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Short communication

Design, synthesis, and evaluation of benzophenone derivatives as novel acetylcholinesterase inhibitors

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Abstract

Starting from a structure-based drug design, new acetylcholinesterase inhibitors were designed and synthesized as analogues of donepezil. The compounds were composed by an aromatic function and a tertiary amino moiety connected by a suitable spacer. In particular, the benzophenone nucleus and the *N*,*N*-benzylmethylamine function were selected. The easily accessible three-step synthesis of these compounds resulted to be significantly less difficult and expensive than that of donepezil. Several compounds possess anti-cholinesterase activity in the order of micro and sub-micromolar. Particularly, compounds **1** and **10** were the most potent inhibitors of the series.

Keywords: Alzheimer's disease; Benzophenone; AChE inhibitors

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by some major pathological signs such as synaptic loss, reduced levels of the neurotransmitter acetylcholine (ACh), β-amyloid senile plaques (SP) and neurofibrillary tangles (NFT). The most dramatic abnormalities are those of the cholinergic system [1]. Acetylcholinesterase (AChE), besides its hydrolyzing activity, also plays a proaggregating (non-catalytic) role, accelerating β-amyloid peptide (Aβ) aggregation and deposition into the fibrils that compose the SP [2]. This action is associated with a specific motif located near the entrance of the active-site gorge termed peripheral anionic site (PAS) [3]. Several efforts have been done for the development of potent and selective AChE inhibitors (AChEI) and to date these drugs (such as tacrine [4], rivastigmine [5], donepezil [6] and galantamine [7]) represent the main therapeutic approach for symptomatic treatment of AD. The crystallographic structure of the *Torpedo californica* AChE gorge complex with donepezil was described by Sussman [8] and clearly showed that the molecule has a distinctive orientation along the active-site gorge, extending from the active anionic site to the PAS, placed at the bottom and at the top, respectively, where it establishes specific interactions (aromatic π−π or cation−π stacking) with conserved aromatic residues. The study allowed characterization of the binding site and identification of the moieties that play a fundamental role in the biological activity of the inhibitor, such as the dimethoxyindanone function, the charged piperidine nitrogen atom and the benzyl group [9]. The synthetic route employed to obtain donepezil [10] was elaborate, quite difficult and also expensive, due to the involvement of many steps and to the employment of unstable or expensive synthetic intermediates or of hazardous reagents.

As a part of our ongoing project aimed at identifying reversible AChEIs [11,12], we developed a new class of compounds endowed with an interesting biological profile and characterized by an easily affordable preparation methodology. The presence of lipophilic moieties and a tertiary amino group represents the key requirement for a good anti-AChE

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activity because of their interaction with the aromatic residues that line the wall of the AChE gorge and with the anionic site of the enzyme. Taking these considerations into account, the examination of the schematic drawing of the molecule of donepezil furnished the rationale for a structure-based drug design as depicted in Fig. 1. Briefly, two main molecular modifications were carried out: (a) ring formation, accomplished by the inclusion of the methylene unit in a six-membered aromatic ring, joining the indanone and the piperidine functions; and (b) opening of the piperidine ring and of the five-membered ring of the indanone cycle. Our efforts culminated in the design of a molecule in which the 3,4-dimethoxybenzophenone nucleus and the *N*,*N*-benzylmethylamine moiety were connected by a methylene spacer (**1**). The positional effect of the substitution of the [(benzylmethylamino)methyl] moiety on the 3,4-dimethoxybenzophenone was then examined, to confirm our working hypothesis, and only the *para*-substituted derivative (**1**) showed high potency, while the corresponding *meta*-substituted analogue proved to be inactive. Therefore, with the aim to identify some essential pharmacophore elements, various portions of the lead molecule (**1**) were modified, while the carbonyl group and the phenyl rings of the benzophenone scaffold, together with the tertiary nitrogen atom, were maintained throughout the present investigation. Several compounds were synthesized and reported in Table 1.

2. Chemistry

Scheme 1 outlines the synthetic route followed for the preparation of the benzophenone derivatives. Friedel–Crafts acylation of the appropriate anisole derivative with *p*-toluoyl chloride in the presence of $SnCl_4$ as Lewis acid yielded the 4-methylbenzophenones **17–19** in good yields. Lithiation of 2-bromonaphtalene with *n*-BuLi and subsequent treatment with *p*-tolunitrile gave, upon acid hydrolytic workup, the naphthalen-2-yl-*p*-tolylmethanone derivative (**20**). Bromination with *N*-bromosuccinimide (NBS) of compounds **17–20** afforded the bromomethyl derivatives **21–24**, that were then subjected to nucleophilic substitution reaction by the selected amines affording the desired final compounds **1–12**, **14–16**. Another part of the synthetic plan led to the insertion of the

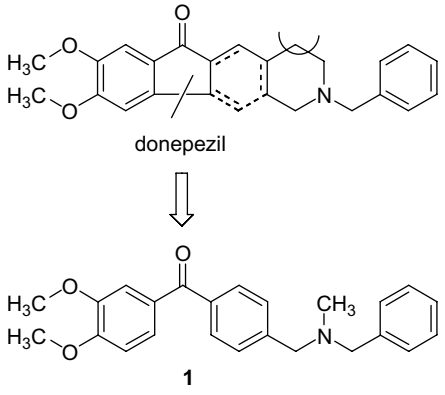
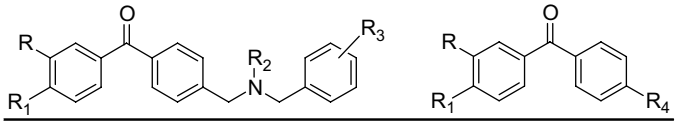
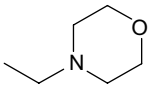
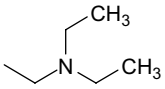
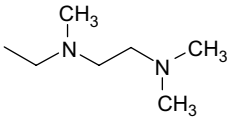
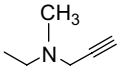
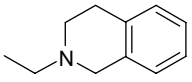
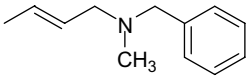


Fig. 1. Schematic drawing of donepezil and structure-based design of compound **1**.

Table 1
Structures of the synthesized compounds **1–16**

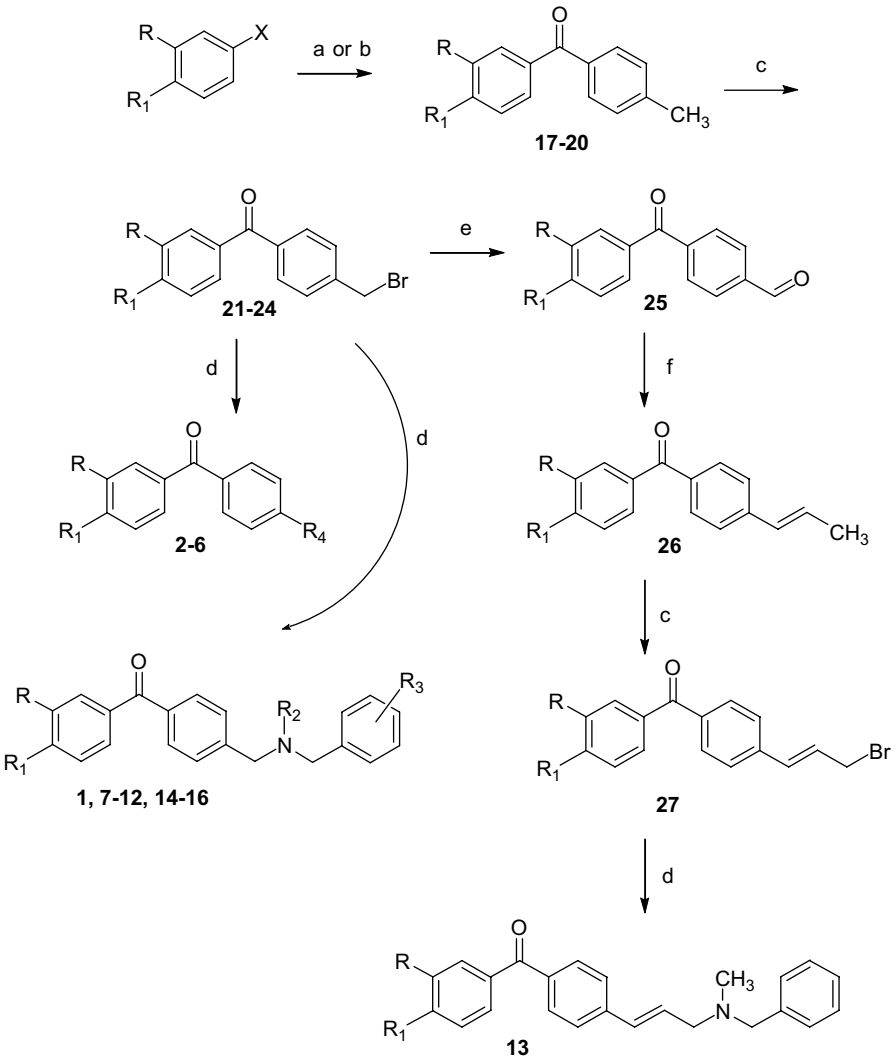


	R	R_1	R_2	R_3	R_4
1	OCH_3	OCH_3	CH_3	H	–
2	OCH_3	OCH_3	–	–	
3	OCH_3	OCH_3	–	–	
4	OCH_3	OCH_3	–	–	
5	OCH_3	OCH_3	–	–	
6	OCH_3	OCH_3	–	–	
7	OCH_3	OCH_3	CH_3	*m*-NO_2	–
8	OCH_3	OCH_3	CH_3	*o*-OCH_3	–
9	OCH_3	OCH_3	CH_3	*m*-CH_3	–
10	OCH_3	OCH_3	C_2H_5	H	–
11	OCH_3	OCH_3	C_2H_4OH	H	–
12	OCH_3	OCH_3	$C_2H_4N(CH_3)_2$	H	–
13	OCH_3	OCH_3	–	–	
14	H	OCH_3	CH_3	H	–
15	F	OCH_3	CH_3	H	–
16	CH=CH–CH=CH		CH_3	H	–

E-propenyl side chain in the 4-position of the benzophenone nucleus: the aldehyde **25**, obtained by treatment of **21** with hexamethylenetetramine, was coupled with the ylide, achieved from triphenylethylphosphonium bromide, according to the classic Wittig procedure, to afford the derivative **26**, this was then brominated with NBS to give **27**, which was treated with *N*,*N*-benzylmethylamine, as reported above, to give the final compound **13**.

3. Results and discussion

The biological profiles of the novel benzophenone derivatives listed in Table 1 toward the enzymes human AChE and butyrylcholinesterase (BChE) were determined and expressed as IC_{50}, values, and the inhibitory activities were reported in Table 2. The ability of compounds **1–16** to inhibit AChE



Scheme 1. Reagents and conditions: (a) X = H; R = H, F, OCH_3; $R_1 = OCH_3$: *p*-toluoyl chloride, $SnCl_4$; (b) X = Br; R, R_1 = CH═CH–CH═CH: *n*-BuLi, THF, −78 °C, 4-methoxybenzonitrile, r.t., then conc. H_2SO_4, H_2O, dioxane, reflux; (c) NBS, $(PhCO)_2O_2$, CCl_4, reflux; (d) selected amine, TEA, toluene, reflux; (e) hexamethylenetetramine, formic acid, EtOH 60%, reflux; (f) triphenylethylphosphonium bromide, NaH, THF, r.t.

and BChE activities was assessed employing the Ellman's method [13] and using donepezil as reference compound.

The compound **1** showed an interesting inhibitory profile with $IC_{50} = 0.46$ μM. Concerning modifications focused on the amino function, some small aliphatic groups were inserted to assess the importance of the *N*,*N*-benzylmethylamino portion: compounds **2**–**5** showed a remarkable decrease in activity, underlying the importance of the benzyl group. We then examined the rigid analogue **6** and found that its potency was in the same way decreased by one order of magnitude. Placement of substituents on the benzyl ring (compounds **7**–**9**) again decreased the inhibitory potency by one order of magnitude in comparison with **1**. A further modification involved the replacement of the methyl group with different alkyl functions (compounds **10**–**12**): only the ethyl moiety maintained the same potency as **1**, while the hydroxyethyl and the dimethylaminoethyl functions decreased the potency by one order of magnitude. Moreover, we explored the effect of elongating the lead: the potency of the vinylog **13** was lower than **1**. Finally, the importance of the 3,4-dimethoxy substitution pattern was investigated: the 3-methoxy group was removed (**14**) or substituted by a fluorine atom (**15**) leading to less potent compounds, and the introduction of a naphthalene moiety (**16**), instead of the 3,4-dimethoxyphenyl function, led to a significant decrease in activity.

Most of the derivatives proved to be selective for AChE with respect to BChE. Particularly, compounds **1** and **10** presented a noteworthy anti-AChE activity, even if lower than that of donepezil, and a remarkable selective behaviour. The structural modifications carried out on compound **1** allowed us to elucidate the nature of the interactions with AChE binding sites. The unmodified benzyl group on the nitrogen atom proved to be an essential requirement for the inhibitory activity. Since both the increase of steric hindrance, due to the presence of substituents, and the elongation of the molecule were poorly tolerated, we could assume that this moiety was properly arranged in such a way as to completely fill the bottom of the AChE gorge. The previous considerations could be

Table 2
Inhibition of human recombinant AChE and BChE from human serum by the studied compounds

Compounds	IC_{50} (μM) ± SEM		IC_{50} ratio BChE/AChE
	AChE	BChE	
1	0.46 ± 0.04	60.7 ± 1.3	132
2	345 ± 74	n.t.	
3	36.8 ± 0.5	n.t.	
4	2.37 ± 0.09	88.8 ± 3.1	33
5	27.2 ± 0.1	n.t.	
6	54.0 ± 9.0	n.t.	
7	4.27 ± 0.52	>100	
8	3.71 ± 0.22	16.2 ± 0.9	4.4
9	1.65 ± 0.26	125 ± 21	76
10	0.57 ± 0.04	71.5 ± 3.7	127
11	1.41 ± 0.14	172 ± 36	122
12	8.64 ± 0.66	33.2 ± 1.3	4
13	2.10 ± 0.09	44.0 ± 1.9	21
14	1.82 ± 0.08	>100	
15	1.57 ± 0.08	>100	
16	19.6 ± 0.11	n.t.	
Donepezil	0.02 ± 0.01[a]	7.42 ± 0.39	371

n.t.: not tested.
[a] Data obtained from Ref. [14].

extended to the *N*-alkyl function: placement of a small alkyl group (methyl or ethyl) resulted favorable, suggesting the importance of the hydrophobic character of the middle region of the enzyme, while compounds bearing an hydrophilic moiety, such as hydroxyethyl or dimethylaminoethyl, could badly interact with the biological counterpart. Moreover, the 3,4-dimethoxyphenyl group proved to be important for the biological activity since structural complication, achieved by inserting an additional phenyl moiety, or simplification, obtained by removing the 3-methoxy group, resulted detrimental for the inhibitory potency.

Molecular modeling studies were carried out to provide a better interpretation of the biological profile of **1** toward hAChE. In particular, docking simulations were carried out with the software GOLD [15] and outcomes were rationalized by means of the clustering algorithm AClAP [16,17]. Compound **1** was studied according to the computational strategy reported in the supplementary data. In Fig. 2, the binding mode of **1** (Fig. 2A) at the hAChE gorge is reported. It can be seen that **1** is properly positioned into the enzyme gorge interacting with the internal residue Trp86 by means of a π−π stacking. Moreover, some aromatic and hydrophobic interactions were also identified between **1** and aromatic residues lining the hAChE gorge. A possible interaction could likely be identified for the carbonyl oxygen of the benzophenone moiety, that in some docking poses was positioned at the right distance for establishing an H-bond with the Phe295 backbone. However, **1** was unable to properly contact Trp286, the key residue of the hAChE PAS. This could be a possible explanation for its lower activity when compared to donepezil, which seems to contact such residue slightly better than our compound (Fig. 2B). Furthermore, such a docking outcome prevented us to embark toward the high costly biological assays to assess the capability of the present series of compounds to interfere with the AChE-induced Aβ

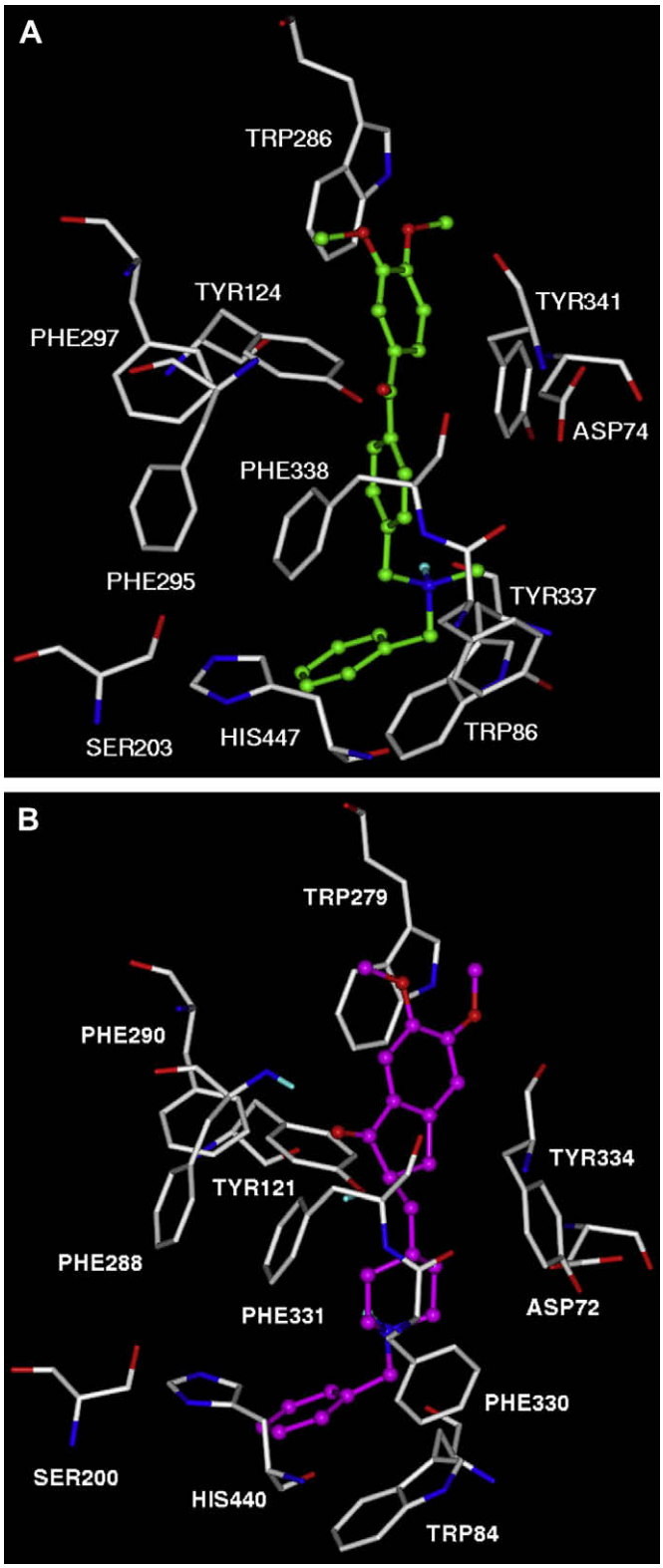


Fig. 2. (A) Docking model of **1** (carbon atoms are green) at the human AChE gorge. It clearly arises that the benzophenone moiety does not properly contact the key PAS residue Trp286. (B) X-ray complex between *Torpedo californica* AChE and donepezil (carbon atoms are magenta). Donepezil seems to contact Trp279 slightly better than **1** (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

aggregation. It is well-established that only inhibitors able to properly interact with AChE PAS and to protrude out of the enzyme gorge have the potential to be inhibitors of the AChE-induced Aβ aggregation.

4. Conclusion

A structure-based drug design allowed us to synthesize a new series of AChEIs as analogues of donepezil. Particularly, the key features for the high inhibitory potency of donepezil, the 3,4-dimethoxy group and the *N*-benzylamino moiety, were inserted on a benzophenone backbone. Compounds **1** and **10** were the most potent inhibitors of the series, while the different structural modifications carried out on compound **1** were not able to improve the AChE inhibitory potency of the new derivatives. Still compound **1**, characterized by a fairly good inhibitory activity and by a simple and affordable synthesis, could be considered as a new lead for further optimization.

5. Experimental protocols

All melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on Merk precoated silica gel F_{254} plates. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Varian 300 MHz spectrometers, peaks positions are given in parts per million downfield from tetramethylsilane (TMS) as internal standard and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet) or br (broad). Mass spectra were obtained with Waters Micromass ZQ 4000 (ES-MS spectra) or V. G. 7070 E (EI-MS spectra) apparatuses. Wherever analyses are only indicated with elements symbols, analytical results obtained for those elements are within 0.4% of the theoretical values. Column chromatography was carried out with silica gel (Kieselgel 40, 0.040–0.063 mm; Merck) using the flash technique. Yields were reported after chromatographic purification. Pure samples were obtained as hydrochloride salts by treating the product with HCl saturated methanol and were crystallized from MeOH/Et_2O. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry.

All elemental analyses and mass spectra are referred to the free base. ^{1}H NMR spectra are referred to the hydrochloride salt, unless otherwise indicated.

5.1. Chemistry

5.1.1. General procedure for the synthesis of benzophenones 17–19

To an ice-cold mixture of the anisole derivative (1 equiv), *p*-toluoyl chloride (1.2 equiv) and anhydrous $SnCl_4$ (1.2 equiv) were added portionwise. After stirring at 60 °C for 4 h, the reaction mixture was quenched with ice/water. Et_2O was added and the organic layer was separated, washed with $NaHCO_3$ saturated solution (10 mL), water (10 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude solid was further purified by crystallization from toluene.

*5.1.1.1. (3,4-Dimethoxyphenyl)-p-tolylmethanone (**17**).* Starting from 1,2-dimethoxybenzene (6.9 g, 50 mmol), compound **17** was obtained, 90% yield, white needles: mp 105–107 °C (lit. [18] 127–128 °C). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H), 3.95 (s, 3H), 3.98 (s, 3H), 6.81 (d, $J = 8.4$ Hz, 1H), 7.38 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.45–7.55 (m, 3H), 7.77 (d, $J = 8.4$ Hz, 2H).

*5.1.1.2. (4-Methoxyphenyl)-p-tolylmethanone (**18**).* Starting from anisole (5.4 g, 50 mmol), compound **18** was obtained, 71% yield, white needles: mp 94–95 °C (lit. [19] 92 °C). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H), 3.95 (s, 3H), 7.10 (d, $J = 8.8$ Hz, 2H), 7.35 (d, $J = 8.4$ Hz, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.70 (d, $J = 8.8$ Hz, 2H).

*5.1.1.3. (3-Fluoro-4-methoxyphenyl)-p-tolylmethanone (**19**).* Starting from 1-fluoro-2-methoxybenzene (1.0 g, 8.0 mmol), compound **19** was obtained, 87% yield, white solid: mp 79–81 °C. ^{1}H NMR ($CDCl_3$) δ 2.45 (s, 3H), 3.98 (s, 3H), 7.02 (t, $J = 8.4$ Hz, 1H), 7.29 (d, $J = 8.4$ Hz, 2H), 7.55–7.72 (m, 4H).

5.1.2. Naphthalen-2-yl-p-tolylmethanone (20)

To a stirred solution of 2-bromonaphthalene (1.0 g, 4.8 mmol) in anhydrous THF (50 mL) under N_2 at −78 °C, *n*-BuLi (1.6 M hexane solution, 9.6 mL) was added dropwise. The mixture was stirred for 1 h at the same temperature, and then 4-methylbenzonitrile (1.20 g, 10.25 mmol) in THF (10 mL) was added dropwise. The solution was stirred for further 1 h at −78 °C and then was allowed to warm to room temperature. On cooling an acidic solution (conc. H_2SO_4 0.75 mL, H_2O 10 mL, dioxane 9 mL) was added. The reaction was then heated at reflux for 2 h, cooled, basified with 2 N NaOH solution, and extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were washed with brine (50 mL), dried over Na_2SO_4 and evaporated under reduced pressure, giving a crude solid which was chromatographed on silica gel with petroleum ether/ethyl acetate (95:5) as eluant, affording **20** (65%), mp 90–92 °C. ^{1}H NMR ($CDCl_3$) δ 2.48 (s, 3H), 7.32 (d, $J = 8.8$ Hz, 2H), 7.50–7.63 (m, 2H), 7.80 (d, $J = 8.0$ Hz, 2H), 7.75–7.98 (m, 4H), 8.26 (s, 1H).

5.1.3. General procedure for the synthesis of bromomethylbenzophenones (21–24, 27)

A mixture of aryl-*p*-tolylmethanone derivative (1 equiv), *N*-bromosuccinimide (1.1 equiv), a catalytic amount of benzoyl peroxide in CCl_4 (10 mL) was heated at 60 °C for 6 h under bright lamp. The reaction mixture was hot filtered and the filtrate was concentrated under reduced pressure. The crude solid was purified by crystallization from ligroin to afford the desired bromomethyl derivative.

*5.1.3.1. (4-Bromomethylphenyl)-(3,4-dimethoxyphenyl)methanone (**21**).* Starting from **17** (2.56 g, 10.0 mmol), compound

21 was obtained, 80% yield, white needles: mp 113–115 °C. ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 3H), 3.98 (s, 3H), 4.53 (s, 2H), 6.80 (d, $J = 8.4$ Hz, 1H), 7.35 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.45–7.55 (m, 3H), 7.74 (d, $J = 8.4$ Hz, 2H).

*5.1.3.2. (4-Bromomethylphenyl)-(4-methoxyphenyl)methanone (**22**).* Starting from **18** (2.25 g, 10.0 mmol), compound **22** was obtained, 85% yield, white needles: mp 108–110 °C (lit. [19] 62 °C). ^{1}H NMR ($CDCl_3$) δ 3.96 (s, 3H), 4.55 (s, 2H), 7.10 (d, $J = 8.8$ Hz, 2H), 7.35 (d, $J = 8.4$ Hz, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.70 (d, $J = 8.8$ Hz, 2H).

*5.1.3.3. (4-Bromomethylphenyl)-(3-fluoro-4-methoxyphenyl)methanone (**23**).* Starting from **19** (2.0 g, 8.2 mmol), compound **23** was obtained, 75% yield, white needles: mp 73–75 °C. ^{1}H NMR (DMSO) δ 3.99 (s, 3H), 4.58 (s, 2H), 7.03 (t, $J = 8.4$ Hz, 1H), 7.51 (d, $J = 8.4$ Hz, 2H), 7.48–7.77 (m, 4H).

*5.1.3.4. (4-Bromomethylphenyl)naphthalen-2-yl-methanone (**24**).* Starting from **20** (0.28 g, 1.14 mmol), compound **24** was obtained, 37% yield, reddish oil. ^{1}H NMR ($CDCl_3$) δ 4.56 (s, 2H), 7.33 (d, $J = 8.4$ Hz, 2H), 7.52–7.62 (m, 2H), 7.78 (d, $J = 8.0$ Hz, 2H), 7.75–8.00 (m, 4H), 8.25 (s, 1H).

*5.1.3.5. [4-(3-Bromopropenyl)phenyl]-(3,4-dimethoxyphenyl)methanone (**27**).* Starting from **26** (0.56 g, 2 mmol), compound **27** was obtained, 45% yield, white needles: mp 132–133 °C. ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 3H), 3.96 (s, 3H), 4.18 (d, $J = 7.5$ Hz, 2H), 6.48–6.58 (m, 1H), 6.72 (d, $J = 16.2$ Hz, 1H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.37 (dd, $J = 2.1$ and 8.4 Hz, 1H), 7.47–7.56 (m, 3H), 7.75 (d, $J = 8.4$ Hz, 2H).

*5.1.4. 4-(3,4-Dimethoxybenzoyl)benzaldehyde (**25**)*

A mixture of **21** (2.0 g, 6.0 mmol), hexamethylentetramine (0.93 g, 6.60 mmol), formic acid (0.6 mL), 60% aqueous EtOH (300 mL) was heated under reflux for 12 h. The solvent was removed and the aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL), the organic layers were dried and evaporated to give a crude solid that was purified by flash chromatography using petroleum ether/ethyl acetate (80:20) as eluant, 67% yield, mp 113–115 °C. ^{1}H NMR ($CDCl_3$) δ 3.96 (s, 3H), 3.98 (s, 3H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.34 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.52 (d, $J = 1.8$ Hz, 1H), 7.88 (d, $J = 8.4$ Hz, 2H), 8.01 (d, $J = 8.4$ Hz, 2H).

*5.1.5. (3,4-Dimethoxyphenyl)-(4-propenylphenyl) methanone (**26**)*

To a stirred suspension of NaH (30 mg, 1.2 mmol) in anhydrous benzene (25 mL) at 0 °C triphenylethylphosphonium bromide (0.45 g, 1.2 mmol) was added; the mixture was stirred under the same conditions for 30 min, then compound **25** (0.5 g, 1.0 mmol) dissolved in benzene (10 mL) was added. The reaction mixture was stirred at r.t. overnight. Ice was added and the organic phase was separated, and dried to afford a crude solid that was purified by flash chromatography using petroleum ether/ethyl acetate (80:20) as eluant, 55% yield, mp 110–112 °C. ^{1}H NMR ($CDCl_3$) δ 1.95 (d, $J = 7.5$ Hz, 3H), 3.95 (s, 3H), 3.97 (s, 3H), 5.91 (dd, $J = 11.7$ and 7.5 Hz, 1H), 6.85 (d, $J = 11.7$ Hz, 1H), 6.91 (d, $J = 8.4$ Hz, 1H), 6.91 (d, $J = 8.7$ Hz, 1H), 7.40 (d, $J = 8.4$ Hz, 2H), 7.49 (d, $J = 2.1$ Hz, 1H), 7.75 (d, $J = 8.4$ Hz, 2H).

*5.1.6. General procedure for the synthesis of compounds **1–16***

To a solution of the bromomethyl intermediate **21–24**, **27** (1 equiv) in toluene (10 mL), the selected amine (1.5 equiv) and triethylamine (1.5 equiv) were added. The resulting mixture was stirred under reflux for 18 h. HCl (3 N, 10 mL) was added dropwise and the aqueous phase was basified with K_2CO_3 and then extracted with dichloromethane (3 × 30 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent was removed under reduced pressure. Unless otherwise indicated HCl saturated methanol was added and the crude solid was crystallized from MeOH/Et_2O.

Starting from **21** the following compounds were obtained.

*5.1.6.1. {4-[(Benzylmethylamino)methyl]phenyl}-(3,4-dimethoxyphenyl)methanone (**1**).* Yield: 75%, hydrochloride salt: mp 158–160 °C. ^{1}H NMR ($CDCl_3$) δ 2.64 (d, $J = 4.4$ Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.04–4.13 (m, 2H), 4.15–4.23 (m, 2H), 6.86 (d, $J = 8.4$ Hz, 1H), 7.30–7.42 (m, 6H), 7.52 (d, $J = 1.8$ Hz, 1H), 7.30–7.40 (m, 4H). ES-MS *m/z*: 376 (M + 1). Anal. $C_{24}H_{25}NO_3$ (C, H, N).

*5.1.6.2. (3,4-Dimethoxyphenyl)-(4-morpholin-4-ylmethylphenyl)methanone (**2**).* Yield: 70%, hydrochloride salt: mp 196–199 °C. ^{1}H NMR ($CDCl_3$) δ 2.80–3.05 (m, 2H), 3.35–3.45 (m, 2H), 3.95–4.05 (m, 8H), 4.20–4.44 (m, 4H), 6.88 (d, $J = 8.4$ Hz, 1H), 7.35 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.53 (d, $J = 8.4$ Hz, 2H), 7.80–7.85 (m, 4H). ES-MS *m/z*: (M + 1). Anal. $C_{20}H_{23}NO_4$ (C, H, N).

*5.1.6.3. (3,4-Dimethoxyphenyl)-{4-[(ethylpropylamino)methyl]phenyl}methanone (**3**).* Yield: 68%, hydrochloride salt: mp 154–156 °C. ^{1}H NMR ($CDCl_3$) δ 3.05–3.25 (m, 4H), 3.96 (s, 3H), 3.98 (s, 3H), 4.24 (d, $J = 4.8$ Hz, 1H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.37 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.52 (d, $J = 8.4$ Hz, 2H), 7.80–7.92 (m, 4H). ES-MS *m/z*: 328 (M + 1). Anal. $C_{20}H_{25}NO_3$ (C, H, N).

*5.1.6.4. (3,4-Dimethoxyphenyl)-(4-{[(2-dimethylaminoethyl)-methylamino]methyl}phenyl)-methanone (**4**).* Yield: 66%, hydrochloride salt: mp 216–218 °C. Base: ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 9H), 2.45–2.65 (m, 4H), 3.61 (s, 2H), 3.95 (s, 3H), 3.96 (s, 3H), 6.89 (d, $J = 8.4$ Hz, 1H), 7.35 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.42 (d, $J = 8.4$ Hz, 2H), 7.48 (d, $J = 1.8$ Hz, 1H), 7.72 (d, $J = 8.4$ Hz, 2H). ES-MS *m/z*: 357 (M + 1). Anal. $C_{21}H_{28}N_2O_3$ (C, H, N).

*5.1.6.5. (3,4-Dimethoxyphenyl)-{4-[(methylprop-2-ynylamino)-methyl]phenyl}methanone (**5**).* Yield: 55%, hydrochloride salt: mp 95–97 °C. ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 1H), 2.92 (d, $J = 4.4$ Hz, 3H), 3.78–3.94 (m, 2H), 3.97 (s, 3H), 3.98

(s, 3H), 4.22–4.29 (m, 2H), 6.90 (d, $J = 8.4$ Hz, 2H), 7.35 (dd, $J = 8.4$ and 1.8 Hz, 1H), 7.53 (d, $J = 1.8$ Hz, 1H), 7.70–7.78 (m, 4H). ES-MS *m/z*: 324 (M + 1). Anal. $C_{20}H_{21}NO_3$ (C, H, N).

*5.1.6.6. [4-(3,4-Dihydro-1H-isoquinolin-2-ylmethyl)phenyl]-(3,4-dimethoxyphenyl)methanone (**6**).* Yield: 77%, hydrochloride salt: mp 122–124 °C. Base: ^{1}H NMR ($CDCl_3$) δ 2.65–2.85 (m, 2H), 2.87–3.00 (m, 2H), 3.68 (s, 2H), 3.78 (s, 2H), 3.96 (s, 3H), 3.98 (s, 3H), 6.90 (d, $J = 8.4$ Hz, 2H), 7.15 (d, 1.8 Hz, 1H), 7.39 (dd, $J = 1.8$ and 8.4 Hz, 1H), 7.50–7.55 (m, 3H), 7.75 (d, $J = 8.4$ Hz, 2H). ES-MS *m/z*: 388 (M + 1). Anal. $C_{25}H_{25}NO_3$ (C, H, N).

*5.1.6.7. (3,4-Dimethoxyphenyl)-(4-{[methyl-(3-nitrobenzyl) amino]methyl}phenyl)methanone (**7**).* Yield: 65%, hydrochloride salt: mp 185–186 °C. ^{1}H NMR ($CDCl_3$) δ 2.72 (d, $J = 4.4$ Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.05–4.18 (m, 2H), 4.20–4.38 (m, 2H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.34 (dd, $J = 1.8$ and 8.4 Hz, 1H), 7.52 (d, $J = 1.8$ Hz, 1H), 7.43 (t, $J = 8.2$ Hz, 1H), 7.80–7.85 (m, 4H), 8.34 (d, $J = 8.2$ Hz, 1H), 8.40 (d, $J = 1.2$ Hz, 1H), 8.50 (d, $J = 8.2$ Hz, 1H). ES-MS *m/z*: 421 (M + 1). Anal. $C_{24}H_{24}N_2O_5$ (C, H, N).

*5.1.6.8. (3,4-Dimethoxyphenyl)-(4-{[(2-methoxybenzyl)methylamino]methyl}phenyl)-methanone (**8**).* Yield: 75%, hydrochloride salt: mp 168–170 °C. ^{1}H NMR ($CDCl_3$) δ 2.65 (d, $J = 4.4$ Hz, 3H), 3.90 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 4.10–4.25 (m, 2H), 4.30–4.43 (m, 2H), 6.89 (d, $J = 7.8$ Hz, 1H), 6.98 (d, $J = 8.4$ Hz, 1H), 7.06 (t, $J = 8.1$ Hz, 1H), 7.35 (d, $J = 8.4$ Hz, 1H), 7.46 (t, $J = 7.8$ Hz, 1H), 7.51 (d, $J = 1.8$ Hz, 1H), 7.63 (d, $J = 7.8$ Hz, 1H), 7.80–7.85 (m, 4H). ES-MS *m/z*: 406 (M + 1). Anal. $C_{25}H_{27}NO_4$ (C, H, N).

*5.1.6.9. (3,4-Dimethoxyphenyl)-(4-{[methyl-(3-methylbenzyl)amino]methyl}phenyl)methanone (**9**).* Yield: 72%, hydrochloride salt: mp 219–221 °C. ^{1}H NMR ($CDCl_3$) δ 2.63 (d, $J = 4.4$ Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.00–4.20 (m, 2H), 4.22–4.32 (m, 2H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.34–7.43 (m, 5H), 7.52 (d, $J = 1.8$ Hz, 1H), 7.78–7.88 (m, 4H). ES-MS *m/z*: 390 (M + 1). Anal. $C_{25}H_{27}NO_3$ (C, H, N).

*5.1.6.10. {4-[(Benzylethylamino)methyl]phenyl}-(3,4-dimethoxyphenyl)methanone (**10**).* Yield: 77%, hydrochloride salt: mp 178–180 °C. ^{1}H NMR ($CDCl_3$) δ 1.52 (t, $J = 6.9$ Hz, 3H), 3.04 (q, $J = 7.0$ Hz, 2H), 3.96 (s, 3H), 3.98 (s, 3H), 4.08–4.22 (m, 2H), 4.25–4.38 (m, 2H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.34 (dd, $J = 1.8$ and 8.4 Hz, 1H), 7.45–7.50 (m, 3H), 7.51 (d, $J = 1.8$ Hz, 1H), 7.65–7.78 (m, 3H), 7.80–7.95 (m, 4H). ES-MS *m/z*: 390 (M + 1). Anal. $C_{25}H_{27}NO_3$ (C, H, N).

*5.1.6.11. (4-{[Benzyl-(2-hydroxyethyl)amino]methyl}phenyl)-(3,4-dimethoxyphenyl)methanone (**11**).* Yield: 76%, hydrochloride salt: mp 183–185 °C. ^{1}H NMR ($CDCl_3$) δ 3.10–3.20 (m, 2H), 3.90–4.10 (m, 8H), 4.20–4.38 (m, 2H), 4.42–4.58 (m, 2H), 4.78–4.85 (br, 1H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.33 (dd, $J = 1.8$ and 8.4 Hz, 1H), 7.42–7.50 (m, 4H), 7.55–7.65 (m, 2H), 7.65–7.78 (m, 3H), 7.78–7.85 (m, 4H). ES-MS *m/z*: 406 (M + 1). Anal. $C_{25}H_{27}NO_4$ (C, H, N).

*5.1.6.12. (4-{[Benzyl-(2-dimethylaminoethyl)amino]methyl}-phenyl)-(3,4-dimethoxyphenyl)methanone (**12**).* Yield: 69%, hydrochloride salt: mp 162–164 °C. Base: ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 6H), 2.45–2.75 (m, 4H), 3.65 (s, 2H), 3.70 (s, 2H), 3.95 (s, 3H), 3.96 (s, 3H), 6.90 (d, $J = 8.4$ Hz, 1H), 7.20–7.55 (m, 9H), 7.72 (d, $J = 7.6$ Hz, 1H). ES-MS *m/z*: 433 (M + 1). Anal. $C_{27}H_{32}N_2O_3$ (C, H, N).

*5.1.6.13. {4-[3-(Benzylmethylamino)propenyl]phenyl}-(3,4-dimethoxyphenyl)methanone (**13**).* Starting from **27** (0.65 g, 0.9 mmol) and *N*,*N*-benzylmethylamine (0.17 mL, 1.35 mmol) compound **13** was obtained, 45% yield, hydrochloride salt: mp 172–174 °C. ^{1}H NMR ($CDCl_3$) δ 3.15–3.24 (m, 2H), 3.65 (s, 2H), 3.95 (s, 3H), 3.97 (s, 3H), 6.40–6.77 (m, 2H), 6.80 (d, $J = 8.4$ Hz, 1H), 7.25–7.41 (m, 5H), 7.43–7.53 (m, 3H). ES-MS *m/z*: 402 (M + 1). Anal. $C_{26}H_{27}NO_3$ (C, H, N).

*5.1.6.14. {4-[(Benzylmethylamino)methyl]phenyl}-(4-methoxyphenyl)methanone (**14**).* Starting from **22** (0.3 g, 1.0 mmol) and *N*,*N*-benzylmethylamine (0.2 mL, 1.5 mmol) compound **14** was obtained as brown oil, 56% yield, hydrochloride salt: mp 170–172 °C. Base: ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 3H), 3.55 (s, 2H), 3.60 (s, 2H), 3.80 (s, 3H), 7.10 (d, $J = 8.8$ Hz, 2H), 7.20–7.40 (m, 5H), 7.35 (d, $J = 8.0$ Hz, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.70 (d, $J = 9.2$ Hz, 2H). ES-MS *m/z*: 346 (M + 1). Anal. $C_{23}H_{23}NO_2$ (C, H, N).

*5.1.6.15. {4-[(Benzylmethylamino)methyl]phenyl}-(3-fluoro-4-methoxyphenyl)methanone (**15**).* Starting from **23** (0.5 g, 1.3 mmol) and *N*,*N*-benzylmethylamine (0.25 mL, 1.95 mmol) compound **15** was obtained, 68% yield, mp 38–40 °C, hydrochloride salt: mp 201–204 °C. ^{1}H NMR ($CDCl_3$) δ 2.63 (d, $J = 4$ Hz, 3H), 3.99 (s, 3H), 4.02–4.20 (m, 2H), 4.22–4.31 (m, 2H), 7.03 (t, $J = 8.4$ Hz, 1H), 7.45–7.53 (m, 3H), 7.44–7.68 (m, 4H), 7.78–7.84 (m, 4H). ES-MS *m/z*: 364 (M + 1). Anal. $C_{23}H_{22}FNO_2$ (C, H, N).

*5.1.6.16. {4-[(Benzylmethylamino)methyl]phenyl}naphthalen-2-yl-methanone (**16**).* Starting from **24** (0.15 g, 0.46 mmol) and *N*,*N*-benzylmethylamine (0.08 mL, 0.69 mmol) compound **16** was obtained, 45% yield, hydrochloride salt: mp 173–175 °C. ^{1}H NMR ($CDCl_3$) δ 2.65 (d, $J = 4.4$ Hz, 3H), 4.10–423 (m, 2H), 4.28–4.42 (m, 2H), 7.45–7.73 (m, 6H), 7.80–8.00 (m, 8H), 8.28 (s, 1H). ES-MS *m/z*: 366 (M + 1). Anal. $C_{26}H_{23}NO$ (C, H, N).

5.2. Determination of inhibitory effect on AChE and BChE activity

The capacity of compounds **1–16** to inhibit AChE activity was assessed using Ellman's method [13]. Initial rate assays were performed at 37 °C with a Jasco V-530 double beam spectrophotometer by following the rate of increase in the

absorbance at 412 nm for 5 min. AChE stock solution was prepared by dissolving human recombinant AChE (E.C. 3.1.1.7) lyophilized powder (Sigma, Italy) in 0.1 M phosphate buffer (pH = 8.0) containing Triton X-100 0.1%. Stock solution of BChE (E.C. 3.1.1.8) from human serum (Sigma, Italy) was prepared by dissolving the lyophilized powder in an aqueous solution of gelatine 0.1%. The final assay solution consisted of 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.02 unit/mL of human recombinant AChE, or BChE from human serum and 550 μM of substrate (acetylthiocholine iodide, ATCh or butyrylthiocholine iodide, BTCh, respectively). Stock solutions of tested compounds were prepared in methanol and diluted in bidistilled water. Five different concentrations of inhibitors were selected in order to obtain inhibition of the enzymatic activity comprised between 20% and 80%. Aliquots (50 μL) of increasing concentration of inhibitor were added to the assay solution and preincubated for 20 min at 37 °C with the enzyme followed by the addition of substrate. Assays were carried out with a blank containing all components except AChE or BChE in order to account for the non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of increasing concentrations of inhibitor was calculated. Each concentration was analyzed in duplicate, and IC_{50} values were determined graphically from log concentration–percent inhibition curves (GraphPad Prism 4.03 software, GraphPad Software Inc.).

5.3. Computational studies. Docking simulations

Docking simulations were carried out by means of the GOLD software [15] (v. 3.0.1) and using the crystal structure of human AChE in complex with fasciculin (PDB code 1B41) [20]. Fasciculin was removed and the ligand binding site was defined as 13 Å from the oxygen of Tyr124 side chain. As suggested by the GOLD authors [15], genetic algorithm default parameters were set: the population size was 100, the selection pressure was 1.1, the number of operations was 10^5, the number of islands was 5, the niche size was 2, migrate was 10, mutate was 95, and crossover was 95. Hundred poses were generated by GOLD and then clusterized by means of AClAP (v. 1.0) [16,17]. Briefly, AClAP is a newly developed clustering protocol implemented in a MATLAB metalanguage program, which combines a hierarchical agglomerative cluster analysis with a clusterability assessment method and a user independent cutting rule [17]. In particular, when applied to docking outcomes, we demonstrated that the combination of the average linkage rule with the cutting function developed by Sutcliffe and co-workers [21] turned out to be an approach that meets all of the criteria required for a robust clustering protocol [16]. The poses reported in Fig. 2 for compound **1** is the one most representative of the most populated cluster (28 poses) that, according to the Chauvenet criterion, was statistically populated. The 3D model of **1** was built using the SYBYL (SYBYL 7.1, Tripos Inc., St. Louis, MO) standard fragment library and then optimized at density functional level of theory (B3LYP/6-31G*) by means of the Gaussian03 software (Gaussian, Inc., Pittsburgh, PA, 2003).

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