 1.40 (os, H-3a), 1.20 (os, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.15 (os, H-3b), 0.96 (os, H-1b), 0.95 (d, *J* = 6.6 Hz, 3H, H-16), 0.87 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.4 (s, C-8), 125.6 (d, C-7), 66.0 (t, C-17), 64.1 (t, C-1′), 52.6 (d, C-9), 50.0 (d, C-5), 42.4 (t, C-3), 41.6 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.6 (t, C-5′), 33.2 (c, C-18), 33.1 (s, C-4), 32.4 (t, C-4′), 31.5 (d, C-13), 28.0 (t, C-2′), 24.8 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20).

4.4.5. Compound 2e

Compound **1** was treated with 1,6-dibromohexane (310 μL) according to general procedure to give compound **2e** as a yellow oil (60% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H, H-7), 4.12 (os, H-17a), 4.06 (os, H-1′), 3.95 (d, *J* = 12.3 Hz, 1H, H-17b), 3.40 (t, *J* = 6.9 Hz, 2H, H-6′), 2.32 (dd, *J* = 6.3 Hz, 14.7 Hz, 1H, H-14a), 2.14 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.92 (os, H-13), 1.92 (os, H-6b), 1.86 (os, H-5′), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.64 (os, H-2′), 1.63 (os, H-12a), 1.51 (os, H-11a), 1.46 (os, H-2), 1.46 (os, H-4′), 1.41 (os, H-3a), 1.38 (os, H-3′), 1.20 (os, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.4 (s, C-8), 125.7 (d, C-7), 66.0 (t, C-17), 64.3 (t, C-1′), 52.6 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.6 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.8 (t, C-6′), 33.2 (c, C-18), 33.1 (s, C-4), 32.8 (t, C-5′), 31.5 (d, C-13), 28.6 (t, C-2′), 27.9 (t, C-4′), 25.3 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20).

4.5. General procedures for the preparation of compounds 3–6

To a solution of **2a**–**2e** (0.1 mmol) in DMF (2 mL) was added the appropriate amine (0.3 mmol) and anhydrous K_2CO_3 (0.2 mmol). After stirring at room temperature (unless otherwise indicated) for 17–24 h, the solvent was removed under vacuum to afford an oily residue that was purified by flash CC with CH_2Cl_2/MeOH mixtures as eluent to afford the desired product.

4.5.1. Compound 3a

Intermediate **2a** was treated with pyrrolidine (25 μL) following the general procedure. The desired product **3a** (53% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H, H-7), 4.22 (t, *J* = 5.7 Hz, 2H, H-1′), 4.13 (d, *J* = 11.7 Hz, 1H, H-17a), 3.94 (d, *J* = 11.7 Hz, 1H, H-17b), 2.77 (t, *J* = 5.7 Hz, 2H, H-2′), 2.62 (m, 4H, H-2″ H-5″), 2.32 (dd, *J* = 7.2 Hz, 15.0 Hz, 1H, H-14a), 2.22 (dd, *J* = 6.6 Hz, 15.0 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.92 (os, H-13), 1.91 (os, H-6b), 1.85 (os, H-1a), 1.81 (os, H-3″, H-4″), 1.77 (os, H-9), 1.70 (os, H-12a), 1.50 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.20 (m, H-5), 1.16 (os, H-11b), 1.15 (os, H-12b), 1.14 (os, H-3b), 0.97 (d, *J* = 6.6 Hz, 3H, H-16), 0.96 (os, H-1b), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.4 (s, C-8), 125.7 (d, C-7), 66.0 (t, C-17), 63.1 (t, C-1′), 54.8 (t, C-2′), 54.7 (t, C-2″, C-5″), 52.6 (d, C-9), 50.0 (d, C-5), 42.4 (t, C-3), 41.6 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 24.7 (t, C-11), 23.8 (t, C-6), 23.6 (t, C-3″, C-4″), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20). HRMS (ESI) *m*/*z*: 420.3483 (calcd for $C_{26}H_{46}N_1O_3$ $[M+H]^+$ 420.3472).

4.5.2. Compound 3b

Intermediate **2b** was treated with pyrrolidine (25 μL) following the general procedure. The desired product **3b** (53% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H, H-7), 4.14 (os, H-1′), 4.11 (os, H-17a), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 2.55 (os, H-3′), 2.54 (os, H-2″, H-5″), 2.23 (m, 2H, H-14), 2.03 (m, 1H, H-6a), 1.92 (os, H-6b), 1.91 (os, H-13), 1.87 (os, H-2′), 1.85 (os, H-1a), 1.80 (os, H-3″, H-4″), 1.76 (os, H-9), 1.65 (os, H-12a), 1.52 (os, H-11a), 1.47 (os, H-2), 1.41 (os, H-3a), 1.21 (m, H-5), 1.16 (os, H-3b), 1.15 (os, H-11b), 1.14 (os, H-12b), 0.97 (d, *J* = 6.9 Hz, 3H, H-16), 0.97 (os, H-1b), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.3 (s, C-8), 124.7 (d, C-7), 65.5 (t, C-17), 62.7 (t, C-1′), 54.3 (t, C-2″, C-5″), 53.3 (t, C-3′), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.9 (t, C-14), 39.2 (t, C-1), 38.8 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.7 (d, C-13), 28.5 (t, C-2′), 24.7 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3″, C-4″), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 434.3642 (calcd for $C_{27}H_{48}N_1O_3$ $[M+H]^+$ 434.3629).

4.5.3. Compound 3c

Intermediate **2c** was treated with pyrrolidine (25 μL) following the general procedure. The desired product **3c** (30% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (93:7). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (os, H-1′), 4.09 (os, H-17a), 3.95 (d, *J* = 12.3 Hz, 1H, H-17b), 2.55 (os, H-2″, H-5″), 2.48 (os, H-4′), 2.25 (dd, *J* = 3.0 Hz, 12.4 Hz, 1H, H-14a), 2.21 (dd, *J* = 1.8 Hz, 12.4 Hz, 1H, H-14a), 2.05 (m, 1H, H-6a), 1.94 (os, H-13), 1.92 (os, H-6b), 1.85 (os, H-1a), 1.80 (os, H-3″, H-4″), 1.75 (os, H-9), 1.65 (os, H-12a), 1.64 (os, H-3′), 1.64 (os, H-2′), 1.52 (os, H-11a), 1.48 (os, H-2), 1.41 (os, H-3a), 1.20 (m, H-5), 1.15 (os, H-3b), 1.14 (os, H-11b), 1.14 (os, H-12b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.4 (s, C-8), 124.4 (d, C-7), 65.3 (t, C-17), 64.1 (t, C-1′), 56.3 (t, C-4′), 54.3 (t, C-2″, C-5″), 53.0 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 42.1 (t, C-14), 39.2 (t, C-1), 39.0 (t, C-12), 37.0 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.7 (d, C-13), 27.0 (t, C-2′), 25.5 (t, C-3′), 24.7 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3″, C-4″), 22.0 (c, C-19), 20.3 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 448.3798 (calcd for $C_{28}H_{50}N_1O_3$ $[M+H]^+$ 448.3785).

4.5.4. Compound 3d

Intermediate **2d** was treated with pyrrolidine (25 μL) following the general procedure. The desired product **3d** (63% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.74 (m, 1H, H-7), 4.10 (os, H-17a), 4.07 (os, H-1′), 3.95 (d, J = 12.3 Hz, 1H, H-17b), 3.80 (br s, 2H, H-2″, H-5″), 3.06 (m, 2H, H-5′), 2.82 (br s, 2H, H-2″/ H-5″), 2.29 (dd, J = 6.9 Hz, 14.7 Hz, 1H, H-14a), 2.18 (os, H-14b), 2.16 (os, H-3″, H-4″), 2.03 (os, H-6a), 1.97 (os, H-4′), 1.95 (os, H-6b), 1.92 (os, H-13), 1.85 (os, H-1a), 1.76 (br s, 1H, H-9), 1.69 (os, H-2′), 1.64 (os, H-12a), 1.52 (os, H-11a), 1.48 (os, H-2), 1.44 (os, H-3′), 1.42 (os, H-3a), 1.20 (m, H-5), 1.18 (os, H-3b), 1.15 (os, H-11b), 1.15 (os, H-12b), 0.97 (os, H-1b), 0.95 (d, J = 6.6 Hz, 3H, H-16), 0.87 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.3 (s, C-8), 125.3 (d, C-7), 65.7 (t, C-17), 63.6 (t, C-1′), 55.5 (t, C-5′), 53.8 (t, C-2″, C-5″), 52.7 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.7 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 28.2 (t, C-2′), 25.3 (t, C-4′), 24.5 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3′), 23.5 (t, C-3″, C-4″), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20). HRMS (ESI) m/z: 462.3953 (calcd for $C_{29}H_{52}N_1O_3$ $[M+H]^+$ 462.3942).

4.5.5. Compound 3e

Intermediate **2e** was treated with pyrrolidine (25 μL) following the general procedure. The desired product **3e** (79% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (95:5). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (os, H-17a), 4.06 (os, H-1′), 3.96 (d, J = 12.3 Hz, 1H, H-17b), 2.56 (br s, 4H, H-2″, H-5″), 2.47 (m, 2H, H-6′), 2.30 (dd, J = 6.6 Hz, 14.7 Hz,1H, H-14a), 2.15 (dd, J = 6.9 Hz, 14.7 Hz, 1H, H-14b), 2.03 (m, 1H, H-6a), 1.92 (os, H-6b), 1.92 (os, H-13), 1.85 (os, H-1a), 1.80 (os, H-3″, H-4″), 1.76 (os, H-9), 1.61 (os, H-2′), 1.58 (os, H-12a), 1.51 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.37 (os, H-5′), 1.36 (os, H-3′), 1.36 (os, H-4′), 1.20 (m, H-5), 1.16 (os, H-11b), 1.16 (os, H-12b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, J = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.5 (s, C-8), 125.0 (d, C-7), 65.7 (t, C-17), 64.4 (t, C-1′), 56.6 (t, C-6′), 54.3 (t, C-2″, C-5″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.5 (d, C-13), 28.7 (t, C-5′), 28.7 (t, C-2′), 27.3 (t, C-4′), 26.0 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3″, C-4″), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) m/z: 476.4099 (calcd for $C_{30}H_{54}N_1O_3$ $[M+H]^+$ 476.4098).

4.5.6. Compound 4a

Intermediate **2a** was treated with diethylamine (31 μL) according to the general procedure. After stirring 6 h at 55 °C, the solvent was removed under vacuum and the oily residue was purified by flash CC with DCM/MeOH (94:6) to give the desired product **4a** as a colorless oil (63% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.26 (t, J = 5.7 Hz, 2H, H-1′), 4.12 (d, J = 12.0 Hz, 1H, H-17a), 3.94 (d, J = 12.0 Hz, 1H, H-17b), 2.88 (t, J = 5.7 Hz, 2H, H-2′), 2.76 (c, J = 7.2 Hz, 4H, H-1″), 2.32 (dd, J = 7.2 Hz, 14.7 Hz, 1H, H-14a), 2.21 (dd, J = 6.9 Hz, 14.7 Hz, 1H, H-14b), 2.06 (m, 1H, H-6a), 1.92 (os, H-13), 1.91 (os, H-6b), 1.85 (os, H-1a), 1.78 (br s, 1H, H-9), 1.70 (os, H-12a), 1.52 (os, H-11a), 1.47 (os, H-2), 1.44 (t, J = 7.2 Hz, 6H, H-2″), 1.42 (os, H-3a), 1.21 (m, H-5), 1.16 (os, H-11b), 1.16 (os, H-12b), 1.16 (os, H-3b), 0.98 (os, H-1b), 0.97 (d, J = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.4 (s, C-15), 139.4 (s, C-8), 125.9 (d, C-7), 66.0 (t, C-17), 61.4 (t, C-1′), 52.6 (d, C-9), 50.8 (t, C-2′), 50.0 (d, C-5), 47.7 (t, C-1″), 42.4 (t, C-3), 41.7 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 24.7 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20), 10.9 (c, C-2″). HRMS (ESI) m/z: 422.3648 (calcd for $C_{26}H_{48}N_1O_3$ $[M+H]^+$ 422.3629).

4.5.7. Compound 4b

Intermediate **2b** was treated with diethylamine (31 μL) according to the general procedure. After stirring 6 h at 55 °C, the solvent was removed under vacuum and the oily residue was purified with flash CC with DCM/MeOH (94:6) to give the desired product **4b** as a colorless oil (43% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.11 (os, H-1′), 4.10 (os, H-17a), 3.96 (d, J = 12.3 Hz, 1H, H-17b), 2.55 (os, 4H, H-1″), 2.53 (os, H-3′), 2.28 (dd, J = 6.9 Hz, 14.7 Hz, 1H, H-14a), 2.18 (dd, J = 6.9 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.91 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.80 (os, H-2′), 1.76 (os, H-9), 1.65 (os, H-12a), 1.50 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.21 (m, H-5), 1.15 (os, H-3b), 1.15 (os, H-11b), 1.15 (os, H-12b), 1.04 (t, J = 7.2 Hz, 6H, H-2″), 0.98 (os, H-1b), 0.96 (d, J = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.3 (s, C-8), 125.0 (d, C-7), 65.6 (t, C-17), 62.8 (t, C-1′), 52.8 (d, C-9), 50.1 (d, C-5), 49.5 (t, C-3′), 46.8 (t, C-1″), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 26.5 (t, C-2′), 24.6 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20), 11.5 (c, C-2″). HRMS (ESI) m/z: 436.3786 (calcd for $C_{27}H_{50}N_1O_3$ $[M+H]^+$ 436.3785).

4.5.8. Compound 4c

Intermediate **2c** treated with diethylamine (31 μL) according to the general procedure. After stirring 6 h at 55 °C, the solvent was removed under vacuum and the oily residue was purified with flash CC with DCM/MeOH (88:12) to give the desired product **4c** as a colorless oil (46% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (os, H-17a), 4.09 (os, H-1′), 3.96 (d, J = 12.0 Hz, 1H, H-17b), 2.59 (c, J = 7.2 Hz, 4H, H-1″), 2.49 (t, J = 7.5 Hz, 2H, H-4′), 2.27 (dd, J = 7.2 Hz, 14.7 Hz, 1H, H-14a), 2.19 (dd, J = 6.6 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.93 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.76 (br s, 1H, H-9), 1.63 (os, H-12a), 1.62 (os, H-2′), 1.58 (os, H-3′), 1.51 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.20 (m, H-5), 1.16 (os, H-3b), 1.15 (os, H-11b), 1.15 (os, H-12b), 1.06 (t, J = 7.2 Hz, 6H, H-2″), 0.97 (os, H-1b), 0.96 (d, J = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.3 (s, C-8), 124.8 (d, C-7), 65.5 (t, C-17), 64.2 (t, C-1′), 52.9 (d, C-9), 52.5 (t, C-4′), 50.1 (d, C-5), 46.8 (t, C-1″), 42.4 (t, C-3), 41.9 (t, C-14), 39.2 (t, C-1), 38.9 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 26.9 (t, C-2′), 24.6 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3′), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20), 11.1 (c, C-2″). HRMS (ESI) m/z: 450.3962 (calcd for $C_{28}H_{52}N_1O_3$ $[M+H]^+$ 450.3942).

4.5.9. Compound 4d

Intermediate **2d** was treated with diethylamine (31 μL) according to the general procedure. After stirring 6 h at 55 °C, the solvent was removed under vacuum and the oily residue was purified with flash CC with DCM/MeOH (92:8) to give the desired product **4d** as a colorless oil (74% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (os, H-17a), 4.07 (os, H-1′), 3.96 (d, J = 12.0 Hz, 1H, H-17b), 3.14 (c, J = 7.2 Hz, 4H, H-1″), 2.99 (m, 2H, H-5′), 2.30 (dd, J = 6.6 Hz, 15.0 Hz, 1H, H-14a), 2.16 (dd, J = 7.2 Hz, 15.0 Hz, 1H, H-14b), 2.08 (os, H-6a), 1.90 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-4′), 1.85 (os, H-1a), 1.75 (br s, 1H, H-9), 1.69 (os, H-2′), 1.64 (os, H-12a), 1.51 (os, H-11a), 1.46 (os, H-2), 1.43 (t, J = 7.2 Hz, 6H, H-2″), 1.42 (os, H-3′), 1.41 (os, H-3a), 1.20 (m, H-5), 1.15 (os, H-11b), 1.15 (os, H-12b), 1.14 (os, H-3b), 0.96 (os, H-1b), 0.95 (d,

J = 6.9 Hz, 3H, H-16), 0.87 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.3 (s, C-8), 125.4 (d, C-7), 65.8 (t, C-17), 63.6 (t, C-1′), 52.7 (d, C-9), 51.5 (t, C-5′), 50.1 (d, C-5), 46.7 (t, C-1″), 42.4 (t, C-3), 41.7 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 28.3 (t, C-2′), 24.5 (t, C-11), 23.8 (t, C-6), 23.6 (t, C-3′), 23.2 (t, C-4′), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20), 8.7 (c, C-2″). HRMS (ESI) *m*/*z*: 464.4114 (calcd for $C_{29}H_{54}N_1O_3$ $[M+H]^+$ 464.4098).

4.5.10. Compound 4e

Intermediate **2e** was treated with diethylamine (31 μL) according to the general procedure. After stirring 6 h at 55 °C, the solvent was removed under vacuum and the oily residue was purified with flash CC with DCM/MeOH (90:10) to give the desired product **4e** as a colorless oil (57% yield). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.11 (os, H-17a), 4.06 (os, H-1′), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 2.66 (c, *J* = 7.2 Hz, 4H, H-1″), 2.53 (m, 2H, H-6′), 2.31 (dd, *J* = 6.6 Hz, 14.7 Hz, 1H, H-14a), 2.15 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.93 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.63 (os, H-2′), 1.63 (os, H-12a), 1.54 (os, H-5′), 1.54 (os, H-11a), 1.47 (os, H-2), 1.41 (os, H-3a), 1.36 (os, H-3′), 1.35 (os, H-4'), 1.20 (os, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.16 (os, H-3b), 1.11 (t, *J* = 7.2 Hz, 6H, H-2″), 0.97 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.4 (s, C-8), 125.3 (d, C-7), 65.8 (t, C-17), 64.4 (t, C-1′), 52.7 (t, C-6′), 52.6 (d, C-9), 50.1 (d, C-5), 46.9 (t, C-1″), 42.4 (t, C-3), 41.7 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.5 (d, C-13), 28.7 (t, C-2′), 27.2 (t, C-4′), 26.2 (t, C-5′), 26.0 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20), 11.0 (c, C-2″). HRMS (ESI) *m*/*z*: 478.4273 (calcd for $C_{30}H_{56}N_1O_3$ $[M+H]^+$ 478.4255).

4.5.11. Compound 5a

Intermediate **2a** was treated with piperidine (30 μL) according to the general procedure. The desired product **5a** (44% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (96:4). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.20 (t, *J* = 6.0 Hz, 2H, H-1′), 4.13 (d, *J* = 12.0 Hz, 1H, H-17a), 3.95 (d, *J* = 12.0 Hz, 1H, H-17b), 2.60 (t, *J* = 6.0 Hz, 2H, H-2′), 2.44 (t, *J* = 4.3 Hz, 4H, H-2″, H-6″), 2.31 (dd, *J* = 6.6 Hz, 14.8 Hz, 1H, H-14a), 2.19 (dd, *J* = 6.9 Hz, 14.8 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.91 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.68 (os, H-12a), 1.58 (os, H-3″, H-5″), 1.52 (os, H-11a), 1.46 (os, H-2), 1.42 (os, H-4″), 1.41 (os, H-3a), 1.20 (m, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.15 (os, H-3b), 0.97 (d, *J* = 6.6 Hz, 3H, H-16), 0.97 (os, H-1b), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.4 (s, C-8), 125.7 (d, C-7), 65.9 (t, C-17), 62.0 (t, C-1′), 57.6 (t, C-2′), 55.0 (t, C-2″, C-6″), 52.6 (d, C-9), 50.0 (d, C-5), 42.4 (t, C-3), 41.6 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 25.9 (t, C-3″, C-5″), 24.6 (t, C-11), 24.3 (t, C-4″), 23.8 (t, C-6), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 434.3646 (calcd for $C_{27}H_{48}N_1O_3$ $[M+H]^+$ 434.3629).

4.5.12. Compound 5b

Intermediate **2b** was reacted with piperidine (30 μL) following the general procedure. The desired product **5b** (63% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.76 (m, 1H, H-7), 4.12 (os, H-1′), 4.09 (os, H-17a), 3.96 (d, *J* = 12.0 Hz, 1H, H-17b), 2.39 (os, H-3′), 2.39 (os, H-2″, H-6″), 2.23 (m, 2H, H-14), 2.05 (m, 1H, H-6a), 1.90 (os, H-6b), 1.90 (os, H-13), 1.85 (os, H-1a), 1.84 (os, H-2′), 1.76 (os, H-9), 1.65 (os, H-12a), 1.59 (os, H-3″, H-5″), 1.53 (os, H-11a), 1.46 (os, H-2), 1.43 (os, H-4″), 1.41 (os, H-3a), 1.20 (os, H-5), 1.15 (os, H-3b), 1.15 (os, H-12b), 1.14 (os, H-11b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.3 (s, C-8), 124.8 (d, C-7), 65.5 (t, C-17), 62.8 (t, C-1′), 56.1 (t, C-3′), 54.7 (t, C-2″, C-6″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.8 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.7 (d, C-13), 26.4 (t, C-2′), 25.8 (t, C-3″, C-5″), 24.6 (t, C-11), 24.4 (t, C-4″), 23.8 (t, C-6), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 448.3807 (calcd for $C_{28}H_{50}N_1O_3$ $[M+H]^+$ 448.3785).

4.5.13. Compound 5c

Intermediate **2c** was reacted with piperidine (30 μL) following the general procedure. The desired product **5c** (41% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.76 (m, 1H, H-7), 4.10 (os, H-1′), 4.10 (os, H-17a), 3.98 (d, *J* = 12.3 Hz, 1H, H-17b), 2.44 (m, 4H, H-2″, H-6″), 2.36 (t, *J* = 6.9 Hz, 2H, H-4′), 2.23 (m, 2H, H-14), 2.06 (m, 1H, H-6a), 1.94 (os, H-13), 1.91 (os, H-6b), 1.86 (os, H-1a), 1.77 (br s, H-9), 1.64 (os, H-2′), 1.63 (os, H-3″, H-5″), 1.63 (os, H-3′), 1.62 (os, H-12a), 1.55 (os, H-4″), 1.48 (os, H-2), 1.46 (os, H-11a), 1.42 (os, H-3a), 1.22 (os, H-5), 1.17 (os, H-3b), 1.16 (os, H-12b), 1.15 (os, H-11b), 0.98 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.4 (s, C-8), 124.6 (d, C-7), 65.4 (t, C-17), 64.2 (t, C-1′), 59.0 (t, C-4′), 54.7 (t, C-2″, C-6″), 52.9 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 42.0 (t, C-14), 39.3 (t, C-1), 38.9 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 27.0 (t, C-2′), 25.9 (t, C-3″, C-5″), 24.6 (t, C-11), 24.3 (t, C-4″), 23.8 (t, C-6), 23.4 (t, C-3′), 22.0 (c, C-19), 20.3 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 462.3962 (calcd for $C_{29}H_{52}N_1O_3$ $[M+H]^+$ 462.3942).

4.5.14. Compound 5d

Intermediate **2d** was reacted with piperidine (30 μL) following the general procedure. The desired product **5d** (43% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (94:6). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.11 (os, H-17a), 4.07 (os, H-1′), 3.96 (d, *J* = 12.7 Hz, 1H, H-17b), 3.43 (br s, 2H, H-2″/ H-6″), 2.93 (m, 2H, H-5′), 2.73 (br s, 2H, H-2″/ H-6″), 2.29 (dd, *J* = 6.9 Hz, 14.8 Hz, 1H, H-14a), 2.16 (dd, *J* = 6.9 Hz, 14.8 Hz, 1H, H-14b), 2.06 (os, H-6a), 2.04 (os, H-3″/ H-5″), 1.96 (os, H-4′), 1.91 (os, H-13), 1.90 (os, H-6b), 1.90 (os, H-4″), 1.87 (os, H-1a), 1.76 (br s, 1H, H-9), 1.68 (os, H-2′), 1.64 (os, H-12a), 1.51 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.41 (os, H-3′), 1.20 (os, H-5), 1.15 (os, H-3b), 1.15 (os, H-11b), 1.14 (os, H-12b), 0.97 (os, H-1b), 0.95 (d, *J* = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.74 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.3 (s, C-8), 125.3 (d, C-7), 65.7 (t, C-17), 63.6 (t, C-1′), 57.5 (t, C-5′), 53.4 (t, C-2″/ C-6″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.2 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 28.2 (t, C-2′), 24.5 (t, C-11), 23.8 (t, C-6), 23.5 (t, C-3′), 23.3 (t, C-4′), 22.6 (t, C-3″/ C-5″), 22.3 (t, C-4″), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.7 (c, C-20). HRMS (ESI) *m*/*z*: 476.4115 (calcd for $C_{30}H_{54}N_1O_3$ $[M+H]^+$ 476.4098).

4.5.15. Compound 5e

Intermediate **2e** was reacted with piperidine (30 μL) following the general procedure. The desired product **5e** (81% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (96:4). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (os, H-17a), 4.05 (os, H-1′), 3.96 (d, *J* = 12.3 Hz, 1H,

H-17b), 2.38 (m, 4H, H-2″, H-6″), 2.30 (os, H-14a), 2.28 (os, H-6′), 2.14 (dd, *J* = 7.5 Hz, 15.0 Hz, 1H, H-14b), 2.02 (m, 1H, H-6a), 1.92 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.62 (os, H-2′), 1.62 (os, H-12a), 1.60 (os, H-3″, H-5″), 1.51 (os, H-5′), 1.50 (os, H-11a), 1.47 (os, H-2), 1.45 (os, H-4″), 1.41 (os, H-3a), 1.34 (os, H-3′), 1.33 (os, H-4′), 1.20 (os, H-5), 1.16 (os, H-11b), 1.16 (os, H-12b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.4 (s, C-8), 125.1 (d, C-7), 65.7 (t, C-17), 64.4 (t, C-1′), 59.5 (t, C-6′), 54.7 (t, C-2″, C-6″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.5 (d, C-13), 28.7 (t, C-2′), 27.4 (t, C-4′), 26.8 (t, C-5′), 26.0 (t, C-3′), 25.9 (t, C-3″, C-5″), 24.5 (t, C-4″), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 490.4274 (calcd for $C_{31}H_{56}N_1O_3$ $[M+H]^+$ 490.4255).

4.5.16. Compound 6a

Intermediate **2a** was reacted with morpholine (26 μL) following the general procedure. The desired product **6a** (72% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (98:2). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.21 (t, *J* = 5.7 Hz, 2H, H-1′), 4.13 (d, *J* = 12.0 Hz, 1H, H-17a), 3.96 (d, *J* = 12.0 Hz, 1H, H-17b), 3.71 (t, *J* = 4.5 Hz, 4H, H-3″, H-5″), 2.62 (t, *J* = 5.7 Hz, 2H, H-2′), 2.51 (t, *J* = 4.5 Hz, 4H, H-2″, H-6″), 2.32 (dd, *J* = 6.6 Hz, 14.7 Hz, 1H, H-14a), 2.18 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.92 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.78 (br s, 1H, H-9), 1.66 (os, H-12a), 1.52 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.20 (os, H-5), 1.18 (os, H-11b), 1.17 (os, H-12b), 1.16 (os, H-3b), 0.97 (d, *J* = 6.6 Hz, 3H, H-16), 0.97 (os, H-1b), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.5 (s, C-15), 139.4 (s, C-8), 125.8 (d, C-7), 67.0 (t, C-3″, C-5″), 66.0 (t, C-17), 61.4 (t, C-1′), 57.4 (t, C-2′), 54.0 (t, C-2″, C-6″), 52.6 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.6 (t, C-14), 39.2 (t, C-1), 38.6 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 24.5 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 436.3437 (calcd for $C_{26}H_{46}N_1O_4$ $[M+H]^+$ 436.3421).

4.5.17. Compound 6b

Intermediate **2b** was reacted with morpholine (26 μL) following the general procedure. The desired product **6b** (43% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (96:4). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.13 (os, H-1′), 4.11 (os, H-17a), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 3.71 (t, *J* = 4.5 Hz, 4H, H-3″, H-5″), 2.44 (os, H-2″, H-6″), 2.42 (os, H-3′), 2.29 (dd, *J* = 6.9 Hz, 14.7 Hz, 1H, H-14a), 2.17 (dd, *J* = 7.2 Hz, 14.7 Hz, 1H, H-14b), 2.05 (m, 1H, H-6a), 1.92 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.82 (os, H-2′), 1.76 (os, H-9), 1.65 (os, H-12a), 1.51 (os, H-11a), 1.46 (os, H-2), 1.41 (os, H-3a), 1.20 (os, H-5), 1.16 (os, H-12b), 1.16 (os, H-11b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.9 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.3 (s, C-8), 125.3 (d, C-7), 67.0 (t, C-3″, C-5″), 65.8 (t, C-17), 62.6 (t, C -1′), 55.7 (t, C-3′), 53.8 (t, C-2″, C-6″), 52.7 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.7 (t, C-14), 39.2 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 26.1 (t, C-2′), 24.5 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 450.3600 (calcd for $C_{27}H_{48}N_1O_4$ $[M+H]^+$ 450.3578).

4.5.18. Compound 6c

Intermediate **2c** was reacted with morpholine (26 μL) following the general procedure. The desired product **6c** (66% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (95:5). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.76 (m, 1H, H-7), 4.10 (os, H-17a), 4.08 (os, H-1′), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 3.71 (t, *J* = 4.5 Hz, 4H, H-3″, H-5′), 2.44 (t, *J* = 4.5 Hz, 4H, H-2″, H-6″), 2.34 (t, *J* = 7.5 Hz, 2H, H-4′), 2.26 (os, H-14a), 2.18 (dd, *J* = 6.9 Hz, 14.7 Hz 1H, H-14b), 2.03 (m, 1H, H-6a), 1.92 (os, H-13), 1.90 (os, H-6b), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.65 (os, H-2′), 1.64 (os, H-12a), 1.57 (os, H-3′), 1.52 (os, H-11a), 1.47 (os, H-2), 1.42 (os, H-3a), 1.21 (os, H-5), 1.16 (os, H-3b), 1.16 (os, H-12b), 1.16 (os, H-11b), 0.98 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.6 (s, C-15), 139.4 (s, C-8), 125.0 (d, C-7), 67.0 (t, C-3″, C-5″), 65.6 (t, C-17), 64.2 (t, C-1′), 58.7 (t, C-4′), 53.9 (t, C-2″, C-6″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.9 (t, C-14), 39.2 (t, C-1), 38.8 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 26.8 (t, C-2′), 24.5 (t, C-11), 23.8 (t, C-6), 23.2 (t, C-3′), 22.0 (c, C-19), 20.2 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 464.3739 (calcd for $C_{28}H_{50}N_1O_4$ $[M+H]^+$ 464.3734).

4.5.19. Compound 6d

Intermediate **2d** was reacted with morpholine (26 μL) following the general procedure. The desired product **6d** (37% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (96:4). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.12 (os, H-17a), 4.08 (os, H-1′), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 3.72 (t, *J* = 4.5 Hz, 4H, H-3″, H-5″), 2.43 (t, *J* = 4.5 Hz, 4H, H-2″, H-6″), 2.33 (os, H-5′), 2.31 (os, H-14a), 2.18 (dd, *J* = 7.2 Hz, 14.7 Hz, 1H, H-14b), 2.06 (m, 1H, H-6a), 1.93 (os, H-13), 1.91 (os, H-6b), 1.86 (os, H-1a), 1.76 (br s, 1H, H-9), 1.65 (os, H-2′), 1.65 (os, H-12a), 1.53 (os, H-4′), 1.52 (os, H-11a), 1.47 (os, H-2), 1.42 (os, H-3a), 1.39 (os, H-3′), 1.20 (os, H-5), 1.17 (os, H-11b), 1.17 (os, H-12b), 1.16 (os, H-3b), 0.98 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.86 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.4 (s, C-8), 125.3 (d, C-7), 67.1 (t, C-3″, C-5″), 65.8 (t, C-17), 64.3 (t, C-1′), 59.1 (t, C-5′), 53.9 (t, C-2″, C-6″), 52.8 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.8 (t, C-14), 39.2 (t, C-1), 38.8 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.6 (d, C-13), 28.7 (t, C-2′), 26.3 (t, C-4′), 24.5 (t, C-11), 24.1 (t, C-3′), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 478.3914 (calcd for $C_{29}H_{52}N_1O_4$ $[M+H]^+$ 478.3891).

4.5.20. Compound 6e

Intermediate **2e** was reacted with morpholine (26 μL) following the general procedure. The desired product **6e** (48% yield) was obtained as colorless oil after purification by flash CC with DCM/MeOH (96:4). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.75 (m, 1H, H-7), 4.10 (d, *J* = 12.3 Hz, 1H, H-17a), 4.06 (t, *J* = 6.6 Hz, 2H, H-1′), 3.96 (d, *J* = 12.3 Hz, 1H, H-17b), 3.71 (t, *J* = 4.5 Hz, 4H, H-3″, H-5″), 2.43 (t, *J* = 4.8 Hz, 4H, H-2″, H-6″), 2.31 (os, H-6′), 2.31 (os, H-14a), 2.14 (dd, *J* = 7.5 Hz, 14.7 Hz, 1H, H-14b), 2.06 (os, H-6a), 1.92 (os, H-13), 1.92 (os, H-6b), 1.85 (os, H-1a), 1.77 (br s, 1H, H-9), 1.63 (os, H-2′), 1.62 (os, H-12a), 1.51 (os, H-11a), 1.49 (os, H-5′), 1.46 (os, H-2), 1.41 (os, H-3a), 1.36 (os, H-3′), 1.35 (os, H-4′), 1.20 (os, H-5), 1.17 (os, H-12b), 1.16 (os, H-11b), 1.15 (os, H-3b), 0.97 (os, H-1b), 0.96 (d, *J* = 6.6 Hz, 3H, H-16), 0.88 (s, 3H, H-19), 0.85 (s, 3H, H-18), 0.75 (s, 3H, H-20); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 173.7 (s, C-15), 139.4 (s, C-8), 125.4 (d, C-7), 67.1 (t, C-3″, C-5″), 65.9 (t, C-17), 64.4 (t, C-1′), 59.2 (t, C-6′), 53.9 (t, C-2″, C-6″), 52.7 (d, C-9), 50.1 (d, C-5), 42.4 (t, C-3), 41.7 (t, C-14), 39.3 (t, C-1), 38.7 (t, C-12), 36.9 (s, C-10), 33.3 (c, C-18), 33.1 (s, C-4), 31.5 (d, C-13), 28.7 (t, C-2′), 27.2 (t, C-4′), 26.6 (t, C-5′), 26.0 (t, C-3′), 24.4 (t, C-11), 23.8 (t, C-6), 22.0 (c, C-19), 20.1 (c, C-16), 18.9 (t, C-2), 13.8 (c, C-20). HRMS (ESI) *m*/*z*: 492.4066 (calcd for $C_{30}H_{54}N_1O_4$ $[M+H]^+$ 492.4047).

4.6. Biological activity

4.6.1. Inhibition assay on AChE and BChE in vitro

Electric eel (*Torpedo californica*) AChE and horse serum BChE were used as sources of both cholinesterases. AChE and BChE inhibitory activities were measured in vitro by the spectrophotometric method developed by Ellman with slight modifications.[33] The lyophilized enzyme, 500 U AChE (300 U BChE), was dissolved in buffer phosphate A (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5 (3) U/mL stock solution. Further enzyme dilution was carried out with buffer phosphate B (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) to produce 0.126 (0.06) U/mL enzyme solution. Samples were dissolved in buffer phosphate B with 2.5% of MeOH as cosolvent. 300 μL of enzyme solution and 300 μL of sample solution were mixed in a test tube and incubated for 60/120 min at room temperature. The reaction was started by adding 600 μL of the substrate solution (0.5 mM DTNB, 0.6 mM ATCI/BTCI, 0.1 M Na_2HPO_4, pH 7.5). The absorbance was read at 405 nm for 180 s at 27 °C. Enzyme activity was calculated by comparing reaction rates for the samples to the blank. All reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Tacrine (99%) was used as the reference AChE and BChE inhibitor.

4.6.2. Kinetic characterization of AChE inhibition

The enzyme reaction was carried out at three fixed inhibitor (**3c**) concentrations (0, 2 and 6 μM). In each case the initial velocity measurements were obtained at varying substrate (S) concentrations ([ATCI] = 15-450 μM) and the reciprocal of the initial velocity (1/v) was plotted as a function of 1/[*S*]. The data were analyzed with GraphPad Prism 5. The Lineweaver–Burk plot showed a pattern of lines with the increasing slopes, characteristic of mixed-type inhibition (K_i = 4.6 ± 0.6 μM). The nonlinear regression of these data fitted with uncompetitive inhibition with a R^2 = 0.9860.

4.6.3. SH-SY5Y human neuroblastoma cells

For standard growth conditions, SH-SY5Y cells were grown in medium consisting of DMEM/Ham F12 nutrient (Gibco) mixture (1:1) supplemented with 10% foetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) at 37 °C in a 5% CO_2. For biochemical determinations, 1×10^5 SH-SY5Y cells were seeded in 35-mm plates and maintained in the conditions described above.

4.6.4. Determination of AChE activity in live cells

We determined the AChE activity according to Ellman's method with minor modifications.[33] Briefly, SH-SY5Y cells grown for 72 h in 35-mm plates were washed three-times with PBS (150 mM NaCl, 10 mM Na_2HPO_4, 10 mM, NaH_2PO_4, pH 7.4) and **3c** was added at final concentration of 1-5 μM. Cells were maintained in the incubator, in presence of DTNB (final concentration 0.31 mM in PBS) and ATChI (final concentration, 0.9 mM in PBS). EtOH was used as vehicle and was kept below 0.13% in all cases. Control cells were incubated in the presence of the vehicle. After 1 h, 1 mL of supernatant was collected from each condition and absorbance was measured. Results are expressed as a percentage of the control.

4.6.5. LDH cytotoxicity assay

Cellular death was spectrophotometrically measured evaluating the activity of the cytoplasmic enzyme LDH released by cells with damaged plasma membranes. These data were compared with that obtained after a complete lysis of cells. SH-SY5Y cells were grown as described above and treated with 1–5 μM **3c** in grown medium. 1–24 h after drug treatment, 100 μL of medium was collected and LDH activity was determined using a kinetic assay according to manufacturer's instructions (Wiener LDH-P UV). LDH release was quantified by comparison with 100% LDH release obtained by treat cells with 0.1% Triton X-100 in PBS.

4.6.6. Light microscopy

To visualize live SH-SY5Y cells, CNh cells grown on coverslips were treated as above and at the indicated times cells were imaged with a 20× phase contrast objective using a Nikon Eclipse E-600 microscope (Nikon, Melville, NY). Images were captured using a K2E Apogee CCD camera driven by CCDOPS software (Santa Barbara Instrument Group, Santa Barbara, CA).

4.6.7. Statistical analysis

All results are presented as mean ± SEM from at least three independent experiments. AChE activity and LDH cytotoxicity were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons The observed differences were considered to be statistically significant when p <0.05. Analysis of data was performed using Origin version 8 OriginLab Graphing Software.

4.7. Molecular docking determinations

Geometry optimization of the derivative **3c**—protonated at physiological pH—was performed with semiempirical calculations (AM1) and the Hartree-Fock method and the 6-31+G (d) basis set incorporated in the Gaussian 03 program.[39–41] The charges of the ligand were obtained using the standard RESP procedure.[42]

Torpedo californica AChE crystal structure was chosen to perform the docking studies given that this was the enzyme used in the in vitro assays. Structure of Protein Data Bank (PDB) entry 2ACE (complexed with ACh) was used for the docking simulations of compound **3c**.[43] The acetylcholine was removed from the active site previously to the modeling, given that compound **3c** is a mixed-type inhibitor. Docking studies were performed with version 4.2.5.1 of the program AutoDock, using the implemented empirical free energy function.[44] The graphical user interface program AutoDock Tools was used to prepare, run and analyze the docking simulations. The simulation space was defined as a 20.25 Å × 24 Å × 34.5 Å box which included the active site and the peripheral site. Atomic interaction energy on a 0.375 Å grid was calculated with the auxiliary program Autogrid 4 using probes corresponding to each map type found in the inhibitor. All rotatable dihedrals in **3c** were allowed to rotate freely. The starting position of **3c** was outside the grid on a random position.

The inhibitor was docked by the Lamarckian genetic algorithm protocol. A total of 256 independent simulations with a population size of 150 members were run for **3c** using AutoDock 4.2.5.1 with default parameters (random starting position and conformation, translation step of 2.0 Å, mutation rate of 0.02, crossover rate of 0.8, local search rate 0.06 and 25,00,000 energy evaluations). After docking, the 256 conformers generated for the inhibitor were assigned to clusters based on a tolerance of 2.0 Å all atom root-mean-square deviation (rmsd) in position from the lowest-energy solution. The clusters were also ranked according to the energies of their representative conformations, which were the lowest-energy solutions within each cluster.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.06.030. These data include MOL files and InChiKeys of the most important compounds described in this article.

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