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Short communication

Synthesis and biological evaluation of helicid analogues as novel acetylcholinesterase inhibitors

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Abstract

A series of helicid analogues were prepared and evaluated in vitro for the cholinesterase (AChE and BuChE) inhibitory activities via UV spectroscopy. The results indicated that compounds **5**, **6d** and **8** exhibited potent AChE inhibitory activities with IC_{50} values of 0.45 ± 0.02 μM, 0.49 ± 0.02 μM, and 0.20 ± 0.01 μM, respectively. High selectivity for AChE over BuChE was also observed. Kinetic study showed that the mechanism of AChE inhibition of compounds **5**, **6d** and **8** was all mixed-type.

Keywords: Helicid analogues; 4-Hydroxybenzaldeyde; Cholinesterase inhibitor

1. Introduction

Alzheimer's disease (AD), a neurodegenerative disorder, is one of the severe health problems of aged population [1]. A deficit in cholinergic neurotransmission was believed to be one of the major causes of the memory impairments in AD patients in the past decades [2,3]. The rational approach to treat AD is to restore the acetylcholine (ACh) levels by inhibiting acetylcholinesterase (AChE) with highly selective inhibitors [4]. Four AChE inhibitors (Fig. 1), tacrine, donepezil, galanthamine, and rivastigmine, have been approved by FDA for the treatment of AD [5,6]. Unfortunately, the potential effectiveness offered by these inhibitors is often limited by the appearance of central and peripheral side effects. Clinical studies have shown that tacrine has hepatotoxic liability [7], physostigmine has poor oral activity [8], and rivastigmine has short half-life [9].

Recent studies focus on the complex nature of AD and disclose the involvement of several other transmitter systems, such as serotonin (5-HT), noradrenalin (NA), dopamine, histamine, excitatory amino acids and neuropeptides [11]. Non-cholinergic neurotransmitter systems undergo severe atrophy throughout the course of the disease. It is also apparent that extensive neuropathology is common in areas normally rich in 5-HT and NA, particularly in the later stages of AD [12]. The multifunctional pathogenesis of AD suggests that molecules with two or more mechanisms of action, acting in a complementary manner, could be more efficacious for AD patients. The present tendency to develop new drugs of AD is to look for inhibitors of AChE endowed with additional therapeutic effects, such as anti-inflammatory and antioxidant agents, activation of other neurotransmitter systems like GABA or monoaminergic systems [13,14].

Natural helicid (**1**), one major active ingredient of Chinese herb medicine, has been used clinically as antalgic and hypnotic for a long time in China, and no obvious side effect has been reported [15,16]. Wang and his co-workers more

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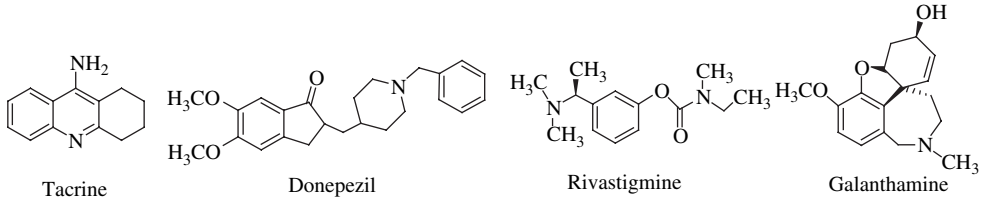


Fig. 1. Structure of AChE inhibitors.

recently indicated that helicid could activate neurotransmitter systems of 5-HT and NA in mice, resulting in an effective antidepressant activity [17]. The aglycon of helicid, 4-hydroxybenzaldehyde, was reported as a potent antioxidant agent and GABA transaminase inhibitor ($IC_{50} = 4.1$ μg/mL) [18]. A further study suggested that the aldehyde group and the hydroxyl group at C-4 are necessary for the actions [19]. Based on our high throughput screening work, 4-hydroxybenzaldehyde also showed weak activity towards AChE, and its IC_{50} value was 37.8 mM. This result prompted us to study 4-hydroxybenzaldehyde glycoside derivatives, analogues of helicid, with the aim to explore new AChE inhibitors with high activity, low toxicity and additional activation property of other neurotransmitter systems like GABA, 5-HT and NA.

2. Chemistry

In general, helicid analogues **3**–**8** were synthesized starting from 4-hydroxybenzaldehyde, followed by glycosidation, deprotection and condensation with amines (Schemes 2–4). In the case of compound **2** (Scheme 1), it was obtained through acetylation of helicid (**1**).

Five alpha glycosyl bromide compounds were firstly prepared using the methods described elsewhere [20–22]. In the presence of NaOH and tetrabutylammonium bromide (TBAB), glycosyl bromide reacted with 4-hydroxybenzaldehyde to afford the corresponding protected phenyl glycosides **3a**–**e**. Beta phenyl glycoside could be obtained as a major product using TBAB as phase-transfer catalyst [23,24]. Subsequent reaction of **3a** and **3e** with sodium methoxide in methanol yielded the sugar-linked helicid analogues **5** and **8**. Schiff base derivatives **4a**–**d**, **6a**–**d** and **7a**–**d** were synthesized, respectively, by the reaction of **3a**, **3e** and **5** with four amines (thiosemicarbazide, semicarbazide, hydroxylamine and methoxyamine).

3. Biology

The inhibitory activities of the prepared compounds against AChE and BuChE were investigated by determining the rate of hydrolysis of acetylthiocholine (ATC) and butylthiocholine (BTC) in comparison with reference compound galanthamine-HBr, using the slightly modified method of Ellman et al. [25].

According to the data in Table 1, the following results could be found.

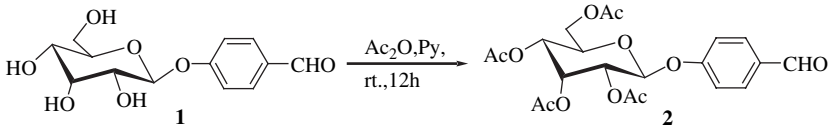
(1) Natural helicid (**1**) was not active up to 500 μM towards AChE, whereas the synthetic helicid analogues **3a**, **4c**, **5**, **6c**, **6d**, **7b**, and **8** showed significant activities with IC_{50} values of less than 10 μM. Helicid analogues **5**, **6d** and **8**, bearing several hydrophilic hydroxyl groups on the sugar moiety, exhibited much higher AChE inhibitory activity than the others, suggesting that the hydrogen atoms of these hydroxyl groups might form hydrogen bond with nitrogen or oxygen atoms of enzyme molecules. The sugar moiety acted like a bridge to link the 4-formylphenoxy moiety and enzyme molecules with these H-bonds, which facilitated the 4-formylphenoxy moiety and enzyme molecules to interact and made the interaction stable.

(2) More interestingly, the configuration of sugar moiety greatly affected the activity towards AChE. Compounds **2** and **3c** were inactive towards AChE, similar to **1**. However, compounds **3a**–**b** and **3d**–**e** exhibited moderate to high activities. It is indicated that the sugar moieties, glucose, galactose, xylose and ribose were much preferable to allose and lactose in inhibition of AChE.

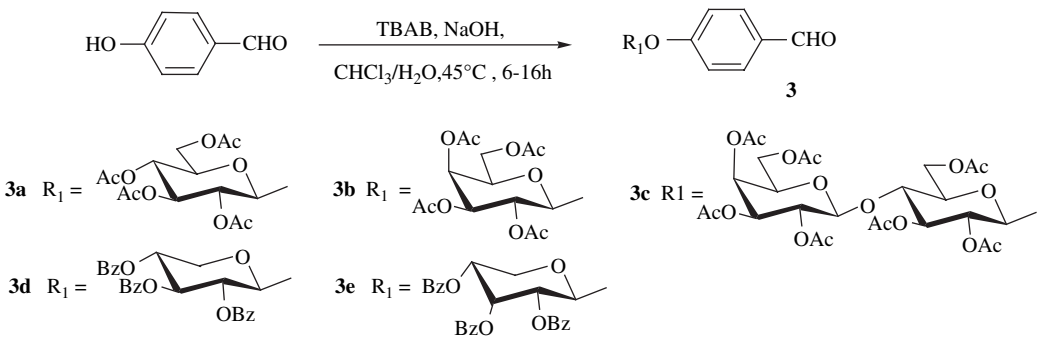
(3) Compound **3a** was 3-fold less active than unprotected glycoside **5** towards AChE, while **3e** was 70-fold less active than **8**, which suggested that the introduction of the acetyl or benzoyl groups on the sugar moieties led to a decrease in AChE inhibitory effect. Benzoyl glycosides were less potent than acetyl glycosides.

(4) The attachment of Schiff base moieties (**4a**–**d**, **6a**–**d** and **7a**–**d**) did not effectively increase activity towards AChE. Even the best one, compound **6d**, was slightly less active than **5**.

(5) Regarding the inhibitory potency of BuChE, most helicid analogues were not active, except benzoyl glycosides **3e** and **7a**–**d** which showed moderate activities. Compounds **5**, **6d** and **8** exhibited strong selectivity for AChE over BuChE.



Scheme 1. Preparation of **2**.



Scheme 2. Preparation of **3a–e**.

We further examined the kinetics of inhibition of AChE by the potent derivatives **5**, **6d** and **8** (Fig. 2). The results demonstrated that the mechanism of AChE inhibition of compounds **5**, **6d** and **8** was all mixed-type, while that of galanthamine-HBr was competitive. Inhibition constant (K_i) was determined from non-linear regression analysis by Dixon plot. Table 2 shows that the K_i values corresponding to the inhibition of AChE with compounds **5**, **6d**, and **8** were 0.73 μM, 0.13 μM, and 0.51 μM, respectively, and the results were close to that of galanthamine-HBr ($K_i = 0.45$ μM) [26].

4. Conclusion

In summary, a series of helicid analogues were synthesized and evaluated as inhibitors of AChE. The most potent inhibitor, compound **8**, which presented an IC_{50} value of 0.20 μM on electric eel AChE, was twice more active than galanthamine-HBr. Our study suggested that helicid analogues might serve as lead compounds for designing new potential AChE inhibitors with high selectivity, low toxicity and additional pharmaceutical effects.

5. Experimental section

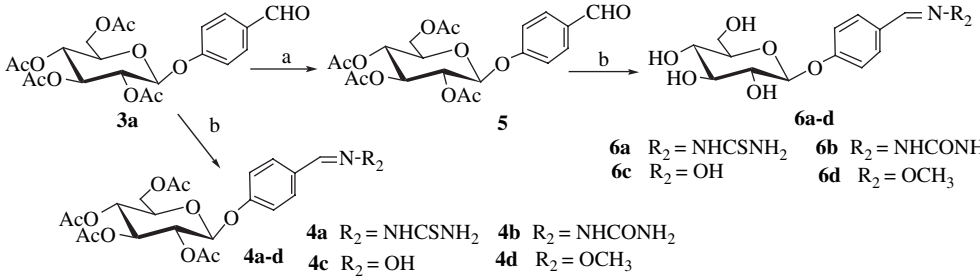
5.1. Chemistry

Melting points were determined with a WRS-1B digital melting point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury-Plus 300 NMR instrument (^{1}H 300 MHz; ^{13}C 75 MHz) in either $CDCl_3$ or DMSO-d_6. Abbreviations for data quoted are s, singlet; br s, broad singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. Mass spectra were recorded on a Thermo Finnigan LCQ DECAXP ion trap mass spectrometer or VS ZAB-HS spectrometer. IR spectra were recorded as potassium bromide pellets on a Bruker Equinox 55 FT/IR spectrometer. Elemental analyses (C, H, N) were carried out on an Elementary Vario EL series elemental analyzer and the results were within ±0.4%. Thin-layer chromatographies were done on pre-coated silica gel 60 F254 plates (Merck). The spots were visualized with UV light or iodine. All reagents were used as received unless otherwise stated.

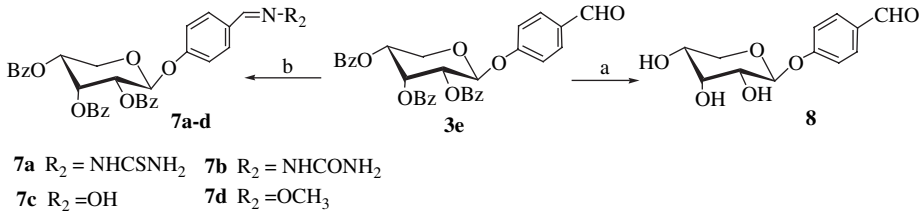
The following glycosyl bromide compounds were prepared according to the standard literature procedure. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide, white solid powder, m.p. 87–89 °C (lit. 88–89 °C) [20]; 2,3,4,6-tetra-*O*-acetyl-α-D-galapyranosyl bromide, white solid powder, m.p. 74–76 °C (lit. 75–76 °C) [20]; 2′,3′,4′,6′,2,3,6-hepta-*O*-acetyl-α-D-lactosyl bromide, white solid powder, m.p. 122–124 °C (lit. 123–124 °C) [20]; 2,3,4-tri-*O*-benzoyl-α-D-xylopyranosyl bromide, white solid powder, m.p. 135–137 °C (lit. 136–137 °C) [21]; 2,3,4-tri-*O*-benzoyl-α-D-ribopyranosyl bromide, white solid powder, m.p. 162–165 °C (lit. 164–166 °C) [22].

5.1.1. Synthesis of 4-formylphenyl (2,3,4,6-tetra-O-acetyl)-β-D-allopyranoside (2)

To a solution of helicid (**1**) (1.0 g, 3.5 mmol) in 2 mL of anhydrous pyridine was added dropwise acetyl anhydride (2.5 g, 25 mmol). The mixture was stirred vigorously at room temperature for 12 h, and then poured into 50 mL of ice water. After 30 min, 20 mL of dichloromethane was added. The organic layer was separated and the aqueous layer was extracted twice with dichloromethane. The combined organic phase was washed with 1 M HCl and brine, dried with anhydrous



Scheme 3. Preparation of **4a–d**, **5** and **6a–d**. Reagents and conditions: (a) NaOMe, MeOH, rt, 3 h; (b) amine, EtOH, reflux or 45 °C, 2–6 h.



Scheme 4. Preparation of **7a–d** and **8**. Reagents and conditions: (a) NaOMe, MeOH, rt, 12 h; (b) amine, EtOH, reflux or 45 °C, 2–6 h.

Na_2SO_4, and the solvent was removed under reduced pressure to give light yellow solid. The crude product was recrystallized in ethanol to yield white solid (**2**), yield 56%, m.p. 135–136 °C. IR (KBr, cm^{-1}) ν: 1752, 1693, 1601, 1506, 1224, 1157, 1127, 1085, 914, 876; ^1H NMR ($CDCl_3$) δ ppm: 9.91 (s, 1H, H–C=O), 7.85 (d, 2H, $J = 8.6$ Hz, ArH), 7.13 (d, 2H, $J = 8.6$ Hz, ArH), 5.75 (t, 1H, $J = 2.9$ Hz, H-3′), 5.48 (d, 1H, $J = 8.1$ Hz, H-1′), 5.19 (dd, 1H, $J = 3.0$ Hz, 8.1 Hz, H-2′), 5.16 (dd, 1H, $J = 2.8$ Hz, 9.9 Hz, H-4′), 4.32–4.27 (m, 1H, H-5′), 4.26–4.24 (m, 2H, H-6′, H-6′), 2.18, 2.08, 2.05, 2.04 ($4 \times$ s, 4×3H, $4 \times CH_3$–C=O); ^{13}C NMR ($CDCl_3$) δ ppm: 191.2, 170.8, 170.6, 170.5, 169.8, 161.7, 132.3, 117.2, 99.0, 71.8, 71.1, 68.8, 67.2, 61.8, 21.2, 21.1, 21.0; ACPI-MS *m/z* (%): 470 (100) [M + NH4$^+$]. Anal. C, H for $C_{21}H_{24}O_{11}$.

5.1.2. Synthesis of compounds *3a–e*

General procedure. A mixture of tetrabutylammonium bromide (4 mmol) in 1:1 water–chloroform (20 mL) was stirred and heated to 45 °C. Glycosyl bromide (0.02 mol) was dissolved in chloroform (20 mL, designated as solution A) and 4-hydroxybenzaldehyde (0.02 mol) was dissolved in 20 mL of water containing 0.88 g (0.022 mol) of NaOH (designated as solution B). Then solutions A and B were simultaneously dropped into the above mixture, while the system was stirred vigorously at 45 °C. After 6–18 h, the organic phase was separated and washed with NaOH (20 mL, 5%), brine, respectively, dried with anhydrous Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by recrystallization or column chromatography on silica gel to afford the corresponding product.

*5.1.2.1. 4-Formylphenyl (2,3,4,6-tetra-O-acetyl)-β-D-glucopyranoside (**3a**)*. White solid product, yield 54%, m.p. 141–142 °C (lit. 142–143 °C) [27]. IR (KBr, cm^{-1}) ν: 2963, 2746, 1750, 1737, 1692, 1601, 1222; ^1H NMR ($CDCl_3$) δ ppm: 9.90 (s, 1H, H–C=O), 7.83 (d, 2H, $J = 8.6$ Hz, ArH), 7.10 (d, 2H, $J = 8.6$ Hz, ArH), 5.54–5.46 (m, 2H, $2 \times$ CH), 5.17 (d, 1H, $J = 8.1$ Hz, H-1′), 5.14–5.10 (m, 1H, CH), 4.25–4.09 (m, 3H, H-5′, H-6′, H-6′), 2.19, 2.06, 2.02 ($3 \times$ s, 4×3H, $4 \times CH_3$–C=O); ACPI-MS *m/z* (%): 470 (100) [M + NH_4^+].

*5.1.2.2. 4-Formylphenyl (2,3,4,6-tetra-O-acetyl)-β-D-galactopyranoside (**3b**)*. White solid product, yield 59%, m.p. 121–122 °C (lit. 121.5–122.0 °C) [28]. IR (KBr, cm^{-1}) ν: 1753,

Table 1
AChE and BuChE inhibitory activities (IC_{50}, μM) of prepared compounds

Compound	R_1	$IC_{50} \pm$ SE AChE (μM)	$IC_{50} \pm$ SE BuChE (μM)	Selectivity for AChE/BuChE
1		na[a]	na	
2		na	na	
3a		1.50 ± 0.22	na	
3b		45.42 ± 4.8	na	
3c		na	na	
3d		34.96 ± 4.1	na	
3e		14.04 ± 1.64	64.27 ± 10	4.58
4a	NH–CS–NH_2	21.47 ± 3.0	na	
4b	NH–CO–NH_2	142.58 ± 20	na	
4c	OH	9.15 ± 1.15	na	
4d	OCH_3	15.09 ± 2.8	na	
5		0.45 ± 0.02	na	
6a	NH–CS–NH_2	22.89 ± 3.1	na	
6b	NH–CO–NH_2	120.70 ± 20	na	
6c	OH	4.79 ± 0.61	na	
6d	OCH_3	0.49 ± 0.03	na	
7a	NH–CS–NH_2	46.43 ± 5.2	71.83 ± 12	1.55
7b	NH–CO–NH_2	7.07 ± 1.05	48.72 ± 8.9	6.89
7c	OH	12.55 ± 2.5	39.60 ± 6.5	3.16
7d	OCH_3	3.08 ± 0.54	45.83 ± 8.4	14.88
8		0.20 ± 0.01	na	
Galanthamine-HBr		0.55 ± 0.02[b]	15.24 ± 3.1	27.70

[a] na = not active up to 500 μM.
[b] IC_{50} values reported in the literature: 0.8–0.3 μM.

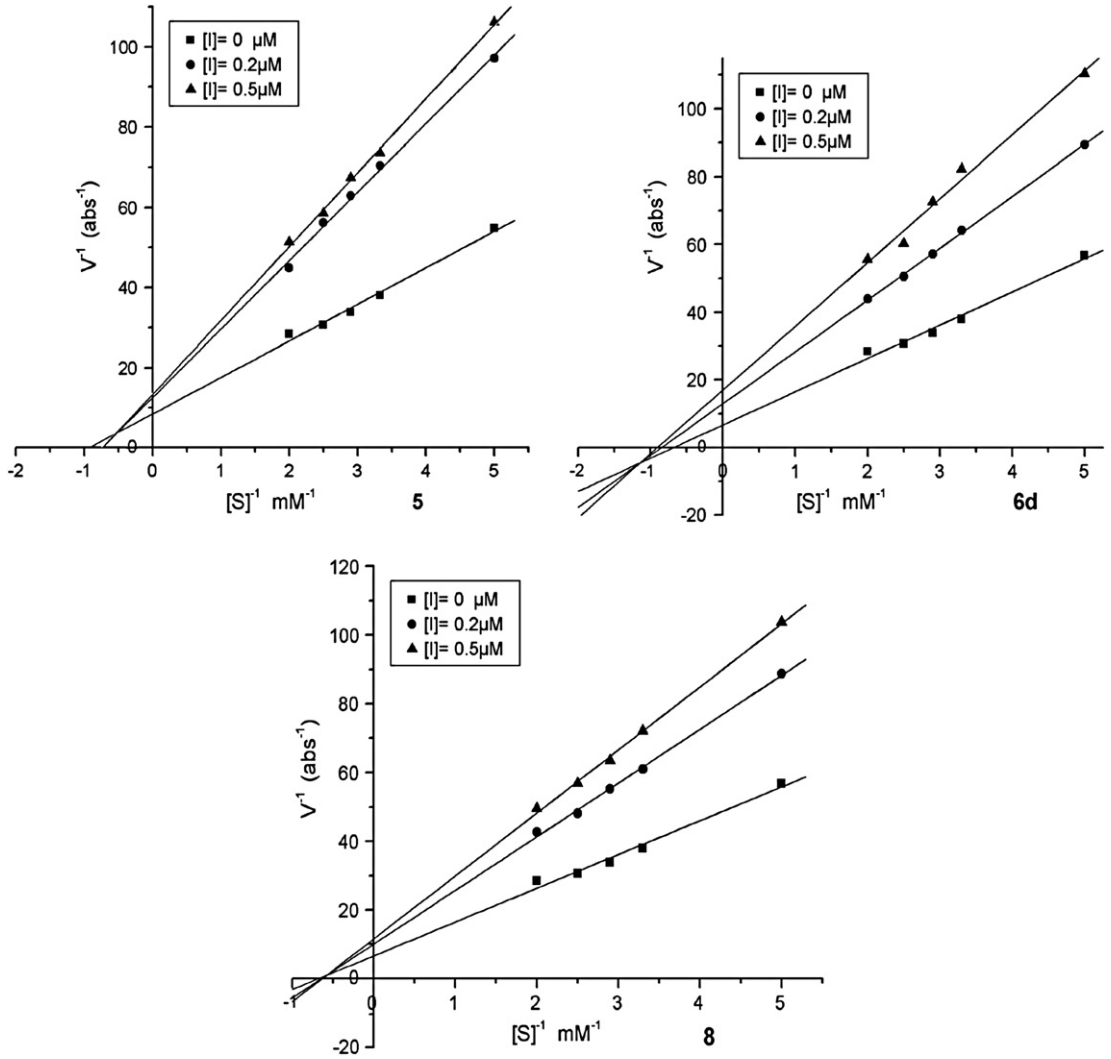


Fig. 2. Double-reciprocal plots of the inhibition kinetics of AChE by compounds **5**, **6d** and **8**.

1694, 1602, 1507, 1371, 1230, 1077, 914, 835; ^{1}H NMR ($CDCl_3$) δ ppm: 9.90 (s, 1H, H–C═O), 7.84 (d, 2H, $J = 8.6$ Hz, ArH), 7.10 (d, 2H, $J = 8.5$ Hz, ArH), 5.45 (dd, 1H, $J = 2.7$ Hz, 10.3 Hz, H-3′), 5.40 (d, 1H, $J = 3.5$ Hz, H-2′), 5.10 (d, 1H, $J = 8.1$ Hz, H-1′), 5.08 (dd, 1H, $J = 3.3$ Hz, 10.5 Hz, H-4′), 4.25–4.10 (m, 3H, H-5′, H-6′, H-6′), 2.19, 2.06, 2.02 (3 × s, 4 × 3H, 4 × CH_3–C═O); ACPI-MS *m/z* (%): 470 (100) [$M + NH_4^+$].

*5.1.2.3. 4-Formylphenyl (2′,3′,4′,6′,2,3,6-hepta-O-acetyl)-β-D-lactoside (**3c**).* White solid product, yield 37%, m.p. 78–79 °C. IR (KBr, cm^{-1}) ν: 1749, 1695, 1602, 1509, 1373, 1231, 1051, 902, 835; ^{1}H NMR ($CDCl_3$) δ ppm: 9.90 (s, 1H, H–C═O), 7.83 (d, 2H, $J = 8.7$ Hz, ArH), 7.07 (d, 2H, $J = 8.7$ Hz, ArH), 5.36 (d, 1H, $J = 3.2$ Hz, CH), 5.21–5.01 (m, 3H, 3 × CH), 4.99–4.95 (m, 1H, CH), 4.53–4.50 (m, 2H, 2 × CH), 4.17–4.05 (m, 4H, 4 × CH), 3.91–3.85 (m, 2H, 2 × CH), 2.17, 2.09, 2.08, 2.07, 1.98 (5 × s, 7 × 3H, 7 × CH_3–C═O); ^{13}C NMR ($CDCl_3$) δ ppm: 191.2, 170.7, 170.6, 170.5, 170.2, 170.0, 169.6, 161.7, 132.2, 117.1, 116.4, 101.6, 98.1, 73.4, 73.1, 71.7, 71.3, 71.2, 69.5, 69.1, 67.0, 62.4, 61.2, 21.2, 21.1, 21.0, 20.9; ESI-MS *m/z* (%): 763 (86) [M + Na]. Anal. C, H for $C_{33}H_{40}O_{19}$.

*5.1.2.4. 4-Formylphenyl (2,3,4-tri-O-benzoyl)-β-D-xylopyranoside (**3d**).* White solid product, yield 46%, m.p. 99 °C. IR (KBr, cm^{-1}) ν: 1726, 1694, 1600, 1506, 1260, 1155, 1092, 1026, 831, 708; ^{1}H NMR ($CDCl_3$) δ ppm: 9.90 (s, 1H, H–C═O), 8.10–7.97 (m, 5H, ArH), 7.85 (d, 2H, $J = 8.70$ Hz, ArH), 7.48–7.29 (m, 10H, ArH), 7.18 (d, 2H, $J = 8.0$ Hz, ArH), 5.73 (t, $J = 5.3$ Hz, 1H, H-2′), 5.65 (d, 1H, $J = 3.5$ Hz, H-1′), 5.50 (dd, 1H, $J = 1.5$ Hz, 5.2 Hz, H-3′), 5.27 (dd, 1H, $J = 3.8$ Hz, 8.5 Hz, H-4′), 4.46 (dd, 1H, $J = 9.5$ Hz, 12.7 Hz, H-5′), 3.90 (dd, 1H, $J = 8.4$ Hz, 12.6 Hz, H-5′); ^{13}C NMR ($CDCl_3$) δ ppm: 191.2, 166.0, 165.6, 165.4, 161.5, 134.1, 132.4, 131.9, 130.4, 128.9, 117.0, 96.9, 69.0, 68.7, 68.3, 61.0; ACPI-MS *m/z* (%): 584 (100) [$M + NH_4^+$]. Anal. C, H for $C_{33}H_{26}O_9$.

*5.1.2.5. 4-Formylphenyl (2,3,4-tri-O-benzoyl)-β-D-ribopyranoside (**3e**).* White solid product, yield 27%, m.p. 129–130 °C. IR (KBr, cm^{-1}) ν: 1727, 1694, 1600, 1505, 1260, 1103, 1025,

Table 2
Inhibition constant and type of inhibition of selected compounds[a]

Compound	$K_i \pm$ SE (μM)	Type of inhibition
5	0.73 ± 0.02	Mixed
8	0.51 ± 0.01	Mixed
6d	0.13 ± 0.004	Mixed
Galanthamine-HBr	0.45 ± 0.01	Competitive

[a] Inhibition constants were calculated from kinetic data.

859, 707; ^{1}H NMR ($CDCl_3$) δ ppm: 9.83 (s, 1H, H–C=O), 7.96–7.94 (m, 4H, ArH), 7.80–7.77 (m, 4H, ArH), 7.49–7.38 (m, 4H, ArH), 7.22–7.17 (m, 7H, ArH), 5.92 (t, 1H, $J = 3.6$ Hz, H-2′), 5.86 (d, 1H, $J = 3.8$ Hz, H-1′), 5.65–5.63 (m, 1H, H-3′), 5.62–5.60 (m, 1H, H-4′), 4.22 (d, 1H, $J = 1.8$ Hz, 12.7 Hz, H-5′), 4.09 (dd, 1H, $J = 1.8$ Hz, 12.7 Hz, H-5′); ^{13}C NMR ($CDCl_3$) δ ppm: 191.3, 166.6, 166.4, 165.7, 161.0, 134.0, 132.4, 132.0, 128.9, 117.1, 96.9, 68.7, 67.7, 66.1, 62.7; ACPI-MS *m/z* (%): 584 (100) $[M + NH_4^+]$. Anal. C, H for $C_{33}H_{26}O_9$.

5.1.3. Synthesis of compounds *4a–d*

*5.1.3.1. 1-(4-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl) benzylidene) thiosemicarbazide (**4a**).* A mixture of compound **3a** (0.45 g, 1 mmol), thiosemicarbazide (0.09 g, 1 mmol) in 15 mL of ethanol was refluxed for 3 h. After being cooled to room temperature, the precipitate was filtered, washed with ether, and recrystallized to get white powder (**4a**), yield 95%, m.p. 136 °C. IR (KBr, cm^{-1}) *ν*: 3452, 1753, 1579, 1503, 1409, 1226, 1092, 1046, 714; ^{1}H NMR (DMSO-d_6) δ ppm: 9.94 (s, 1H, N–H), 7.86 (s, 1H, –CH=N), 7.59 (d, 2H, $J = 8.6$ Hz, ArH), 7.20 (s, 1H, N–H), 7.00 (d, 2H, $J = 8.6$ Hz, ArH), 6.50 (s, 1H, N–H), 5.33–5.28 (m, 2H, 2 × CH), 5.21–5.14 (m, 2H, 2 × CH), 4.30 (dd, 1H, $J = 7.2$ Hz, 12.3 Hz, CH), 4.18 (dd, 1H, $J = 10.3$ Hz, 12.0 Hz, CH), 3.94–3.89 (m, 1H, CH), 2.09, 2.07, 2.06, 2.05 (4 × s, 4 × 3H, 4 × CH_3–C=O); ESI-MS *m/z* (%): 526 (13) [M + 1], 548 (100) [M + Na]. Anal. C, H, N for $C_{22}H_{27}N_3O_{10}S$.

*5.1.3.2. 1-(4-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl) benzylidene) semicarbazide (**4b**).* To a solution of compound **3a** (0.45 g, 1 mmol) in 15 mL of ethanol was added semicarbazide hydrochloride (0.11 g, 1 mmol) which had been adjusted with 2M NaOH to pH = 6–7. The mixture was refluxed for 6 h. After being cooled, the precipitate was filtered, washed with ether, and recrystallized to get white powder **4b**, yield 85%, m.p. 211–212 °C. IR (KBr, cm^{-1}) *ν*: 3423, 1716, 1572, 1409, 1232, 1093, 1052, 709; ^{1}H NMR (DMSO-d_6) δ ppm: 10.19 (s, 1H, N–H), 7.79 (s, 1H, –CH=N), 7.67 (d, 2H, $J = 8.7$ Hz, ArH), 6.98 (d, 2H, $J = 8.7$ Hz, ArH), 6.45 (br s, 2H, N–H), 5.62 (d, 1H, $J = 8.0$ Hz, H-1′), 5.40 (t, 1H, $J = 9.5$ Hz, CH), 5.08–4.96 (m, 2H, 2 × CH), 4.25–4.04 (m, 3H, 3 × CH), 2.01, 1.97 (2 × s, 4 × 3H, 4 × CH_3–C=O); ESI-MS *m/z* (%): 510 (3) [M + 1], 532 (100) [M + Na]. Anal. C, H, N for $C_{22}H_{27}N_3O_{11}$.

*5.1.3.3. 1-(4-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl) benzylidene) hydroxylamine (**4c**).* To a solution of compound **3a** (0.45 g, 1 mmol) in 15 mL of ethanol was added hydroxylamine hydrochloride (0.07 g, 1 mmol) which had been adjusted with 2 M NaOH to pH = 6–7. The mixture was stirred for 2 h at 45 °C. The precipitate was filtered off, the solvent was removed under reduced pressure, and the residue was washed with ether and recrystallized in ethanol to give **4c**. White powder, yield 78%, m.p. 161–162 °C. IR (KBr, cm^{-1}) *ν*: 3454, 1752, 1687, 1376, 1224, 1099, 1054, 952; ^{1}H NMR ($CDCl_3$) δ ppm: 8.07 (s, 1H, –CH=N), 7.50 (d, $J = 8.6$ Hz, 2H, ArH), 6.98 (d, 2H, $J = 8.6$ Hz, ArH), 5.31–5.27 (m, 2H, 2 × CH), 5.20–5.10 (m, 2H, 2 × CH), 4.28 (dd, 1H, $J = 7.0$ Hz, 12.3 Hz, CH), 4.17 (dd, 1H, $J = 10.3$ Hz, 12.3 Hz, CH), 3.91–3.86 (m, 1H, CH), 2.07, 2.06, 2.05, 2.04 (4 × s, 4 × 3H, 4 × CH_3–C=O); ESI-MS *m/z* (%): 468 (5) [M + 1], 490 (100) [M + Na]. Anal. C, H, N for $C_{21}H_{25}NO_{11}$.

*5.1.3.4. 1-(4-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl) benzylidene) methoxyamine (**4d**).* The procedure was the same as described in the preparation of **4c**, starting from methoxyamine hydrochloride. White solid powder, yield 68%, m.p. 132 °C. IR (KBr, cm^{-1}) *ν*: 1748, 1608, 1509, 1236, 1046, 917; ^{1}H NMR ($CDCl_3$) δ ppm: 8.00 (s, 1H, –CH=N), 7.50 (d, 2H, $J = 8.6$ Hz, ArH), 6.97 (d, 2H, $J = 8.6$ Hz, ArH), 5.31–5.26 (m, 2H, 2 × CH), 5.19–5.09 (m, 2H, 2 × CH), 4.26 (dd, 1H, $J = 7.0$ Hz, 12.3 Hz, CH), 4.17 (dd, 1H, $J = 10.4$ Hz, 12.4 Hz, CH), 3.95 (s, 3H, OCH_3), 3.91–3.85 (m, 1H, CH), 2.08, 2.06, 2.05, 2.04 (4 × s, 4 × 3H, 4 × CH_3–C=O); ESI-MS *m/z* (%): 482 (6) [M + 1], 504 (100) [M + Na]. Anal. C, H, N for $C_{22}H_{27}NO_{11}$.

5.1.4. Synthesis of 4-formylphenyl-O-β-D-glucopyranoside (*5*)

To a solution of compound **3a** (4.52 g, 10 mmol) in 20 mL of methanol was added 20 mL of methanol solution of 0.2 mol/L sodium methoxide. The mixture was stirred for 3 h at room temperature. The methanol was removed under reduced pressure, and the residue was purified by chromatography (MeOH/$CHCl_3$, 1/9, v/v) to give pure **5**. White powder, yield 96%, m.p. 157–158 °C (lit. 155–157 °C) [29]. IR (KBr, cm^{-1}) *ν*: 3455, 2930, 1691, 1590, 1415, 1240, 1066, 841, 658; ^{1}H NMR (DMSO-d_6) δ ppm: 9.87 (s, 1H, H–C=O), 7.86 (d, 2H, $J = 8.5$ Hz, ArH), 7.19 (d, 2H, $J = 8.5$ Hz, ArH), 5.43 (br s, 1H, –OH), 5.16 (br s, 1H, –OH), 5.04 (d, 1H, $J = 6.9$ Hz, H-1′), 4.60 (s, 1H, CH), 3.69 (d, 1H, $J = 10.9$ Hz, CH), 3.45–3.42 (m, 1H, CH), 3.32–3.25 (m, 2H, 2 × CH), 3.20 (dd, 1H, $J = 8.5$ Hz, 16.7 Hz, CH); ESI-MS *m/z* (%): 283 (26) [M − 1]. Anal. C, H for $C_{13}H_{16}O_7$.

5.1.5. Synthesis of compounds *6a–d* and *7a–d*

The procedure was the same as described in Section 5.1.3 (synthesis of **4a–d**).

*5.1.5.1. 1-(4-(O-β-D-Glucopyranosyl) benzylidene) thiosemicarbazide (**6a**).* Light orange powder, yield 76%, m.p. 212 °C. IR (KBr, cm^{-1}) *ν*: 3425, 1569, 1500, 1412, 1201, 1076, 956; ^{1}H NMR (DMSO-d_6) δ ppm: 11.31 (s, 1H,

N–H), 8.10 (s, 1H, N–H), 7.97 (s, 1H, –CH=N), 7.92 (s, 1H, N–H), 7.71 (d, 2H, $J = 8.5$ Hz, ArH), 7.01 (d, 2H, $J = 8.5$ Hz, ArH), 4.92 (d, 1H, $J = 7.0$ Hz, H-1′), 3.69–3.67 (m, 1H, CH), 3.46–3.43 (m, 1H, CH), 3.35–3.15 (m, 4H, 4 × CH); ESI-MS *m/z* (%): 358 (5) [M + 1], 380 (100) [M + Na]. Anal. C, H, N for $C_{14}H_{19}N_3O_6S$.

*5.1.5.2. 1-(4-(O-β-D-Glucopyranosyl) benzylidene) semicarbazide (**6b**).* Light yellow powder, yield 78%, m.p. 207–208 °C. IR (KBr, cm^{-1}) *ν*: 3381, 2917, 1676, 1579, 1507, 1457, 1243, 1172, 1034, 926, 872; ^{1}H NMR (DMSO-d_6) *δ* ppm: 10.09 (s, 1H, N–H), 7.77 (s, 1H, –CH=N), 7.62 (d, 2H, $J = 8.5$ Hz, ArH), 7.00 (d, 2H, $J = 8.5$ Hz, ArH), 6.42 (br s, 2H, N–H), 4.89 (d, 1H, $J = 7.0$ Hz, H-1′), 3.69–3.67 (m, 1H, CH), 3.48–3.42 (m, 1H, CH), 3.35–3.15 (m, 4H, 4 × CH); ESI-MS *m/z* (%): 342 (3) [M + 1], 366 (100) [M + Na]. Anal. C, H, N for $C_{14}H_{19}N_3O_7$.

*5.1.5.3. 1-(4-(O-β-D-Glucopyranosyl) benzylidene) hydroxylamine (**6c**).* Light yellow powder, yield 80%, m.p. 162–163 °C. IR (KBr, cm^{-1}) *ν*: 3384, 2913, 1606, 1513, 1242, 1082, 965; ^{1}H NMR (DMSO-d_6) *δ* ppm: 11.02 (br s, 1H, –OH), 8.05 (s, 1H, –CH=N), 7.50 (d, 2H, $J = 8.5$ Hz, ArH), 7.02 (d, 2H, $J = 8.5$ Hz, ArH), 4.89 (d, 1H, $J = 7.0$ Hz, H-1′), 3.36–3.31 (m, 2H, 2 × CH), 3.29–3.23 (m, 2H, 2 × CH), 3.19–3.13 (m, 2H, 2 × CH); ESI-MS *m/z* (%): 300 (3) [M + 1], 322 (100) [M + Na]. Anal. C, H, N for $C_{13}H_{17}NO_7$.

*5.1.5.4. 1-(4-(O-β-D-Glucopyranosyl) benzylidene) methoxyamine (**6d**).* White powder, yield 68%, m.p. 103–104 °C. IR (KBr, cm^{-1}) *ν*: 3355, 2930, 1608, 1509, 1236, 1046, 916; ^{1}H NMR (DMSO-d_6) *δ* ppm: 8.14 (s, 1H, –CH=N), 7.52 (d, 2H, $J = 8.5$ Hz, ArH), 7.03 (d, 2H, $J = 8.4$ Hz, ArH), 4.91 (d, 1H, $J = 7.3$ Hz, H-1′), 3.84 (s, 3H, OCH_3), 3.68 (br s, 0.5H, –OH), 3.65 (br s, 0.5H, –OH), 3.47–3.41 (m, 1H, CH), 3.35–3.17 (m, 5H, 5 × CH); ESI-MS *m/z* (%): 314 (8) [M + 1], 336 (100) [M + Na]. Anal. C, H, N for $C_{14}H_{19}NO_7$.

*5.1.5.5. 1-(4-(2,3,4-Tri-O-benzoyl-β-D-ribopyranosyl) benzylidene) thiosemicarbazide (**7a**).* Light yellow powder, yield 88%, m.p. 155–156 °C. IR (KBr, cm^{-1}) *ν*: 3429, 3324, 1726, 1597, 1270, 1098, 711; ^{1}H NMR (DMSO-d_6) *δ* ppm: 11.35 (s, 1H, N–H), 8.14 (s, 1H, N–H), 7.97 (s, 1H, –CH=N), 7.94 (s, 1H, N–H), 7.88–7.84 (m, 6H, ArH), 7.75 (d, 2H, $J = 7.9$ Hz, ArH), 7.67–7.58 (m, 3H, ArH), 7.50–7.40 (m, 6H, ArH), 7.03 (d, 2H, $J = 8.0$ Hz, ArH), 5.93 (t, 1H, $J = 3.5$ Hz, CH), 5.78 (d, 1H, $J = 5.1$ Hz, CH), 5.66–5.60 (m, 2H, 2 × CH), 4.25 (d, 1H, $J = 12.9$ Hz, CH), 4.07 (d, 1H, $J = 13.3$ Hz, CH); ESI-MS *m/z* (%): 640 (4) [M + 1], 662 (18) [M + Na]. Anal. C, H, N for $C_{34}H_{29}N_3O_8S$.

*5.1.5.6. 1-(4-(2,3,4-Tri-O-benzoyl-β-D-ribopyranosyl) benzylidene) semicarbazide (**7b**).* Light yellow powder, yield 86%, m.p. 133–134 °C. IR (KBr, cm^{-1}) *ν*: 3476, 1728, 1687, 1266, 1101, 711; ^{1}H NMR (DMSO-d_6) *δ* ppm: 10.18 (s, 1H, N–H), 7.92 (d, 4H, $J = 7.8$ Hz, ArH), 7.82 (s, 1H, –CH=N), 7.75 (dd, 4H, $J = 4.7$ Hz, 13.3 Hz, ArH), 7.63–7.52 (m, 4H, ArH), 7.42–7.32 (m, 6H, ArH), 7.22 (d, $J = 8.1$ Hz, 2H, ArH), 6.45 (br s, 2H, N–H), 6.11 (t, 1H, $J = 3.5$ Hz, CH), 5.96 (d, 1H, $J = 5.1$ Hz, CH), 5.68–5.60 (m, 2H, 2 × CH), 4.32 (d, 1H, $J = 12.6$ Hz, CH), 4.13 (d, 1H, $J = 11.8$ Hz, CH); FAB-MS *m/z* (%): 624 (5) [M + 1], 646 (15) [M + Na]. Anal. C, H, N for $C_{34}H_{29}N_3O_9$.

*5.1.5.7. 1-(4-(2,3,4-Tri-O-benzoyl-β-D-ribopyranosyl) benzylidene) hydroxylamine (**7c**).* Light orange foam-like solid, yield 88%, m.p. 107–108 °C. IR (KBr, cm^{-1}) *ν*: 3443, 1727, 1271, 1097, 711; ^{1}H NMR ($CDCl_3$) *δ* ppm: 8.01 (s, 1H, –CH=N), 7.96 (t, 4H, $J = 6.6$ Hz, ArH), 7.80 (d, 2H, $J = 8.0$ Hz, ArH), 7.49–7.39 (m, 5H, ArH), 7.23–7.16 (m, 6H, ArH), 7.06 (d, 2H, $J = 8.4$ Hz, ArH), 5.93 (t, 1H, $J = 3.2$ Hz, CH), 5.78 (d, 1H, $J = 4.6$ Hz, CH), 5.66–5.60 (m, 2H, 2 × CH), 4.25 (d, 1H, $J = 12.9$ Hz, CH), 4.07 (d, 1H, $J = 13.3$ Hz, CH); ESI-MS *m/z* (%): 582 (5) [M + 1], 604 (100) [M + Na]. Anal. C, H, N for $C_{33}H_{27}NO_9$.

*5.1.5.8. 1-(4-(2,3,4-Tri-O-benzoyl-β-D-ribopyranosyl) benzylidene) methoxyamine (**7d**).* Light yellow powder, yield 76%, m.p. 78–79 °C. IR (KBr, cm^{-1}) *ν*: 1728, 1264, 1101, 1062, 712; ^{1}H NMR ($CDCl_3$) *δ* ppm: 8.03–7.82 (m, 5H, –CH=N, 4 × ArH), 7.78 (d, 2H, $J = 7.9$ Hz, ArH), 7.45–7.38 (m, 5H, ArH), 7.22–7.18 (m, 6H, ArH), 7.04 (d, 2H, $J = 8.0$ Hz, ArH), 5.93–5.91 (m, 1H, CH), 5.76 (d, 1H, $J = 5.1$ Hz, CH), 5.64–5.60 (m, 2H, 2 × CH), 4.23 (d, 1H, $J = 12.3$ Hz, CH), 4.05 (d, 1H, $J = 12.7$ Hz, CH), 3.86 (s, 3H, OCH_3); ESI-MS *m/z* (%): 596 (8) [M + 1], 618 (100) [M + Na]. Anal. C, H, N for $C_{34}H_{29}NO_9$.

*5.1.6. Synthesis of 4-formylphenyl-O-β-D-ribopyranoside (**8**)*

The procedure was the same as described in Section 5.1.4 (synthesis of **5**). Light yellow solid, yield 85%, m.p. 143–144 °C. IR (KBr, cm^{-1}) *ν*: 3405, 1683, 1582, 1415, 1240, 1109, 1000, 825, 650; ^{1}H NMR (DMSO-d_6) *δ* ppm: 9.85 (s, 1H, H–C=O), 7.84 (d, 2H, $J = 8.4$ Hz, ArH), 7.17 (d, 2H, $J = 8.5$ Hz, ArH), 5.41 (d, 1H, $J = 5.2$ Hz, H-1′), 5.22 (br s, 1H, –OH), 5.03 (br s, 1H, –OH), 3.89 (s, 1H, CH), 3.69–3.59 (m, 4H, 4 × CH); ^{13}C NMR (DMSO-d_6) *δ* ppm: 192.5, 162.5, 133.0, 132.6, 131.4, 117.4, 116.8, 99.1, 71.0, 69.0, 68.6, 65.0; FAB-MS *m/z* (%): 255 (3) [M + 1], 277 (15) [M + Na]. Anal. C, H for $C_{12}H_{14}O_6$.

5.2. Biological evaluation

5.2.1. Inhibition of AChE and BuChE

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from electric eel), butylcholinesterase (BuChE, E.C. 3.1.1.8, from horse serum), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), butylthiocholine chloride (BTC) and acetylthiocholine chloride (ATC) were purchased from Sigma–Aldrich (Steinheim, Germany). Galanthamine hydrobromide was obtained from Sigma–Aldrich (Sintra, Portugal).

The assay was performed as described in the following procedure [25]. Five different concentrations (normally in the

range of 10^{-4}–10^{-9} M) of each compound were measured at 412 nm for 1 min, each concentration in triplicate.

For buffer preparation, 0.1 M dipotassium hydrogen phosphate was adjusted to pH = 8.0 with 1 M potassium dihydrogen phosphate. Enzyme solutions were prepared to give 2.5 units/mL in 1.5 mL aliquots. Furthermore, 0.01 M DTNB solution, 0.075 M ATC and BTC solutions, respectively, were used. A cuvette containing 840 μL of phosphate buffer, 10 μL of the respective enzyme, 50 μL of DTNB and 50 μL of the test compound solution was allowed to pre-incubate for 15 min at 37 °C, and the reaction was started by addition of 50 μL of the substrate solution (ATC/BTC). The reaction was monitored by measuring the absorbance at 412 nm. For the reference value, 50 μL of DMSO replaced the test compound solution. For determining the blank value, additionally 10 μL of buffer replaced the enzyme solution.

5.2.2. Kinetic characterization of AChE inhibition

Kinetic characterization of AChE was performed using a reported method [30]. Five different concentrations of AChE and inhibitors were mixed in the assay buffer (pH = 8.0), containing 40 μM of 5,5′-dithio-bis (2-nitrobenzoic acid), 0.035 units/mL AChE, and 550 μM acetylthiocholine chloride. Test compound was added into the assay solution and pre-incubated with the enzyme at 37 °C for 15 min, followed by the addition of substrate. Kinetic characterization of the hydrolysis of acetylthiocholine catalyzed by AChE was done spectrometrically at 412 nm. A parallel control with no inhibitor in the mixture, allowed adjusting activities to be measured at various times.

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