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RESEARCH ARTICLE

New *N,N*-dimethylcarbamate inhibitors of acetylcholinesterase: design synthesis and biological evaluation

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Abstract

A series of *N,N*-dimethylcarbamates containing a *N,N*-dibenzylamino moiety was synthesized and tested to evaluate their ability to inhibit Acetylcholinesterase (AChE). The most active compounds **4** and **8**, showed 85 and 69% of inhibition at 50 μM, respectively. Furthermore, some basic SAR rules were outlined: an alkyl linker of six methylene units is the best spacer between the carbamoyl and dibenzylamino moieties; electron-withdrawal substituents on aromatics rings of the dibenzylamino group reduce the inhibitory power. Compound **4** produces a slow onset inhibition of AChE and this is not due to the carbamoylation of the enzyme, as demonstrated by the time-dependent inhibition assay of AChE with compound **4** and by MALDI-TOF MS analysis of trypsinized AChE inhibited by compound **4**. Instead, compound **4** could act as a slow-binding inhibitor of AChE, probably because of its high conformational freedom due to the linear alkyl chain.

Keywords

Acetylcholinesterase inhibitors, carbamate, MALDI-TOF-MS

History

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Introduction

Acetylcholinesterase (AChE) plays a pivotal role in the termination of synaptic transmission by rapid hydrolysis of the neurotransmitter acetylcholine (ACh) into acetate and choline in the synaptic cleft, after the neurotransmitter release from the presynaptic nerve terminal[1]. AChE is present in both the central and peripheral nervous systems, including at the neuromuscular junction[2–4].

AChE is a serine hydrolase belonging to the esterase family. Its active site contains two subsites, the ''esterasic'' and ''anionic'' subsites. The esterasic site, where ACh is hydrolyzed, contains the catalytic triad of three amino acids: Ser203 (the amino acids numbering, unless otherwise specified, refer to the human AChE), His447 and Glu334[5]. The contiguous anionic subsite, called catalytic ''anionic'' site (CAS), recognizes the quaternary group of ACh via π-cation interactions with aromatic residues; here, among the aromatic amino acids, Trp86 is critical for this role[6]. A second anionic-binding site for ACh, known as ''peripheral'' anionic site (PAS), lies on the surface of AChE approximately 20 Å distant from the active site. The PAS consists of five residues (Tyr72, Asp74, Tyr124, Trp286 and Tyr337) involved in the allosteric modulation of catalysis at the active site and is the target of various anti-cholinesterase drugs. It is also implicated in a number of non-classical functions, in particular, amyloid deposition, cell adhesion and neurite outgrowth[7]. A gorge, surrounded by aromatic amino acidic residues and with a length of about 20 Å, connects the PAS to the active site[8].

The inhibition of AChE, by compounds that interact with the CAS and or with the PAS, has, as a consequence, a prolongation of the persistency of acetylcholine in the synaptic cleft and hence an increase in cholinergic tone. For this reason, AChE is still a therapeutic target for the symptomatic treatment of Alzheimer's disease (AD) because it is associated with a cholinergic deficit[9]. AChE inhibitors (AChEIs) have also been suggested for other possible therapeutic applications, i.e. Wernicke–Korsakoff syndrome[10] and posttraumatic cognitive impairments[11].

Furthermore, besides of its cholinergic role, in the AD the AChE plays a non-cholinergic role accelerating beta-amyloid plaques assembly[12]. Thus, the AChEIs can now be seen in new perspectives, being able to restore the compromised cholinergic tone and at the same time interfering with the AChE-induced amyloid deposition.

In Chart 1 the main AChEIs approved for the treatment of AD are represented[13]. Tacrine was described as reversible inhibitor, interacting with Trp86 at CAS; its use has actually been abandoned due to the high incidence of the side effects. Donepezil (Aricept®) is a reversible inhibitor that binds the PAS, it is indicated for the use in the palliative treatment of mild to moderate AD. Galantamine (Razadyn®, Nivalin®) is a reversible inhibitor that interacts with anionic subsite and with the aromatic gorge; it is indicated for the treatment of mild to moderate AD and other memory impairments. Rivastigmine (Exelon®) is a slow-acting inhibitor that covalently binds to Ser203 blocking esterasic activity; it has been approved for the treatment of mild to moderate AD.

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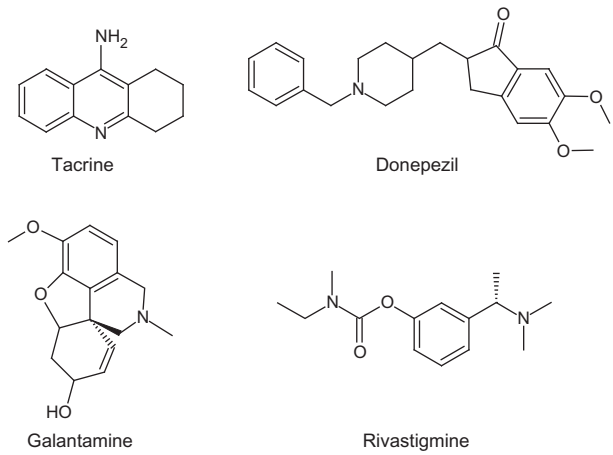
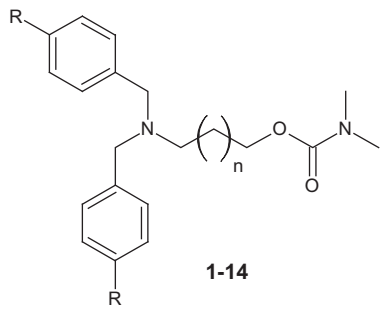


Chart 1. Main AChEIs approved for the treatment of AD.

Chart 2. Synthesized carbamates **1–14**.



1 : R = H; n = 1
2 : R = H; n = 2
3 : R = H; n = 3
4 : R = H; n = 4
5 : R = CH_3; n = 1
6 : R = CH_3; n = 2
7 : R = CH_3; n = 3
8 : R = CH_3; n = 4
9 : R = F; n = 1
10 : R = F; n = 2
11 : R = F; n = 4
12 : R = CF_3; n = 1
13 : R = CF_3; n = 2
14 : R = CF_3; n = 4

Agents as Rivastigmine, that contain a carbamyl group, are typical pseudo-irreversible AChEIs[14,15] that give carbamylation of active site serine residue and form a carbamoyl–enzyme intermediate that will be slowly hydrolyzed in order to give back the native enzyme.

Here, we report the synthesis, the characterization and the AChE inhibition properties of new series of carbamate derivatives (Chart 2). For this series of compounds, we have chosen a small dimension *N,N*-dimethylcarbamic function, in order to allow its entrance through the cramped catalytic gorge. With the aim of optimize the interaction of synthesized compounds with aromatic amino acids characterizing the PAS and the access gorge, we have added an *N,N*-dibenzylamino moiety. These two fragments were linked by means of a flexible alkyl chain with variable length, comprised between 3 and 6 methylenic units. The synthesized compounds should inhibit AChE through combined interactions: the carbamic function could interact with the amino acid residues of the catalytic site (esterasic and anion subsite); in addition, the *N,N*-dibenzylamino group should also interact with the aromatic amino acid residues of the PAS. Because of the difficulty to predict the conformational behavior of linear alkyl chains, we chose to use different length chains to connect the aromatic and the carbamic moieties. Furthermore, we also evaluate the effects of electron withdrawal or electron donor substituents on the aromatic groups.

The synthesized compounds were evaluated for the AChEIs activity and the most active compound was studied, by UV and MALDI mass spectrometry, in order to demonstrate if it is really able to produce the carbamylation of AChE.

Materials and methods

Chemical synthesis

All reagents, solvents and deuterated were purchased from Sigma-Aldrich (Milano, Italy). Acetonitrile was freshly purified by distillation over calcium hydride. ^{1}H and ^{13}C NMR spectra were recorded on AVANCE200 Bruker spectrometer operating at 200 and 50 MHz, respectively; chemical shifts (δ) are given in ppm, relatively to TMS as internal reference, coupling constant are given in Hertz. The following abbreviations were used, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Mass spectra were acquired on a GC-MS mass spectrometer Hewlett-Packard 5890 series II equipped with a mass selective detector Hewlett-Packard 5971 series II.

Synthesis of carbamates 1–14

The synthesis of carbamates **1**–**14** was carried out following two different strategies (due to the low yields): *N,N*-dibenzylation of the amino alcohol followed by dimethylcarbamoylation (Route A) or dimethylcarbamoylation of the amino alcohol and subsequent *N,N*-dibenzylation (Route B). The higher yields in carbamates were obtained following Procedure B.

Route A. To a solution of amino alcohol (1.0 mmol) in 5.0 mL MeCN, benzyl bromide (3.0 mmol) was added dropwise and the mixture was kept under stirring at room temperature for 24 h. Then, Et_3N (6.0 mmol) was added dropwise at room temperature and the mixture was kept under stirring at room temperature for 48 h. The solvent was evaporated under reduced pressure and the

residue was extracted using ethyl acetate. After evaporation of the solvent under reduced pressure, usually pure dibenzylamino alcohol was obtained (48–79% yields). Dibenzylamino alcohol thus obtained (0.7 mmol), *N,N*-dimethylaminocarbamoyl chloride (1.0 mmol) and freshly distilled pyridine (1.0 mmol) in CH_2Cl_2 (2.0 mL) were kept under stirring at room temperature for three days; then the solvent was evaporated under reduced pressure and the product was purified by flash column chromatography (*n*-hexane: ethyl acetate from 9:3 to 7:3 as eluent). Yields: 5–15%.

Route B. To a solution of amino alcohol (1.0 mmol) in 5.0 mL MeCN, HCl gas was bubbled for 3–4 min; then N_2 gas was bubbled into the mixture to eliminate HCl excess. *N,N*-dimethylaminocarbamoyl chloride (1.0 mmol) was added to the mixture and the solution was refluxed for 12 h. The product was used in the following reaction without further purification. Benzyl bromide (3.0 mmol) was added to the solution and the mixture was kept at room temperature under stirring for 3 h; the mixture refluxed for 12 h, then triethylamine (3.0 mmol) was added and the mixture refluxed for 2 days. The solvent was evaporated under reduced pressure and the product was purified by flash column chromatography (*n*-hexane: ethyl acetate from 9:3 to 7:3 as eluent). Yields: 15–40%.

Characterization data

The characterization data for the synthesized compounds are given below.

3-(Dibenzylamino)propan-1-ol (**15a**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.73–1.83 (m, 2H), 2.67 (*t*, $J = 5.9$ Hz, 2H), 3.61 (s, 4H), 3.64 (*t*, $J = 5.4$ Hz, 2H), 7.25–7.35 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.9, 52.9, 58.4, 63.5, 126.8, 128.4, 129.0, 137.8; EIMS, *m/z*: $M^{\cdot+}$ absent, 210 (17%), 91 (100%), 65 (24%), 44 (36%).

4-(Dibenzylamino)butan-1-ol (**15b**) ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.55–1.64 (m, 2H), 1.71–1.77 (m, 2H), 2.57 (*t*, $J = 6.3$ Hz, 2H), 3.58 (*t*, $J = 5.7$ Hz, 2H), 3.70 (s, 4H), 7.27–7.42 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.9, 30.9, 53.4, 58.1, 62.1, 127.5, 128.4, 129.5, 137.5; EIMS, *m/z*: $M^{\cdot+}$ absent, 210 (13%), 148 (18%), 91 (100%), 65 (20%).

5-(Dibenzylamino)pentan-1-ol (**15c**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25–1.61 (m, 6H), 2.47 (*t*, $J = 7.0$ Hz, 2H), 3.56–7.62 (m, 6H), 7.25–7.41 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.3, 26.5, 32.4, 53.0, 58.2, 62.8, 127.0, 128.2, 129.0, 139.0; EIMS, *m/z*: 283 ($M^{\cdot+}$, 1%), 210 (32%), 91 (100%), 65 (11%).

6-(Dibenzylamino)hexan-1-ol (**15d**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25–1.30 (m, 2H), 1.44–1.56 (m, 2H), 2.45 (*t*, $J = 7.2$ Hz, 2H), 3.56–7.63 (m, 6H), 7.25–7.42 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.4, 26.8, 32.7, 52.9, 58.1, 62.9, 127.0, 128.2, 129.0, 139.0; EIMS, *m/z*: $M^{\cdot+}$ absent, 210 (30%), 91 (100%), 73 (25%).

3-(Bis(4-methylbenzyl)amino)propan-1-ol (**15e**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.86–1.91 (m, 2H), 2.37 (s, 6H), 2.77–2.80 (m, 2H), 3.69–3.74 (m, 6H), 7.12–7.29 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 27.9, 52.7, 58.3, 63.0, 126.4, 128.3, 128.4, 130.1, 137.1, 138.0; EIMS, *m/z*: $M^{\cdot+}$ absent, 238 (19%), 105 (100%), 77 (12%).

4-(Bis(4-methylbenzyl)amino)butan-1-ol (**15f**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.58–1.74 (m, 4H), 2.34 (s, 6H), 2.52 (*t*, $J = 6.1$ Hz, 2H), 3.58 (*t*, $J = 5.4$ Hz, 2H), 3.61 (s, 4H), 7.07–7.28 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 24.2, 31.1, 53.4, 58.1, 62.5, 126.5, 128.1, 128.3, 130.3, 137.2, 137.9; EIMS, *m/z*: 297 ($M^{\cdot+}$, 1%), 238 (19%), 105 (100%), 84 (23%).

6-(Bis(4-methylbenzyl)amino)hexan-1-ol (**15 h**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.28–1.33 (m, 4H), 1.43–1.64 (m, 4H), 2.36 (s, 6H), 2.49–2.56 (m, 2H), 3.60 (*t*, $J = 6.4$ Hz, 2H), 3.69 (s, 4H), 7.09–7.12 (m, 4H), 7.23–7.27 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.5, 25.6, 26.5, 27.0, 32.7, 53.1, 58.1, 62.5, 126.2, 127.8, 128.1, 129.9, 137.7, 138.6; EIMS, *m/z*: $M^{\cdot+}$ absent, 238 (1%), 105 (100%), 79 (21%).

3-(Bis(4-fluorobenzyl)amino)propan-1-ol (**15i**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.77–1.89 (m, 2H), 2.73 (*t*, $J = 6.0$ Hz, 2H), 3.65 (m, 6H), 7.09–7.00 (m, 4H), 7.38–7.31 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.1, 52.5, 57.5, 63.1, 115.2 (d, $J = 21.3$ Hz), 130.7 (d, $J = 8.0$ Hz), 133.6 (d, $J = 3.2$ Hz), 162.0 (d, $J = 245.3$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 231 (7%), 136 (25%), 123 (34%), 109 (100%), 95 (30%).

4-(Bis(4-fluorobenzyl)amino)butan-1-ol (**15 l**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.60–1.63 (m, 2H), 2.64 (*t*, $J = 5.4$ Hz, 2H), 3.55 (*t*, $J = 5.0$ Hz, 2H), 3.71 (s, 4H), 6.93–7.01 (m, 4H), 7.21–7.28 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.3, 32.2, 49.2, 53.1, 62.4, 115.2 (d, $J = 21.2$ Hz), 129.8 (d, $J = 8.0$ Hz), 134.9 (d, $J = 3.2$ Hz), 162.0 (d, $J = 245.0$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 246 (11%), 109 (100%), 83 (18%).

6-(Bis(4-fluorobenzyl)amino)hexan-1-ol (**15m**):^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25–1.32 (m, 2H), 1.40–1.58 (m, 2H), 2.37–2.49 (m, 2H), 3.57–3.64 (m, 6H), 6.99–7.07 (m, 4H), 7.30–7.37 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.5, 26.7, 27.0, 32.6, 53.0, 57.3, 62.6, 114.9 (d, $J = 21.2$ Hz), 130.3 (d, $J = 7.8$ Hz), 134.9 (d, $J = 2.9$ Hz), 161.9 (d, $J = 244.4$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 246 (1%), 109 (100%), 83 (21%).

3-(Bis(4-(trifluoromethyl)benzyl)amino)propan-1-ol (**15n**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.75–1.87 (m, 2H), 2.65 (*t*, $J = 6.1$ Hz, 2H), 3.64 (s, 4H), 3.68 (*t*, $J = 5.6$ Hz, 2H), 7.45 (d, $J = 8.2$ Hz, 4H), 7.60 (d, $J = 8.2$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.4, 52.9, 58.2, 63.1, 124.1 (q, $J = 271.9$ Hz), 125.5 (q, $J = 3.8$ Hz), 129.2, 129.7 (q, $J = 32.4$ Hz), 142.3; EIMS, *m/z*: $M^{\cdot+}$ absent, 159 (100%), 140 (8%), 109 (41%), 69 (5%).

4-(Bis(4-(trifluoromethyl)benzyl)amino)butan-1-ol (**15o**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.55–1.71 (m, 4H), 2.50 (*t*, $J = 6.2$ Hz, 2H), 3.61 (*t*, $J = 5.7$ Hz, 2H), 3.65 (s, 4H), 7.48 (d, $J = 8.2$ Hz, 4H), 7.60 (d, $J = 8.2$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.9, 30.7, 53.8, 58.0, 62.4, 124.2 (q, $J = 271.8$ Hz), 125.2 (q, $J = 3.8$ Hz), 129.1, 129.3 (q, $J = 32.2$ Hz), 143.1; EIMS, *m/z*: $M^{\cdot+}$ absent, 346 (21%), 159 (100%), 140 (9%), 109 (31%).

6-(Bis(4-(trifluoromethyl)benzyl)amino)hexan-1-ol (**15p**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27–1.33 (m, 4H), 1.50–1.57 (m, 4H), 2.43 (t, $J = 6.9$ Hz, 2H), 3.64–3.58 (m, 6H), 7.47 (d, $J = 8.0$ Hz, 4H), 7.58 (d, $J = 8.0$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.5, 26.9, 32.6, 53.7, 58.0, 62.6, 124.3 (q, $J = 273.3$ Hz), 125.4 (q, $J = 3.8$ Hz), 128.8, 129.2 (q, $J = 32.2$ Hz), 144.0; EIMS, *m/z*: $M^{\cdot+}$ absent, 346 (12%), 159 (100%), 140 (7%), 109 (19%).

3-(Dibenzylamino)propyl dimethylcarbamate[1]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.81–1.88 (m, 2H), 2.50–2.62 (m, 2H), 2.62 (bs, 3H), 2.87 (bs, 3H), 3.58 (s, 4H), 4.11 (*t*, $J = 6.3$ Hz, 2H), 7.26–7.40 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 26.6, 35.6, 36.3, 49.5, 58.3, 63.1, 126.7, 128.2, 128.7, 139.7, 156.5; EIMS, *m/z*: $M^{\cdot+}$ absent, 145 (100%), 117 (35%), 102 (15%), 91 (62%), 72 (41%).

4-(Dibenzylamino)butyl dimethylcarbamate[2]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.60–1.63 (m, 4H), 2.43–2.47 (m, 2H), 2.88 (s, 6H), 3.57 (bs, 4H), 4.01 (*t*, $J = 6.0$ Hz, 2H), 7.23–7.40 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.5, 26.9, 35.9, 36.3, 53.0, 58.3, 65.2, 126.8, 128.2, 128.7, 139.8, 156.7; EIMS, *m/z*: $M^{\cdot+}$ absent, 158 (68%), 118 (29%), 91 (100%), 72 (52%).

4-(Dibenzylamino)pentyl dimethylcarbamate[3]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.34–1.43 (m, 2H), 1.49–1.59 (m, 4H), 2.45 (*t*, $J = 6.9$ Hz, 2H), 2.90 (s, 6H), 3.57 (bs, 4H), 4.04 (*t*, $J = 6.4$ Hz, 2H), 7.24–7.41 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.6, 26.6, 28.9, 35.9, 36.2, 53.1, 58.3, 65.3, 126.8, 128.1, 128.8, 139.8, 156.8; EIMS, *m/z*: $M^{\cdot+}$ absent, 263 (13%), 210 (51%), 91 (100%), 72 (17%).

6-(Dibenzylamino)hexyl dimethylcarbamate[4]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (bm, 4H), 1.56 (bm, 4H), 2.44 (bm, 2H), 2.89 (s, 6H), 3.58 (bs, 4H), 4.01 (*t*, $J = 6.6$ Hz, 2H), 7.27–7.37 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.7, 26.8, 29.0, 35.9, 36.2, 53.9, 58.0, 65.3, 127.1, 128.3, 129.1, 139.7, 156.8; EIMS, *m/z*: $M^{\cdot+}$ absent, 210 (11%), 91 (100%), 72 (30%), 42 (26%).

3-(Bis(4-methylbenzyl)amino)propyl dimethylcarbamate[5]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.58 (bs, 8H), 2.35 (m, 2H), 2.88 (bs, 6H), 3.52 (s, 4H), 4.02 (*t*, $J = 6.3$ Hz, 2H), 7.06–7.43 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 26.6, 35.7, 36.3, 49.5, 58.3, 63.3, 125.9, 127.6, 128.1, 129.6, 137.7, 139.5, 156.6; EIMS, *m/z*: $M^{\cdot+}$ absent, 105 (100%), 72 (59%), 42 (53%).

4-(Bis(4-methylbenzyl)amino)butyl dimethylcarbamate[6]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.61–1.63 (m, 4H), 2.36 (s, 6H), 2.46 (m, 2H), 2.88 (s, 6H), 3.55 (bs, 4H), 4.02 (*t*, $J = 6.1$ Hz, 2H), 7.08–7.27 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 26.9, 29.7, 35.8, 36.1, 52.8, 58.1, 65.1, 125.9, 127.6, 128.1, 129.7, 137.8, 139.6, 156.7; EIMS, *m/z*: $M^{\cdot+}$ absent, 263 (3%), 238 (8%), 105 (100%), 91 (9%).

6-(Bis(4-methylbenzyl)amino)pentyl dimethylcarbamate[7]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.29–1.41 (m, 2H), 1.55–1.641 (m, 4H), 2.37 (s, 6H), 2.41–2.44 (m, 2H), 2.91 (s, 6H), 3.55 (s, 4H), 4.05 (*t*, $J = 6.4$ Hz, 2H), 7.06–7.09 (m, 2H), 7.19–7.23 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 23.6, 26.6, 28.9, 35.9, 36.2, 53.1, 58.3, 65.4, 125.9, 127.5, 128.0, 129.6, 137.6, 139.7, 156.8; EIMS, *m/z*: 382 ($M^{\cdot+}$, 1%), 277 (16%), 238 (82%), 105 (100%).

6-(Bis(4-methylbenzyl)amino)hexyl dimethylcarbamate[8]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.28–1.33 (m, 4H), 1.51–1.64 (m, 4H), 2.36 (s, 6H), 2.43 (*t*, $J = 6.8$ Hz, 2H), 2.91 (s, 6H), 3.54 (s, 4H), 4.04 (*t*, $J = 6.6$ Hz, 2H), 7.04–7.07 (m, 4H), 7.19–7.26 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.4, 25.8, 26.9, 29.1, 35.8, 36.3, 53.2, 58.2, 65.4, 125.9, 127.5, 128.0, 129.6, 137.6, 139.7, 156.9; EIMS, *m/z*: $M^{\cdot+}$ absent, 238 (23%), 133 (18%), 105 (100%).

3-(Bis(4-fluorobenzyl)amino)propyl dimethylcarbamate[9]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.77–1.84 (m, 2H), 2.45–2.51 (m, 2H), 2.63 (bs, 3H), 2.87 (bs, 3H), 3.50 (s, 4H), 4.08 (*t*, $J = 6.2$ Hz, 2H), 6.94–7.03 (m, 4H), 7.26–7.33 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 26.5, 35.5, 36.2, 49.2, 57.5, 62.9, 115.0 (d, $J = 21.3$ Hz), 130.1 (d, $J = 7.8$ Hz), 135.1, 156.5, 161.9 (d, $J = 244.5$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 109 (100%), 89 (4%), 72 (24%), 42 (17%).

4-(Bis(4-fluorobenzyl)amino)butyl dimethylcarbamate[10]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.56–1.61 (m, 4H), 2.40–2.42 (m, 2H), 2.88 (bs, 6H), 3.49 (s, 4H), 4.00 (t, $J = 6.2$ Hz, 2H), 6.96–7.04 (m, 4H), 7.27–7.33 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.5, 26.9, 35.9, 36.2, 52.9, 57.5, 65.1, 114.9 (d, $J = 21.4$ Hz), 130.0 (d, $J = 4.7$ Hz), 135.2 (d, $J = 1.6$ Hz), 156.8, 162.0 (d, $J = 260.9$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 246 (3%), 109 (100%), 72 (30%), 42 (19%).

6-(Bis(4-fluorobenzyl)amino)hexyl dimethylcarbamate[11]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.28–1.31 (m, 4H), 1.50–1.62 (m, 4H), 2.36 (s, 6H), 2.38 (*t*, $J = 6.6$ Hz, 2H), 2.90 (s, 6H), 3.49 (s, 4H), 4.02 (*t*, $J = 6.6$ Hz, 2H), 6.95–7.04 (m, 4H), 7.27–7.33 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.7, 26.9, 29.0, 35.9, 36.3, 53.2, 57.4, 65.3, 114.9 (d, $J = 21.3$ Hz), 130.0 (d, $J = 7.5$ Hz), 135.4 (d, $J = 2.0$ Hz), 156.8, 161.8 (d, $J = 242.4$ Hz); EIMS, *m/z*: $M^{\cdot+}$ absent, 246 (4%), 109 (100%), 72 (21%), 42 (10%).

3-(Bis(4-(trifluoromethyl)benzyl)amino)propyl dimethylcarbamate[12]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.71–1.88 (m, 2H), 2.48–2.55 (m, 2H), 2.55 (bs, 3H), 2.86 (bs, 3H), 3.61 (s, 4H), 4.12 (*t*, $J = 6.1$ Hz, 2H), 7.47 (d, $J = 8.2$ Hz, 4H), 7.57 (d, $J = 8.2$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 26.5, 35.4, 36.2, 49.7, 58.1, 62.7, 124.1 (q, $J = 271.8$ Hz), 125.2 (q, $J = 3.7$ Hz), 128.8 (q, $J = 52.2$ Hz), 128.9, 143.6, 156.4; EIMS, *m/z*: $M^{\cdot+}$ absent, 346 (10%), 303 (35%), 214 (38%), 159 (100%), 109 (17%), 72 (19%).

4-(Bis(4-(trifluoromethyl)benzyl)amino)butyl dimethylcarbamate[13]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.59–1.63 (m, 4H), 2.47 (bs, 2H), 2.87 (bs, 6H), 3.61 (s, 4H), 4.03 (*t*, $J = 6.2$ Hz, 2H), 7.47 (d, $J = 8.2$ Hz, 4H), 7.57 (d, $J = 8.2$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 23.6, 26.9, 35.8, 36.3, 53.4, 58.0, 65.0, 124.1 (q, $J = 272.7$ Hz), 125.2, 128.8, 129.0 (q, $J = 52.2$ Hz), 143.6, 156.6; EIMS, *m/z*: $M^{\cdot+}$ absent, 332 (100%), 186 (66%), 159 (76%), 69 (17%).

6-(Bis(4-(trifluoromethyl)benzyl)amino)hexyl dimethylcarbamate[14]: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27–1.33 (m, 4H), 1.53–1.58 (m, 4H), 2.42 (t, $J = 7.0$ Hz, 2H), 2.89 (s, 6H), 3.60 (s, 4H), 4.03 (t, $J = 6.7$ Hz, 2H), 7.47 (d, $J = 8.2$ Hz, 4H), 7.57 (d, $J = 8.2$ Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.8, 26.9, 26.9, 29.0, 35.8, 36.2, 53.7, 58.0, 65.2, 124.2 (q, $J = 271.7$ Hz), 125.1 (q, $J = 3.8$ Hz),128.8, 129.2 (q, $J = 32.2$ Hz), 143.9, 156.8; EIMS, *m/z*: $M^{\cdot+}$ absent, 346 (94%), 159 (100%), 90 (4%).

Enzymatic tests

The spectrophotometric method of Ellman[16] with minor modifications[17,18] was used to evaluate the inhibition of AChE. Electric eel AChE (AChE, EC 3.1.1.7), acetylthiocholine iodide, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), donepezil, used as reference standard, and carbaryl (1-naphthyl methylcarbamate) were purchased from Sigma-Aldrich (Milan, Italy). The sequencing grade modified trypsin was purchased from Promega (Milan, Italy). The assay was carried out by double beam UV-Vis lambda 40 Perkin Elmer spectrophotometer, using optical polystyrene cuvettes ($10 \times 10 \times 45$ mm, 340–800 nm optical transparency). Each compound was dissolved in DMSO in the opportune quantity in order to obtain a final cuvette DMSO content $< 0.05\%$, that does not affect the enzyme activity.

Percent inhibition of AChE

Aliquots of 3.0 mL of 0.1 M (pH 7.4) phosphate buffer containing DTNB (0.25 mM) and AChE (0.083 U mL^{-1}) were placed in a polystyrene cuvette of 1.0 cm path length; 1 µL of a solution 150 mM in DMSO of the tested compound was added. To start the reaction 30 µL of an aqueous solution in phosphate buffer (pH = 7.4) of acetylthiocoline (10 mM) were added. The increase in the absorbance, due to the production of the yellow 5-nitro-2-tio benzoic anion, was recorded at 412 nm and 25 °C between 0.5 and 1.5 min. As a control, an identical solution of the enzyme without the inhibitor was processed following the same protocol to determine the 100% of enzyme activity.

Each experiment was repeated at least in triplicate. The potency of each compound to inhibit AChE activity was expressed as percent inhibition calculated using the following equation:

$$\text{Inhibition } (\%) = \frac{A_c - A_i}{A_c} \times 100$$

where A_i and A_c represent the change in the absorbance in the presence of inhibitor and without inhibitor, respectively.

*Time-dependent inhibition assay for compound **4***

Aliquots of 3.0 mL of 0.1 M (pH 7.4) phosphate buffer containing DTNB (0.25 mM) and AChE (0.083 U mL^{-1}) were mixed in a polystyrene cuvette of 1 cm path length with 1 µL of a solution 15 mM in DMSO of the compound **4**, obtaining a 5 µM final concentration. Immediately after, acetylthiocoline (30 µL, 10 mM) was added. Then, the change in the absorbance was recorded. In order to obtain a reference enzymatic activity (negative control), a solution containing the enzyme without the inhibitor was processed in the same way. After 40 min of incubation, the inhibition percentage was measured again, both for AChE

incubated with **4** and for the negative control, using the protocol above described. As a positive control of the carbamylation reaction, a 1 μM carbaryl solution was used.

At the same time a solution of AChE (0.083 U mL^{-1}) in 25.0 mL of 0.1 M phosphate buffer pH 7.4 was prepared. From this stock solution of AChE, three aliquots of 2.0 mL each were taken: solution A, containing compound **4** (40 μM); solution B, with carbaryl (4 μM); solution C, that was left as it was for having a negative control. Each solution was incubated at 37 °C for 2 h. The buffer solution with residual inhibitor, if presents, was removed from the incubation mixture by centrifugation and ultrafiltration, using Vivaspin 2 device with membrane 3000 MWCO PES (Sartorius Stedim Biotech GmbH, Goettingen, Germany); the residual content was washed with 2 mL of phosphate buffer and then taken up with 3.0 mL of phosphate buffer containing DTNB (0.25 mM) to measure the inhibition percentage as described above.

Mass spectrometry analysis

Preparation of AChE

Fifty micrograms of AChE in 500 μL of 50 mM sodium phosphate buffer at pH 7.4 were incubated with compound **4** (200 μM) or carbaryl (200 μM). The control sample of AChE was incubated only with buffer. After incubation at 37 °C for 2 h, residual inhibitor was removed from the incubation mixture by centrifugation and ultrafiltration, using Vivaspin 2 device. The buffer was exchanged to 50 mM ammonium bicarbonate buffer. Each sample was concentrated and then taken up with MeCN to denature the enzyme. The samples were subjected to proteolytic digestion by overnight incubation with sequencing grade modified trypsin, 0.02 μg mL^{-1} in 50 mM acetic acid (1:50 protein ratio of trypsin/AChE), at room temperature. The digested samples were concentrated under reduce pressure and then dissolved in 200 μL of H_2O-MeCN-TFA (100:20:0.02) for the mass spectrometry analysis.

MALDI-TOF-MS analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PE Biosystems Voyager DE mass spectrometer (Houston, TX). Mass spectra were acquired in positive-ion linear mode under delayed extraction conditions, using an acceleration voltage of 23 kV and laser intensity of 3500 V with a 337 nm pulsed nitrogen laser. External calibration was performed using human insulin (5807.57 Da). Trypsinized AChE were mixed with a matrix of 5 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile/water 0.3% (v/v) TFA, pH 2.2. Aliquot of 1 μL peptidematrix mixture was spotted on a polished MALDI-TOF MS target plate and dried by evaporation at ambient temperature. Analyte-matrix co-crystals appeared homogeneous in nature. The mass spectra were the average of 256 laser shots collected from multiple locations on the target spot and monitored by a digital oscilloscope during acquisition.

Molecular docking study

The crystal structure of *Torpedo californica* AChE (TcAChE) complexed with donepezil (code: 1EVE, resolution of 2.50 Å) was downloaded from PDB (http://www.rcsb.org). Prior to performing molecular docking, the protein PDB file was prepared using the Dock Prep tool available in the free software package UCSF Chimera 1.10.1[19]. This involved the addition of hydrogen atoms, removal of water molecules and assigning partial charges (using the AMBER99 force field). Docking calculations were carried out using SwissDock, an online server that docks ligand to protein using EADock DSS software[20,21]. Docking runs were performed using the ''Accurate'' parameters option, which is the most exhaustive in terms of the number of binding modes sampled. Output clusters were obtained after each docking run and were ranked according to the FullFitness (FF) scoring function specified by the SwissDock algorithm (cluster 0 being the cluster with the best FF score). A greater negative FF score indicates a more favorable binding mode with a better fit. Within each cluster, the individual binding poses were further arranged and ranked based on their FF score. Results of the SwissDock were visualized by UCSF Chimera package. Re-docking experiment with natural ligand (Donepezil) was carried out in order to evaluate the ability of the software to correctly recognize the AChE-binding site.

Results and discussion

Chemistry

The synthesis of carbamates **1–14** was firstly achieved following a two steps procedure (Route A, Scheme 1): *N,N*-dibenzylation of an α,ω-amino alcohol followed by the *N,N*-dimethylcarbamoylation at the oxygen atom. Unfortunately, the yields of the second step were very low (5–15%). Consequently, the synthesis of carbamates **1–14** was achieved (in some cases in higher yields) following a different pathway (Route B, Scheme 1), in which an α,ω-amino alcohol was transformed into the corresponding hydrochloride by reaction with gaseous HCl, and then carbamoylated at the oxygen atom by reaction with *N,N*-dimethylcarbamoyl chloride. The following reaction with benzyl bromide led to the desired carbamates (15–40% yields). The spectroscopic data are in good agreement with the proposed structures.

Enzymatic assays

All synthesized compounds were tested to evaluate their ability to inhibit AChE. The results are reported in Table 1. The most potent inhibitor[4] presents a percent inhibition equal to 85% at 50 μM.

The reported data clearly indicate that two main features influence the enzyme inhibition: (i) the nature of the R substituent at the aromatic ring, which plays a key role in the inhibitory potency; (ii) the length of the alkyl spacer, which also significantly affects the activity. As shown by the data in Table 1, the most active compounds are **4** (R = H; $n = 4$) and **8** (R = CH_3; $n = 4$) with a percent inhibition, at 50 μM, of 85 and 69%, respectively. These compounds are characterized by unsubstituted aromatic rings[4] or substituted with a para-methyl group[8]; their electron density is suitable to allow interactions with the aromatic residues in the active enzymatic site. When the aromatic ring is substituted by electron withdrawing groups[11,14] a substantial decrease in the activity is observed (inhibition of 12% and 31%, respectively). Moreover, **4** and **8** have an alkyl linker separating the dibenzylamino and the carbamic moieties with six methylene units. Therefore, this alkyl chain probably represents the best spacer in terms of length and flexibility.

For compound **4**, the inhibition assay on AChE shows a time-dependent activity: at 5 μM concentration of compound **4**, the percentage of inhibition increases from 50%, without incubation, to 95%, with 40 min of incubation. This strong dependence of inhibitory potency on incubation time suggests that compound **4** could act as pseudo-irreversible inhibitor, forming a carbamoyl-AChE complex by covalent interaction with serine in the catalytic site. Furthermore, the removal of the carbamic function reduced the inhibitory potency from 85% to 32% in the same experimental conditions (**4** versus **15d**).

Scheme 1. Synthetic routes to carbamates **1–14**.

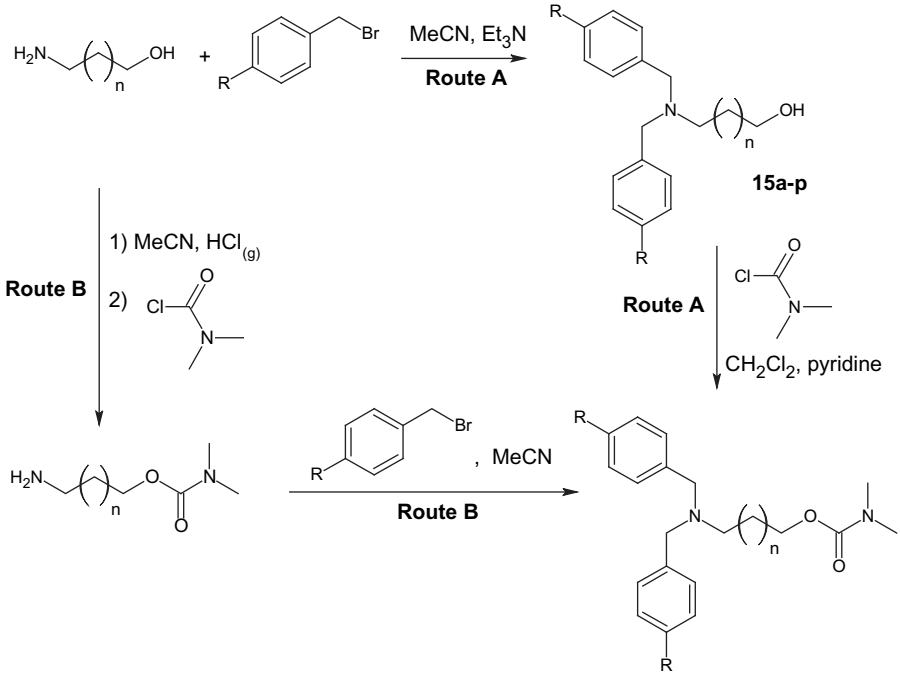
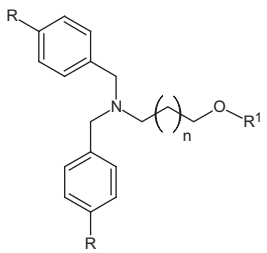


Table 1. AChE inhibition activity of synthesized carbamates **1–14** and alcohol **15d**. The data represent the inhibition percentage at different inhibitor concentration (see experimental).



Compound	R	R^1	*n*	Inhibition % at 50 μM	at 25 μM	at 12.5 μM
1	H	$CON(CH_3)_2$	1	57	–	–
2	H	$CON(CH_3)_2$	2	41	36	–
3	H	$CON(CH_3)_2$	3	60	–	–
4	H	$CON(CH_3)_2$	4	85	–	–
5	CH_3	$CON(CH_3)_2$	1	–	8	–
6	CH_3	$CON(CH_3)_2$	2	–	27	–
7	CH_3	$CON(CH_3)_2$	3	26	–	–
8	CH_3	$CON(CH_3)_2$	4	69	–	–
9	F	$CON(CH_3)_2$	1	–	–	25
10	F	$CON(CH_3)_2$	2	13	–	–
11	F	$CON(CH_3)_2$	4	12	–	–
12	CF_3	$CON(CH_3)_2$	1	31	–	–
13	CF_3	$CON(CH_3)_2$	2	7	–	–
14	CF_3	$CON(CH_3)_2$	4	31	–	–
15d	H	H	4	32		

To verify the role of the carbamic function on the activity of the compound **4**, we have carried out experiments to evaluate the reversibility of the enzymatic inhibition.

In particular, we prepared a stock solution of AChE in phosphate buffer, from which three aliquots were taken to obtain the solution A (added with compound **4**), the solution B (added with carbaryl, a known carbamylating agent) and the solution C (without inhibitor). Each solution was incubated for 2 h in the same conditions, then the inhibition percentages were determined and they were of the 95% for the solution A and 65% for the solution B (the activity of the solution C was used as 100% of enzyme activity).

After the incubation, from the solutions A and B the residual free inhibitor was removed, the enzyme was washed, and the activity was determined again. In the case of the compound **4**, the enzyme activity was totally recovered, instead, in the case of Carbaryl the inhibition percentage became 90%. This result suggests that the compound **4** does not act as a pseudo-irreversible inhibitor, because it does not produce the carbamylation of the enzyme.

We also carried out a mass spectrometry experiment to obtain another proof of the non-covalent inhibition mechanism of the compound **4**, according to a literature procedure[22]. Also in this case, we have prepared three AChE solutions: the first incubated

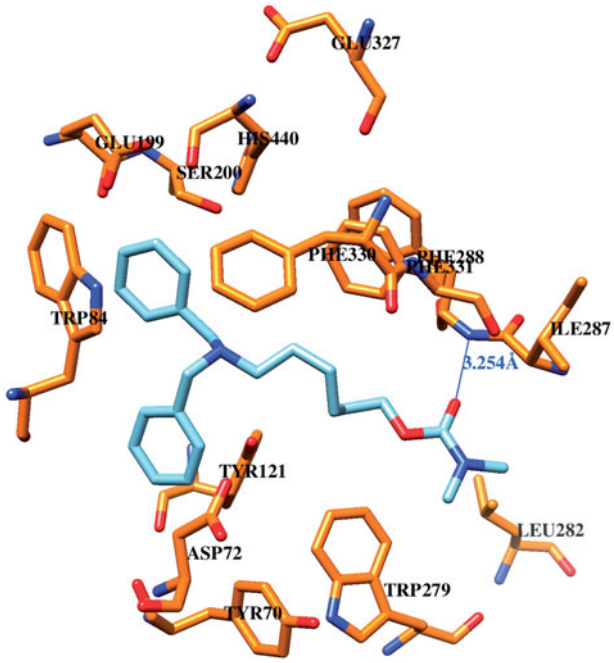


Figure 1. Best binding pose of compound **4** (light blue colored) in the active site of TcAChE (1EVE). The selected amino acids are orange colored.

with compound **4**, the second incubated with Carbaryl (positive control) and the third without inhibitor (negative control). After incubation and removal of inhibitor excess, the enzyme was treated with trypsine and the digested peptides were analyzed by MALDI-TOF-MS. The negative control, AChE without inhibitor, was analyzed to identify the peaks of trypsin digested AChE; several peaks appeared in the range of 2000–4000 mass unit.

The comparison of the mass spectra obtained for the three solutions revealed the presence of a peak at 2659.8 *m/z* in all samples, corresponding to the active site peptide (containing Ser225); in the AChE incubated with Carbaryl also appeared a peak at 2717.3 *m/z*, corresponding to the addition of the $CONHCH_3$ group (the carbamylated active site fragment). On the contrary, for the compound **4** any carbamylation peak was not detected.

Overall, the data obtained by MALDI-TOF MS experiments and those on enzyme inhibition reversibility indicate that the carbamate **4** does not act through the enzyme carbamoylation. Rather, it would seem to behave as a slow-binding inhibitor, which does not bind covalently to the enzyme, but simply requires a longer time interval to establish the optimal interactions with the enzyme[23].

Molecular docking studies

To support the experimental results obtained by enzymatic inhibition, we carried out a molecular docking study using SwissDock freely available on Swiss Institute of Bioinformatics Website. Three-dimensional crystal structure of TcAChE complexed with donepezil was obtained by RCSB Protein Data Bank (PDB code: 1EVE). The protein preparation and the molecular docking results visualization were carried out using the UCSF Chimera package. The EADock DSS software was able to identify the native-binding mode of donepezil as the best FF score binding conformation.

Figure 1 shows the best binding pose of the carbamate **4** in the active site of TcAChE; it can be observed that the dibenzylamine moiety is directed inside of the active site and one of the two aromatic rings approaches the Trp84 (numbering of the TcAChE). Moreover, the alkyl chain drives the carbamate group toward the access gorge allowing the H bond interaction between the carbonyl group of the carbamate and the amide NH of Phe288.

All studied carbamates **1**–**14** share this binding orientation but for those compounds having a shorter alkyl linker the H bond interaction was lost, or worse alignment of the aromatic portions was observed.

Conclusion

A set of carbamates, derivatives of *N,N*-dibenzylamine, were synthesized and tested as AChE inhibitor. In these compounds, the aromatic portion is linked to the carbamic one by means of an alkyl linker of variable length. The obtained inhibition data may allow us to outline a preliminary set of Structure-Activity Relationship: (a) the presence of carbamic function greatly increases the inhibition activity (compare **4** versus **15d**); (b) the optimal alkyl linker contains six methylene units, as indicated by compounds **4** and **8**; (c) the presence of electron-withdrawal substituents on the aromatic moiety, such as fluorine or trifluoromethyl, reduces the inhibitory potency, as demonstrated by compounds **9**–**14**.

As the carbamate inhibitors of AChE usually act through the carbamylation of the active site serine, we decided to verify if also the most active compound **4**, acts according to this mechanism of action. In the time-dependent inhibition experiment, we found a slow onset inhibition activity, which was not related to the serine carbamylation; in fact, the reversibility study of the inhibition shows that the AChE readily recovered its activity when the inhibitor **4** was removed, thus suggesting the establishment of weak interactions between inhibitor and enzyme. Furthermore, the MALDI-TOF MS experiment confirmed this data, as no carbamoylation peaks were found in the trypsin digested AChE incubated with **4**.

The slow onset inhibition showed by the carbamate **4** could be explained with the high conformational freedom of both its alkyl linker and *N,N*-dibenzylamine moieties. This is a typical behavior observed for slow-binding inhibitors with great steric hindrance or with great conformational freedom, which requires a delay time in order that the enzyme and inhibitor adapt their conformations to each other for the optimal interactions[23].

A slow-binding inhibitor presents pharmacological advantages over classical reversible inhibitors, because it shows long target-residence time, and, as a consequence, a prolonged efficacy and minimal side effects. Slow-binding inhibitors of AChE could be considered promising new drugs for the treatment of neurodegenerative disorders such as Alzheimer disease and myasthenia.

Declaration of interest

The authors report that they have no conflicts of interest. The present investigation was supported by the grant of ''Sapienza University of Rome''.

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