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Design, synthesis and evaluation of 2,4-disubstituted pyrimidines as cholinesterase inhibitors

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ABSTRACT

A group of 2,4-disubstituted pyrimidine derivatives (**7a–e**, **8a–e** and **9a–d**) that possess a variety of C-2 aliphatic five- and six-membered heterocycloalkyl ring in conjunction with a C-4 arylalkylamino substituent were designed, synthesized and evaluated as cholinesterase (ChE) inhibitors. The steric and electronic properties at C-2 and C-4 positions of the pyrimidine ring were varied to investigate their effect on ChE inhibitory potency and selectivity. The structure–activity relationship (SAR) studies identified *N*-benzyl-2-thiomorpholinopyrimidin-4-amine (**7c**) as the most potent cholinesterase inhibitor (ChEI) with an IC_{50} = 0.33 μM (acetylcholinesterase, AChE) and 2.30 μM (butyrylcholinesterase, BuChE). The molecular modeling studies indicate that within the AChE active site, the C-2 thiomorpholine substituent was oriented toward the cationic active site region (Trp84 and Phe330) whereas within the BuChE active site, it was oriented toward a hydrophobic region closer to the active site gorge entrance (Ala277). Accordingly, steric and electronic properties at the C-2 position of the pyrimidine ring play a critical role in ChE inhibition.

Alzheimer's disease (AD) is a progressive neuro degenerative disorder that affects regions of the brain that control cognition, memory, language, speech and awareness to one's physical surroundings.[1] The characteristics of AD include the progressive loss of cholinergic neural transmission, formation of abeta-amyloid plaques (Aβ-plaques) and neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, all of which support the cholinergic, β-amyloid and tau hypotheses of AD.[2] Although the cholinergic hypothesis is one of the earliest and most studied pathways, recent findings suggests a link between diminished cholinergic neural transmission and Aβ-plaque toxicity in AD pathogenesis generating a renewed interest in the cholinergic hypothesis.[3]

According to the cholinergic hypothesis, the pathogenesis of AD is a result of the progressive decline of cholinergic transmission mediated via the neurotransmitter acetylcholine (ACh).[2] The ChE enzymes, AChE and BuChE are hydrolytic enzymes that act on ACh to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline and acetate.[4] Recent studies have shown that AD pathogenesis is characterized by the rapid loss of AChE activity in the early stages of the disease along with the increasing ratio of BuChE to AChE as the disease progresses.[5,6] These findings support the need to control the activity of the ChE enzymes at different stages of AD progression.

In this regard, several fused heterocyclic ring templates were developed as ChE inhibitors (ChEIs) (Fig. 1). The acridine derivative tacrine (**1**) was one of the earliest ChEI developed to treat AD.[7] The related derivative bis-7-tacrine (**2**), is a highly potent ChEI.[8] Furthermore, β-carbolines (**3**) and phenothiazines (**4**) were developed as dual AChE and BuChE inhibitors.[9–11] As part of our program to develop novel ChEIs, we herein report the design, synthesis and evaluation of a class of 2,4-disubstituted pyrimidine derivatives (**7a–e**, **8a–e** and **9a–d**) based on a non-fused heterocyclic ring

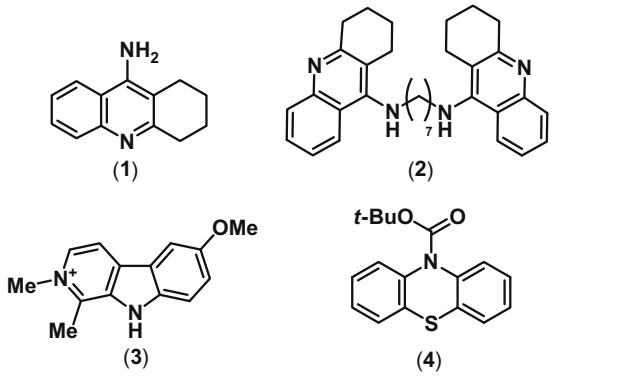

Figure 1. Structures of some fused heterocyclic ring templates that exhibit ChE inhibition (tacrine **1**; bis-tacrine **2**; β-carboline **3**; phenothiazine **4**).

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template. Their synthetic protocol, AChE/BuChE inhibition, structure–activity relationship (SAR) studies and molecular modeling investigations are described.

The *N*-benzyl, *N*-phenethyl and naphthyl methyl 2-chloropyrimidin-4-amine intermediates (**6a**–**c**) were synthesized from 2,4-dichloropyrimidine starting material (**5**) by a nucleophilic aromatic substitution reaction using a base such as *N*,*N*-diisopropylethylamine (DIPEA) in moderate yield (60–65%) (Scheme 1).[12] In the next step, the C-2 chlorine is displaced by various cyclic secondary amines (R^1 = pyrrolidine, morpholine, thiomorpholine, 1-methylpiperazine, 4-methylpiperidine) under rigorous conditions in a sealed pressure vessel using *n*-butanol as a solvent to afford the target 2,4-disubstituted pyrimidine derivatives (**7a**–**e**, **8a**–**e** and **9a**–**d**) in good yield (75–85%) (Scheme 1).[13,14]

The potency and selectivity of the synthesized pyrimidine derivatives (**7a**–**e**, **8a**–**e** and **9a**–**d**) were evaluated in vitro using an inhibition assay with AChE (electric eel) and BuChE (equine serum). The inhibition data (IC_{50} values) are reported in Table 1.[15–17]

Amongst the *N*-benzyl series of compounds (**7a**–**e**), varying the C-2 substituents modulated the ChE inhibitory profile (Table 1). The presence of a C-2 five-membered pyrrolidine substituent in **7a** led to the inhibition of both AChE (IC_{50} = 9.80 μM) and BuChE (IC_{50} = 26.40 μM), although **7a** was not as potent as the reference compound tacrine (**1**, AChE IC_{50} = 0.173 μM; BuChE IC_{50} = 0.019 μM; Table 1). In contrast, the presence of a six-membered C-2 substituent such as a morpholine (**7b**), a thiomorpholine (**7c**), 1-methylpiperazine (**7d**) and a 4-methylpiperidine (**7e**) afforded compounds that exhibited potent AChE inhibition (IC_{50} values = 0.33–0.56 μM range). However, **7b** and **7d** exhibited either weak (**7b**, BuChE IC_{50} = 68.30 μM) or no inhibition of BuChE (**7d**, IC_{50} > 100 μM) whereas, compounds **7c** (AChE IC_{50} = 0.33 μM; BuChE IC_{50} = 2.30 μM) and **7e** (AChE IC_{50} = 0.39 μM; BuChE IC_{50} = 2.90 μM) exhibited dual inhibition of both AChE and BuChE. From the *N*-benzyl series (**7a**–**e**), **7c** (*N*-benzyl-2-thiomorpholinopyrimid-4-amine) was identified as the most potent ChE inhibitor (AChE IC_{50} = 0.33 μM; BuChE IC_{50} = 2.30 μM; Table 1) with superior AChE inhibitory potency and selectivity.

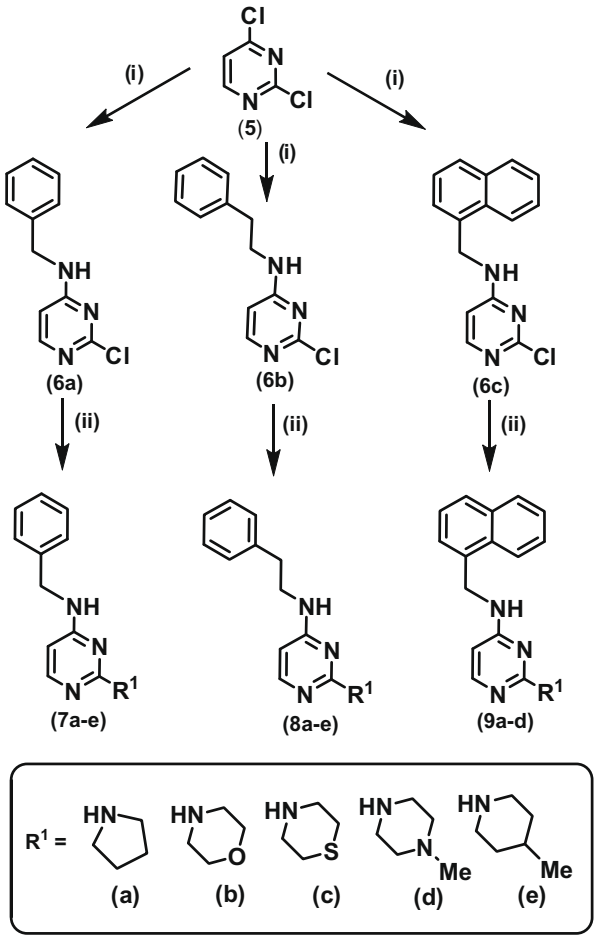
Amongst the *N*-phenethyl series (**8a**–**e**), **8a** with a C-2 five-membered pyrrolidine substituent (AChE IC_{50} = 12.00 μM; BuChE IC_{50} = 13.80 μM) and **8e** with a C-2 six-membered 4-methylpiperidine substituent (AChE IC_{50} = 7.90 μM; BuChE IC_{50} = 17.70 μM) exhibited dual ChE inhibition with **8e** exhibiting superior AChE selectivity, although they were not as potent compared to the *N*-benzyl counterpart **7e** (Table 1). Interestingly, the presence of a six-membered C-2 substituent such as a morpholine (**8b**), thiomorpholine (**8c**) and a 1-methylpiperazine (**8d**) led to a loss of BuChE inhibition (BuChE IC_{50} > 100 μM, Table 1). In general, the *N*-phenethyl derivatives (**8a**–**e**) exhibited decreased AChE inhibitory potency compared to their *N*-benzyl counterparts (**7a**–**e**) and the AChE inhibitory potency was dependent on the electronic and steric parameters of C-2 substituents and the activity was of the order: morpholine > 4-methylpiperidine > pyrrolidine > 1-methylpiperazine > thiomorpholine.

Amongst the naphthyl methyl series (**9a**–**d**), **9a** containing a C-2 five-membered pyrrolidine substituent (AChE IC_{50} = 5.80 μM; BuChE IC_{50} = 8.90 μM; Table 1) exhibited dual ChE inhibition whereas **9b** (AChE IC_{50} = 15.70 μM; BuChE IC_{50} = 28.0 μM) and **9c** (AChE IC_{50} = 15.50 μM; BuChE IC_{50} = 34.70 μM) possessing a C-2 six-membered morpholine or a thiomorpholine substituent were equipotent AChE inhibitors with weak BuChE inhibition. It was interesting to note that compound **9d** with a C-2 methylpiperazine substituent exhibited 6.7-fold selectivity toward BuChE (AChE IC_{50} = 17.60 μM; BuChE IC_{50} = 2.60 μM; SI = 6.77) although it was not as potent relative to tacrine (AChE IC_{50} = 0.173 μM; BuChE IC_{50} = 0.019 μM; SI = 9.11; Table 1). These in vitro data indicate that the ChE inhibition was also dependent on the volume of

Table 1
In vitro ChE (AChE and BuChE) inhibition assay data for compounds **7a**–**e**, **8a**–**e** and **9a**–**d**

Compound	AChE IC_{50}[a] (μM)	BuChE IC_{50}[a] (μM)	SI[b]	C log P[c]
7a	9.80	26.40	0.37	2.96
7b	0.50	68.30	0.007	2.14
7c	0.33	2.30	0.14	2.97
7d	0.56	>100	<0.006	2.70
7e	0.39	2.90	0.13	4.04
8a	12.00	13.80	0.87	3.61
8b	4.80	>100	<0.05	2.79
8c	25.00	>100	<0.25	3.62
8d	14.40	>100	<0.14	3.35
8e	7.90	17.70	0.45	4.69
9a	5.80	8.90	0.65	4.14
9b	15.70	28.00	0.56	3.31
9c	15.50	34.70	0.45	4.15
9d	17.60	2.60	6.77	3.87
Tacrine (**1**)	0.173	0.019	9.11	3.27
Bis-tacrine (**2**)	0.0018	0.0105	0.17	10.09

[a] The in vitro test compound concentration required to produce 50% inhibition of AChE (electric eel) or BuChE (equine serum). The result (IC_{50}, μM) is the mean of two separate determinations (n = 4) and the deviation from the mean is <10% of the mean value.
[b] Selectivity index (SI) = AChE IC_{50}/BuChE IC_{50}.
[c] The *C* log *P* value was calculated using the ChemDraw Ultra program, Version 11.0, CambridgeSoft company.



Scheme 1. Reagents and conditions: (i) DIPEA, phenylmethanamine (**6a**) or 2-phenylethanamine (**6b**) or (naphthalen-1-yl)methanamine (**6c**), EtOH, 0 to 70–80 °C, reflux 3 h; (ii) *n*-BuOH, R^1 = pyrrolidine, morpholine, thiomorpholine, 1-methylpiperazine and 4-methylpiperidine, respectively, 145–150 °C, 30–40 min.

pyrimidine derivatives (**7a**–**e**, **8a**–**e** and **9a**–**d**) with the naphthyl methyl derivatives (**9a**–**d**) exhibiting BuChE inhibition as well due to their increased volume/size compared to *N*-phenethyl series. Furthermore, theoretical log *P* values for the synthesized compounds correlate well (*C* log *P* = 2.14–4.69 range; Table 1) with the reference compound tacrine (*C* log *P* = 3.27) supporting their ability to reach the central nevous system (CNS).

The enzyme–ligand binding interactions of the most potent ChE inhibitor, **7c** (*N*-benzyl-2-thiomorpholinopyrimidin-4-amine, AChE IC_{50} = 0.33 μM; BuChE IC_{50} = 2.30 μM; SI = 0.14), was investigated by molecular modeling studies (Fig. 2).[18] The docking study of **7c** within the active site of *Tc*AChE (Fig. 2) indicates that the pyrimidine ring was oriented closer to the catalytic triad (His440) at the bottom of the active site and was stacked between Trp84 and Phe330 in the cationic active site (CAS) (distance < 5 Å). This observation is consistent with the binding pattern of the central pyridine ring in tacrine.[7] The pyrimidine *N*-1 of **7c** was about 2.68 Å away from N*H* of indole (Trp84) and was about 2.88 Å away from backbone C=*O* (Gly441), respectively. The C-2 thiomorpholine ring was oriented in a non-polar region comprised of Gly80, Ser81, Trp84, Phe330, Leu333, Tyr334, Trp432, Ile439, Met436 and Tyr442 (distance < 5 Å). The nitrogen atom of thiomorpholine was undergoing a hydrogen-bonding interaction with indole N*H* of Trp84 (distance = 3.05 Å) whereas the distance between O*H* of Tyr442 and nitrogen atom of thiomorpholine was about 3.90 Å. The sulphur atom of thiomorpholine was undergoing hydrophobic interactions with the side chains of Met436 and Leu333 (distance < 5 Å). Interestingly, the sulphur atom of thiomorpholine was also forming a weak hydrogen bond with O*H* of Tyr334 (distance = 3.59 Å). The C-4 *N*-benzyl ring was oriented in a hydrophobic region comprised of Gly117, Gly118, Gly123, Leu127 and Tyr130 closer to the 'peripheral' anionic site (PAS, Trp279) (distance < 5 Å).

A similar docking experiment was conducted to investigate the binding interactions of **7c** within the active site of mammalian BuChE (Fig. 3). The BuChE active site lacks the PAS present in AChE and its gorge entry is lined by smaller aliphatic amino acid residues whereas in AChE it is lined by bulkier aromatic amino acids. This makes the volume of BuChE active site about 60% larger than that of AChE.[6,19] In contrast to *Tc*AChE binding, the pyrimidine ring of **7c** binds in the centre of BuChE active site such that the C-4 *N*-benzyl ring was oriented toward the catalytic triad (His438). The pyrimidine was in a region comprised of polar amino acids such as Asp70,

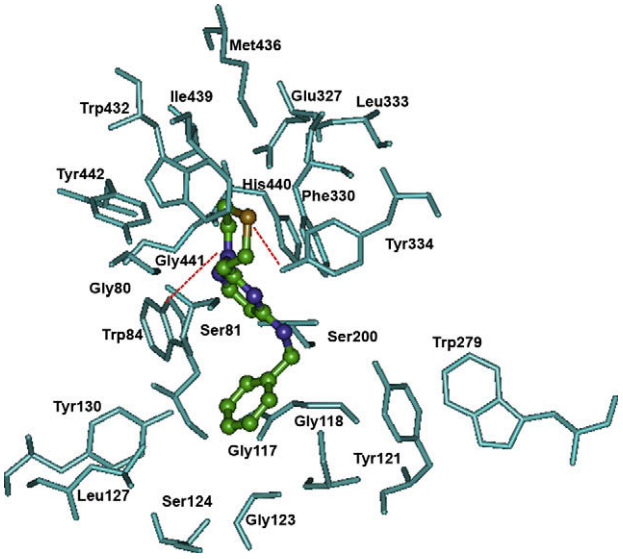


Figure 2. Docking of *N*-benzyl-2-thiomorpholinopyrimidin-4-amine (**7c**) in the active site of *Tc*AChE (PDB code: 1ACJ). Red lines represent hydrogen-bonding interactions. Hydrogen atoms are not shown for clarity.

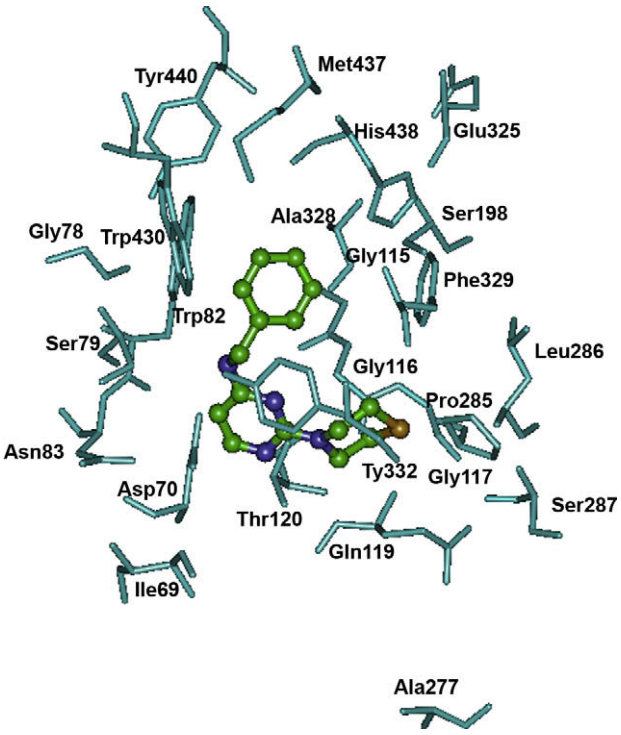


Figure 3. Docking of *N*-benzyl-2-thiomorpholinopyrimidin-4-amine (**7c**) in the active site of mammalian BuChE (PDB code: 1P0I). Hydrogen atoms are not shown for clarity.

Ser79, Asn83 and Thr120 (distance < 5 Å). The *N*-1 of pyrimidine was about 3.91 Å away from O*H* of Thr120. The C-2 thiomorpholine ring was in a hydrophobic region closer to the active site gorge comprised of Gly116, Gly117, Leu286 and Pro285 (distance < 5 Å). The sulphur atom of the thiomorpholine ring was about 3.94 Å away from backbone C=*O* of Pro285. The distance between Ala277, which is present at the entrance of the active site, and C-2 of the thiomorpholine ring was about 11.89 Å. In addition, The C-4 *N*-benzyl substituent was in a hydrophobic region closer to the CAS comprised of Trp82, Ala328, Phe329, Trp430, Met437, His438 and Tyr440 (distance < 5 Å). These binding interaction studies are consistent with the ChE inhibitory data obtained for **7c** (Table 1).

In general, the presence of either a C-4 *N*-benzyl (**7a**–**c** and, **7e**) or naphthyl methyl (**9a**–**d**) substituent with varying C-2 groups provided dual ChE (AChE/BuChE) inhibition. In contrast, the presence of a C-4 *N*-phenethyl substituent as in **8b**–**d** led to a loss in BuChE inhibition. These studies indicate that a non-fused pyrimidine based ring template can be developed as a dual ChE inhibitor by modulating the steric and electronic parameters at C-2 and C-4 positions.

In summary, the current study supports the hypothesis that (i) a pyrimidine ring serves as a suitable template in the design of novel ChE inhibitors; (ii) the ChE inhibition was sensitive to substituent electronic and steric parameters at C-2 and C-4 position of the pyrimidine ring and (iii) compound **7c** (AChE IC_{50} = 0.33 μM; BuChE IC_{50} = 2.30 μM; SI = 0.14) with a C-4 *N*-benzyl substituent and a C-2 six-membered thiomorpholine substituent was identified as a dual ChE inhibitor.

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14. *General procedure for the synthesis of 4-substituted-2-chloropyrimidin-4-amines (**6a–c**):* To a mixture of **5** (2 g, 13.4 mmol) and the respective amine [13.4 mmol, phenylmethanamine, 2-phenylethanamine or (naphthalen-1-yl)methanamine, respectively] in 25 mL of EtOH, kept at 0 °C (ice-bath), DIPEA (2.43 mL, 14.7 mmol) was added. The reaction was allowed to stir on the ice-bath for 5 min and was refluxed at 75–80 °C for 3 h. After cooling to 25 °C, the EtOH was evaporated in vacuo and the residue was re-dissolved in a solvent mixture of EtOAc and dichloromethane (DCM) in ~3:1 ratio and washed successively with saturated $NaHCO_3$ and NaCl solution (1× 15 mL), respectively. Aqueous layer was washed with EtOAc (3× 15 mL) and the combined organic layer was dried over anhydrous $MgSO_4$ then filtered. The organic layer was evaporated in vacuo and the resulting solid or oily residue was further purified by silica gel column chromatography using EtOAc/hexanes (3:1) as eluent to afford the respective intermediate (**6a–c**).
    General procedure for the synthesis of 2,4-disubstituted-pyrimidin-4-amines (**7a–e**, **8a–e** and **9a–d**)*:* To a solution of **6a–c** (0.9–1.0 mmol) in 3 mL of *n*-BuOH kept in a pressure vessel (PV) with stirring, the respective secondary amine (1.20 mmol, pyrrolidine, morpholine, thiomorpholine, 1-methyl piperazine or 4-methylpiperidine, respectively) was added. The sealed PV was placed in an oil bath at 145–150 °C and stirred for 30–40 min. The *n*-BuOH was evaporated in vacuo and the residue was re-dissolved in a solvent mixture of EtOAc and DCM in ~3:1 ratio and washed successively with saturated $NaHCO_3$ and NaCl solution (1× 15 mL), respectively. The aqueous layer was washed with EtOAc (3× 5 mL) and the organic layer was dried over anhydrous $MgSO_4$ then filtered. The solution was evaporated in vacuo and the residue obtained was further purified as needed by a silica gel column chromatography using EtOAc/hexanes (3:1) as eluent to afford the respective 2,4-disubstituted pyrimidine derivatives (**7a–e**, **8a–e** and **9a–d**).
    *Analytical data for N-benzyl-2-thiomorpholinopyrimidin-4-amine (**7c**):* Yield, 77%; brown solid; mp 85–87 °C; IR (film): 3258 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.54–2.58 (m, 4H, thiomorpholine S-CH_2), 4.03–4.07 (m, 4H, thiomorpholine N-CH_2), 4.47 (d, J = 5.6 Hz, 2H, CH_2-NH), 5.03 (br s, 1H, CH_2-N*H*), 5.66 (d, J = 5.7 Hz, 1H, pyrimidine H-5), 7.24–7.33 (m, 5H, benzyl H-2, H-3, H-4, H-5 and H-6), 7.84 (d, J = 5.7 Hz, 1H, pyrimidine H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.76 (S-CH_2), 45.11 (CH_2-NH), 46.32 (N-CH_2), 94.11 (pyrimidine C-5), 127.32, 127.37 and 128.60 (benzyl C-2, C-3, C-4, C-5 and C-6), 138.76 (benzyl C-1), 155.94 (pyrimidine C-2), 160.90 (pyrimidine C-6), 162.97 (pyrimidine C-4); HREIMS Calcd for $C_{15}H_{18}N_4S$ (M^+) m/z 286.3952, found m/z 286.1872.
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17. *Cholinesterase inhibition assay:* The ability of the test compounds (**7a–e**, **8a–e** and **9a–d**) to inhibit electric eel AChE (product number C3389, Sigma, Ann Arbor, MI) and equine serum BuChE (product number C1057, Sigma, St. Louis, MO) was determined using Ellman's method (IC_{50} values, μM). Stock solutions of test compounds were dissolved in a minimum volume of DMSO (1%) and were diluted using the buffer solution (50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 0.02 M $MgCl_2{\cdot}6H_2O$). In 96-well plates, 160 μl 5,5′-dithiobis(2-nitrobenzoic acid) (1.5 mM DTNB), 50 μl of AChE (0.22 U/mL prepared in 50 mM Tris–HCl, pH 8.0, 0.1% w/v bovine serum albumin, BSA) or 50 μl of BuChE (0.12 U/mL prepared in 50 mM Tris–HCl, pH 8.0, 0.1% w/v BSA) were incubated with 10 μl of various concentrations of test compounds (0.001–100 μM) at room temperature for 5 min followed by the addition of the substrates (30 μl) acetylthiocholine iodide (15 mM ATCl) or *S*-butyrylthiocholine iodide (15 mM BTCl) and the absorbance was measured at different time intervals (0, 60, 120 and 180 s) at a wavelength of 405 nm. Percent inhibition was calculated by the comparison of compound-treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve (duplicate to quadruplicate determinations).
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