

## NEW MULTIFUNCTIONAL AGENTS AND THEIR INHIBITORY EFFECTS ON THE ACETYL CHOLINESTERASE ENZYME

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A novel series of 2-((-1-substituted phenyl-1*H*-tetrazol-5-yl)thio)-2,3-dihydro-1*H*-inden-1-one compounds were designed, synthesized, and evaluated as multi-potent anti-Alzheimer drug candidates. First, treatment of various organic isothiocyanates with sodium azide in the presence of pyridine gave corresponding 1-substituted phenyl-1*H*-tetrazol-5-thiol compounds. Then, novel 2-((-1-substituted phenyl-1*H*-tetrazol-5-yl)thio)-2,3-dihydro-1*H*-inden-1-one compounds were synthesized by treatment of 2,3-dihydro-1*H*-inden-1-one with the 1-substituted phenyl-1*H*-tetrazol-5-thiol in the presence of I<sub>2</sub>. The synthesized compounds were characterized by spectroscopic methods. The inhibitory effects of the synthesized compounds on the acetyl cholinesterase enzyme (AChE) were then tested. An *o*-iodo substituent displayed higher activity compared to the other analogs. The *o*-iodo substituent showed a mixed-type of the AChE with an IC<sub>50</sub> value of 1.75 µM. For the studied compounds all IC<sub>50</sub> values for AChE were in the micromolar range.

**Key words:** Alzheimer's disease; tetrazole; inhibition

## НОВИ МУЛТИФУНКЦИОНАЛНИ СРЕДСТВА И НИВНО ИНХИБИТОРНО ДЕЈСТВО ВРЗ АЦЕТИЛ ХОЛИНЕСТЕРАЗНИОТ ЕНЗИМ

Беше дизајнирана, синтетизирана и евалуирна една нова серија соединенија на 2-((-1-супституирани фенил-1*H*-тетразол-5-ил)тио)-2,3-дихидро-1*H*-инден-1-он како потенцијални мултипотентни кандидати за лекови против Алцхајмерова болест. Најпрво, третирањето на разни органски изотиоцијанати со натриумазид во присуство на пиридин ги дава соодветните соединенија 1-супституиран фенил-1*H*-тетразол-5-тиол. Потоа, беа синтетизирани новите соединенија 2-((-1-супституиран фенил-1*H*-тетразол-5-ил)тио)-2,3-дихидро-1*H*-инден-1-он со третирање на 2,3-дихидро-1*H*-инден-1-он со 1-супституиран фенил-1*H*-тетразол-5-тиол во присуство на I<sub>2</sub>. Синтетизираните соединенија беа карактеризирани со спектроскопски методи. Беа тестирани инхибиторните ефекти на синтетизираните соединенија врз ацетил холинестеразниот ензим (AChE). Најголема активност во споредба со сите аналози покажа *o*-јодо супституентот. Овој супституент покажа мешан тип на AChE со вредност на IC<sub>50</sub> од 1,75 µM. За сите испитувани соединенија сите вредности на IC<sub>50</sub> беа во микромоларен опсег.

**Клучни зборови:** Алцхајмерова болест; тетразол; инхибиција

### 1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease and the main cause of dementia in the elderly (especially people >65 years-old). Although the definite causes of AD remain unknown, low levels of acetylcholine (ACh), the inflamma-

tion of neurons, Tau-protein aggregation and β-amyloid (Aβ) deposits are thought to play definitive roles in this disease [1]. There are currently two major hypotheses regarding AD, including (1) the cholinergic hypothesis, and (2) the amyloid hypothesis. AD drug therapy is based on the cholinergic hypothesis. The cholinergic hypothesis

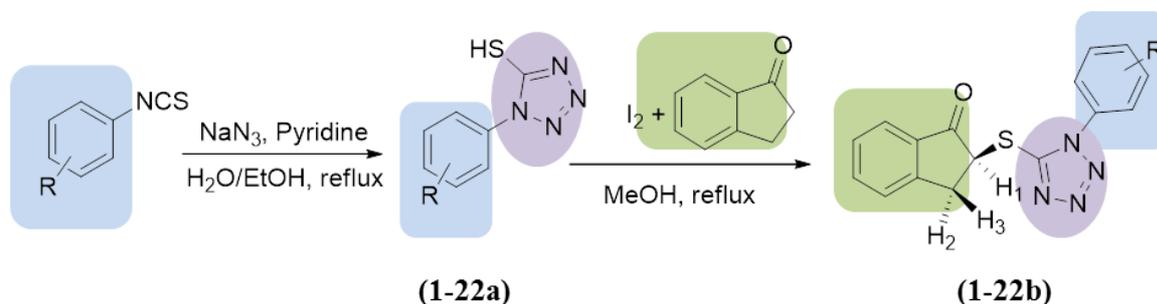
includes the following four acetyl cholinesterase (AChE) inhibitors: tacrine, rivastigmine, galanthamine and donepezil [2, 3]. However, these AChE inhibitors have side effects, including hepatotoxicity, gastrointestinal tract excitement and hallucination. Therefore, the identification of new and better AChE inhibitors remains an important goal.

Some research groups including Badia et al., Roman et al., and Camps et al., have synthesized anticholinesterasic drugs. These drugs show high selectivity and potent inhibitory action on AChE in both in vitro and ex vivo studies [4, 5]. Coumarin has also been shown to be effective for treating cognitive deficit and delaying the neurodegenerative process (6). N. Guzior et al. have developed a new series of AChE inhibitors that were designed as dual binding site cholinesterase inhibitors able to bind to both the catalytic anionic site (CAS) and the peripheral anionic site (PAS) [6]. Guzior et al. developed a new series of AChE inhibitors that were designed as dual binding site cholinesterase (ChE) inhibitors able to bind to both the catalytic anionic site (CAS) and the peripheral anionic site (PAS) [6].

Some reviews indicate that Donepezil is a new class of ChE inhibitor having N-benzylpiperidine and indanone moiety [7, 3]. Donepezil-like multifunctional agents have been widely employed to improve the performance of AChE inhibitors.

Tetrazole derivatives, including indanone have attracted interest as excellent candidates to improve the performance of AChE inhibitors. Tetrazole chemistry has steadily developed since the 1980s. Tetrazoles are important heterocyclic compounds, known as the nitrogen analogs of the carboxylic acids [8]. Tetrazoles possess various

biological activities, such as antibacterial, antituberculosis, antifungal, antiviral, antiallergic, antiulcer, anti-inflammatory and anticonvulsant activities [9, 10]. Furthermore, 1,5-disubstituted tetrazoles, such as cardiazole, are known to have a stimulating effects on the central nervous system [11–13]. The sulfur-containing 5-thio substituted tetrazole compounds have been the focus of recent research interest regarding their use in medicinal chemistry, especially for cancer, acquired immune deficiency syndrome (AIDS), hypertension treatments as well as for their HIV (Human Immunodeficiency Virus)-protease inhibitory activity [14, 15]. The 2,3 dihydroinden-containing skeleton compounds are also important in medicinal chemistry. The 2,3-dihydroinden derivatives are commonly used as antibacterial agents, anti-inflammatory agents, cerebral activators and in the treatment of amnesia [9]. In addition, the inden derivative compounds have been used as calcium channel modulators and neuroactive dopamine  $\beta$ -hydroxylase inhibitors [16–18]. Thus, there is considerable interest in the synthesis and characterization of multifunctional agents involving indanone and tetrazole (Fig. 1). Here we synthesized novel thiotetrazole-containing compounds to provide communication between the nerves, which show inhibition of the AChE. The structures of these compounds were evaluated using Fourier Transform Infrared (FT-IR), Proton Nuclear Magnetic Resonance ( $^1\text{H-NMR}$ ), Carbon-13 Nuclear Magnetic Resonance ( $^{13}\text{C-NMR}$ ) and High Resolution Mass Spectrometry (HR-MS). Finally, we examined the inhibition effect of these compounds against AChE, purified from *Electrophorus electricus* (electric eel) type V-S.



R = -H, *o*-[-F], *m*-[-F], *p*-[-F], *o*-[-Cl], *m*-[-Cl], *p*-[-Cl], *o*-[-Br], *m*-[-Br], *p*-[-Br], *o*-[-I], *m*-[-I], *p*-[-I], *o*-[-CH<sub>3</sub>], *m*-[-CH<sub>3</sub>], *p*-[-CH<sub>3</sub>], *o*-[-OCH<sub>3</sub>], *m*-[-OCH<sub>3</sub>], *p*-[-OCH<sub>3</sub>], *o*-[-NO<sub>2</sub>], *m*-[-NO<sub>2</sub>], *p*-[-NO<sub>2</sub>]

Fig. 1. Synthesis rotation of multifunctional agents containing thiotetrazole

## 2. EXPERIMENTAL

The organic isothiocyanates and other chemicals were obtained from Merck, Fluka Sigma-Aldrich, and Alfa Aesar. All melting points were determined in sealed capillaries and are reported without correction. FT-IR spectra were recorded on a Mattson 1000 spectrometer as KBr pellets or on a Thermo Nicolet 6700 spectrometer.  $^1\text{H-NMR}$  spectra were recorded on a Varian Gemini 300 (300 MHz) NMR spectrometer in dimethyl sulfoxide (DMSO)- $d^6$  and  $(\text{CHCl}_3)$ - $d^1$ .  $^{13}\text{C-NMR}$  spectra were recorded on a Varian Gemini 300 (75MHz) NMR spectrometer in (DMSO)- $d^6$  and  $(\text{CHCl}_3)$ - $d^1$ . Mass spectra measurements were recorded on a Thermo Finnigan Trace DSQ. Ultraviolet-visible (UV) spectra were recorded on a Shimadzu UV Spectrophotometer, UV 1800 Model.

### 2.1. Preparation of 1-substituted phenyl-1H-tetrazol-5-thiol from organic phenylisothiocyanates (1-22a)

Sodium azide ( $\text{NaN}_3$ , 2.5 mmol) was dissolved in 10.0 ml of  $\text{H}_2\text{O}:\text{EtOH}$  (3:1). Then, pyridine (3.0 mmol) and a substituted phenyl isothiocyanate derivative (1.0 mmol) were added to the solution. The mixture was refluxed for ~16 h, and the reaction was monitored by Thin Layer Chromatography (TLC). The EtOH of the obtained mixture was removed under vacuum. Ethyl acetate (10.0 ml) was added to the mixture, and the organic layer was extracted with water three times. The aqueous layer was collected and acidified with 3M HCl to pH 1–2. The 1-substituted phenyl-1H-tetrazole-5-thiol was precipitated as a white/yellow solid. The solid was filtered, dried, and recrystallized with ethyl acetate and *n*-hexane.

### 2.2. Preparation of 2-((1-substituted phenyl-1H-tetrazol-5-yl)thio)-2,3-dihydro-1H-inden-1-one (1-22b)

The 1-substituted phenyl-1H-tetrazole-5-thiol (1.0 mmol) was dissolved in 3.0 ml of MeOH. Then,  $\text{I}_2$  (1.5 mmol) and 2,3-dihydro-1H-inden-1-one (1.5 mmol) were added over the solution, and the mixture refluxed for ~18 h. Then, 4.5 ml of  $\text{H}_2\text{O}$  were added over the mixture and extracted with ethyl acetate ( $3 \times 10.0$  ml). The collected organic layer was extracted with 10% anhydrous sodium thiosulfate solution ( $2 \times 10.0$  ml) to remove excess  $\text{I}_2$ . The organic layer was then dried over

anhydrous sodium sulfate, filtered, and the solvent was removed under vacuum. The obtained solid was separated by column chromatography with ethyl acetate/*n*-hexane (1/3) and recrystallized with ethyl acetate/*n*-hexane.

### 2.3. Inhibition studies of Acetyl Cholinesterase Enzyme (AChE)

AChE activity measurements were performed at room temperature according to the spectrophotometric assay of Ellman et al. [20]. The principle of the Ellman method, which is a calorimetric procedure, is that the AChE enzyme catalyzes the hydrolysis reaction of Acetylthiocholine iodide (ATCh) (Fig. 2). Thyocholine, the product of this reaction, forms a yellow-colored compound with 5,5'-ditiyobis-(2-nitrobenzoic acid) (DTNB), which is an Ellman reactive and plays a role as a coloring agent. AChE enzyme activity was measured by observing the formed yellow compound (5-thio-2-nitrobenzoic acid) spectrophotometrically at a wavelength of 412 nm. ATCh was used as the substrate for all experiments. Stock inhibitor solutions were prepared in DMSO. The solvent concentration was never more than 0.4% (v/v) in the reaction mixture. The reaction mixtures for the determination of the  $IC_{50}$  values were composed of inhibitor ( $1.0 \times 10^{-4}$  M to  $1.0 \times 10^{-8}$  M), ATCh ( $1.0 \times 10^{-4}$  M), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) ( $0.67 \times 10^{-4}$  M), AChE (5.0 unit/ml), and 0.1 M phosphate buffer (pH = 8.0). After 30 min incubation, the absorbance of the mixture was monitored using a spectrophotometer at 412 nm. Control experiments were performed in the presence of DMSO. To detect the inhibition type, Lineweaver-Burke graphics were plotted using absorbance values that were obtained in the presence ( $1.0 \times 10^{-5}$ ,  $5.0 \times 10^{-6}$ , and  $1.0 \times 10^{-7}$  M) and absence of inhibitor. ATCh concentrations used were  $3.0 \times 10^{-3}$ ,  $6.0 \times 10^{-3}$ ,  $3.0 \times 10^{-2}$ , and  $6.0 \times 10^{-2}$  M. The type of inhibition was determined from the  $K_M$  and  $V_{\max}$  values found in the Lineweaver-Burke plot obtained for the indicated inhibitor concentrations. Inhibition types were determined in the absence and presence of inhibitor using  $3.0 \times 10^{-3}$  to  $6.0 \times 10^{-2}$  M ATCh from the Lineweaver-Burke plots. Each determination was repeated three times, and the mean values obtained,  $IC_{50}$  and  $K_i$  values, were calculated using GraphPad Prism 6 (GraphPad Software). To allow comparison of our results with the literature, Donepezil HCl was used as the reference compound. The  $IC_{50}$ ,  $K_i$  constants, and the inhibition

type of the synthesized compounds and Donepezil HCl are given in Table 1.

### 3. RESULTS AND DISCUSSION

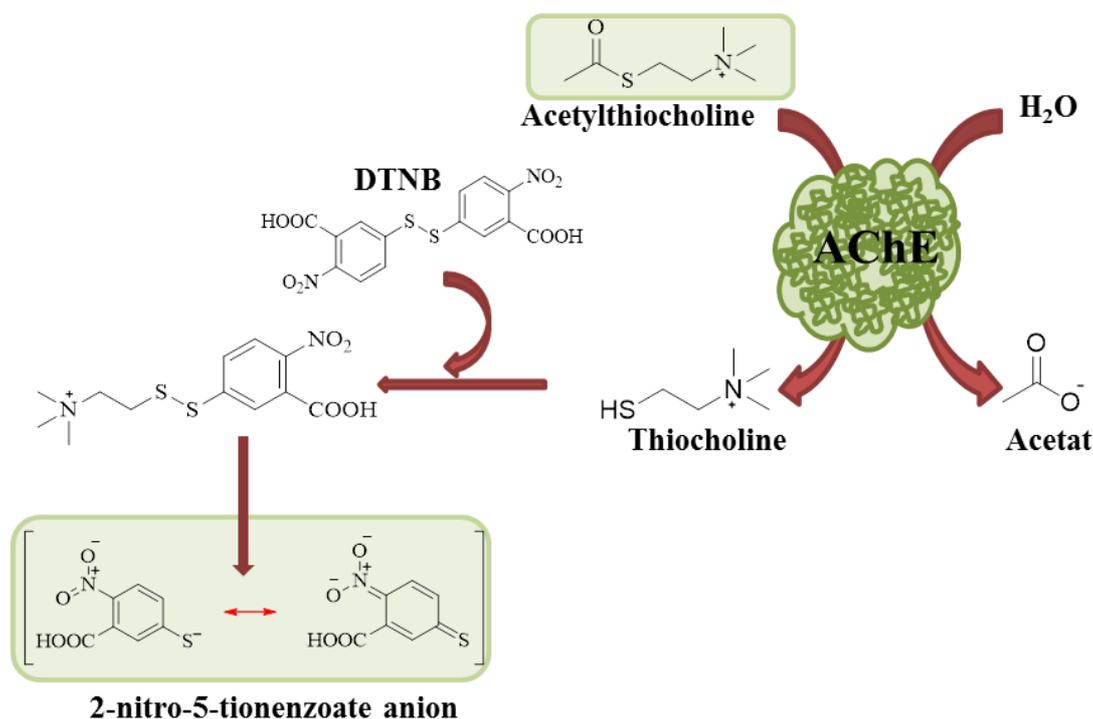
#### 3.1. Chemistry

First, we synthesized 1-substituted phenyl-1*H*-tetrazol-5-thiol by reaction of substituted phenylisothiocyanate with sodium azide in water/ethanol in the presence of pyridine (Fig. 1, 1-22a) and compared the structures of the synthesized compounds against the literature (melting point and FT-IR) [19–23]. We found that the synthesized compounds were consistent with the literature. We then synthesized novel 2-((1-substituted phenyl-1*H*-tetrazol-5-yl)thio)-2,3-dihydro-1*H*-inden-1-one compounds by reaction of 1-substituted phenyl-1*H*-tetrazol-5-thiol with 2,3-dihydro-1*H*-inden-1-one in the presence of  $I_2$  in methanol (Fig. 1, 1-22b). All of the synthesized compounds were characterized by IR,  $^1H$ -NMR,  $^{13}C$ -NMR spectra, and

mass spectrometry. In the IR spectrum, the characteristic C=O absorption band of the 1*H*-inden-1-one was observed between 1713 and 1728  $cm^{-1}$ , and the N=N characteristic absorption band was observed between 1498 and 1528  $cm^{-1}$ . The  $^1H$ -NMR and  $^{13}C$ -NMR spectra of the compounds (Fig. 1, 1-22b) are also in agreement with the proposed structures.

#### 3.2. *In vitro* inhibition of AChE

The inhibitory effects of the synthesized compounds on AChE were evaluated using the Ellman method (Fig. 2 and Fig. 3) [20–25], with Donepezil HCl as the positive control. We examined the inhibition effect of the synthesized compounds against AChE, purified from *Electrophorus electricus* (electric eel) type V-S, with an activity of 100 unit/mL. The  $IC_{50}$  values for AChE inhibition and the selectivity for AChE are summarized in Table 1. We found that all of the synthesized compounds exhibit inhibitory activities.



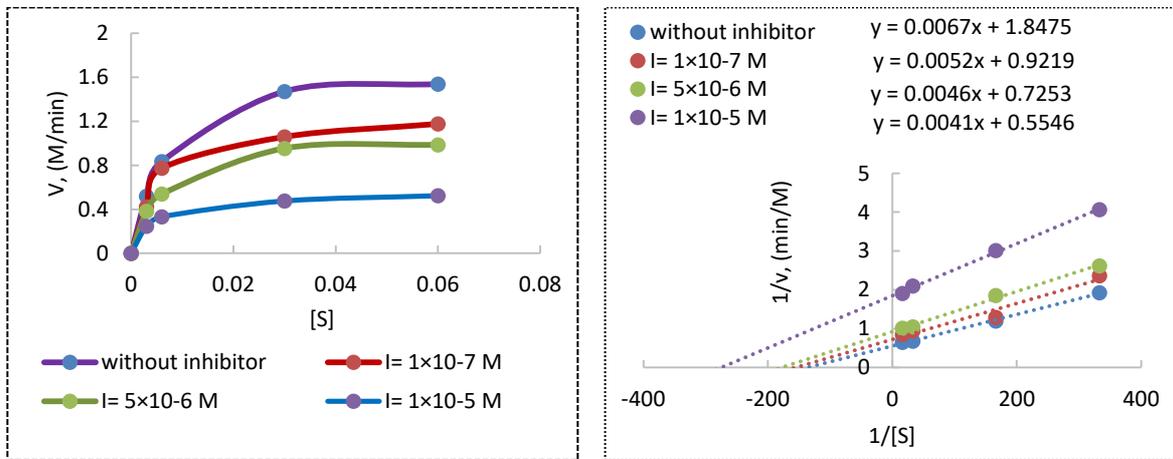
**Fig. 2.** Hydrolysis mechanism of acetylcholine (ACh) catalyzed by acetylcholinesterase (AChE); DTNB: 5,5'-dithiobis(2-nitrobenzoic acid)

Michaelis-Menten and Lineweaver-Burke graphics were plotted for all compounds (Fig. 3). When Figure 3 is examined, it can be seen that  $K_m$  and  $V_{max}$  values decrease for each compound when compared to plots without inhibitor. This type of

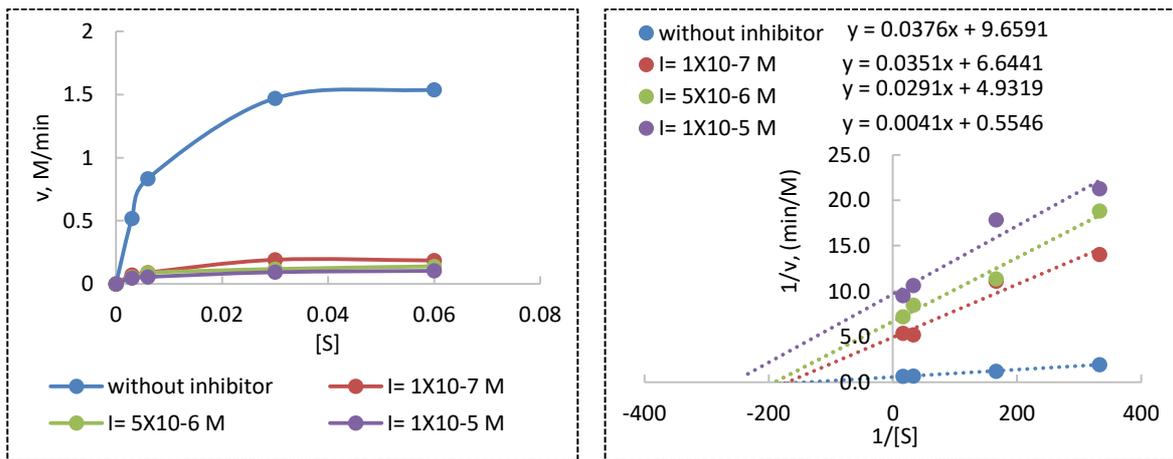
inhibition is called mixed inhibition. Because of this, the inhibition types of the compounds were detected as mixed inhibition.  $IC_{50}$  and  $K_i$  values were calculated using GraphPad Prism 6 (GraphPad Software).

Figure 3

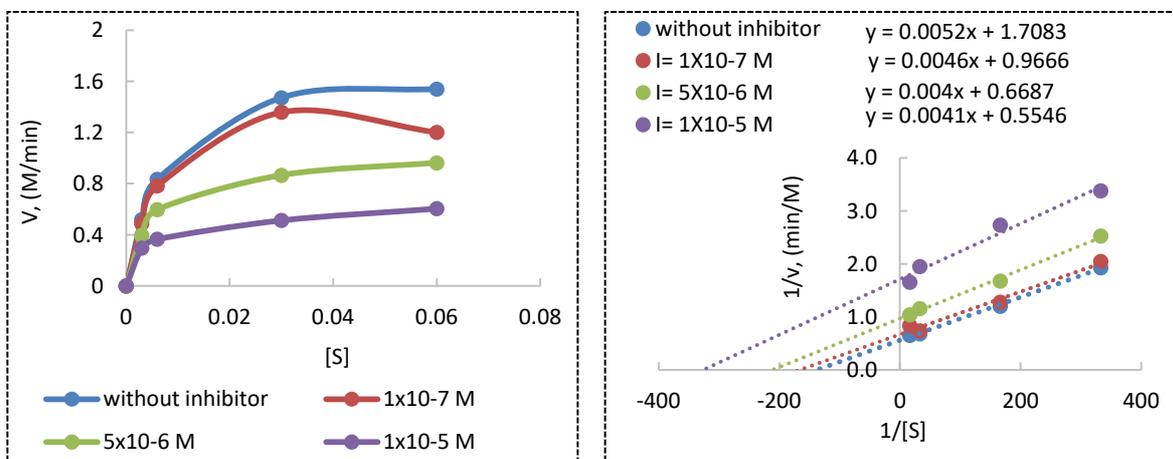
Michaelis-Menten and Lineweaver-Burke plots for 1b



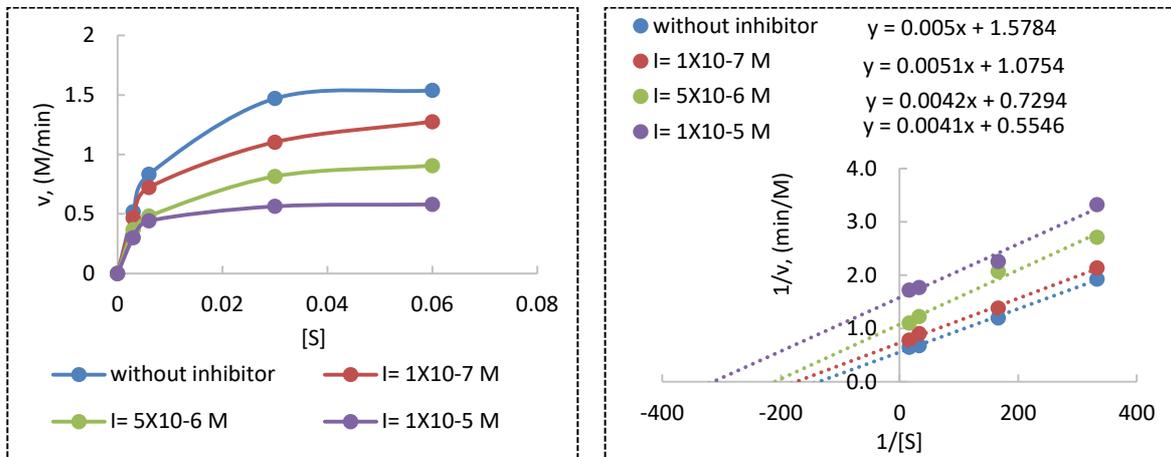
Michaelis-Menten and Lineweaver-Burke plots for 2b



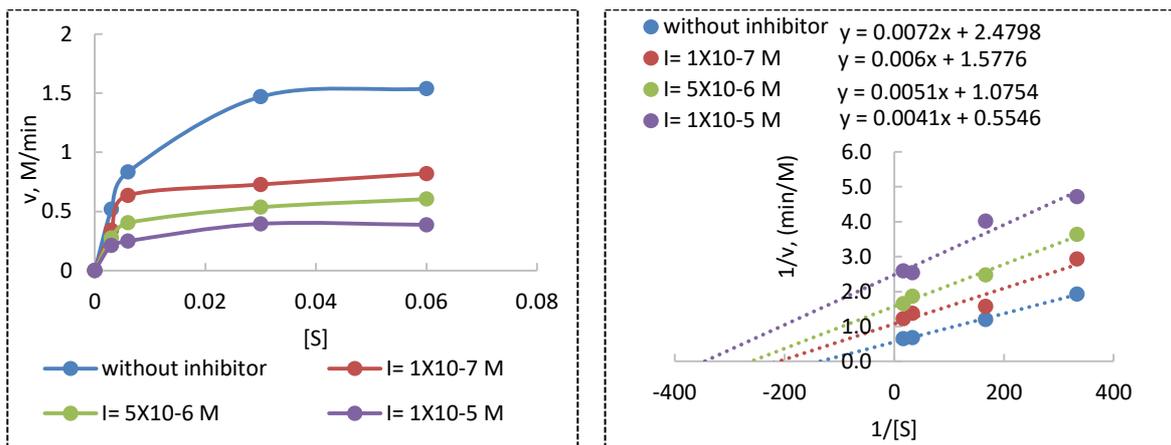
Michaelis-Menten and Lineweaver-Burke plots for 3b



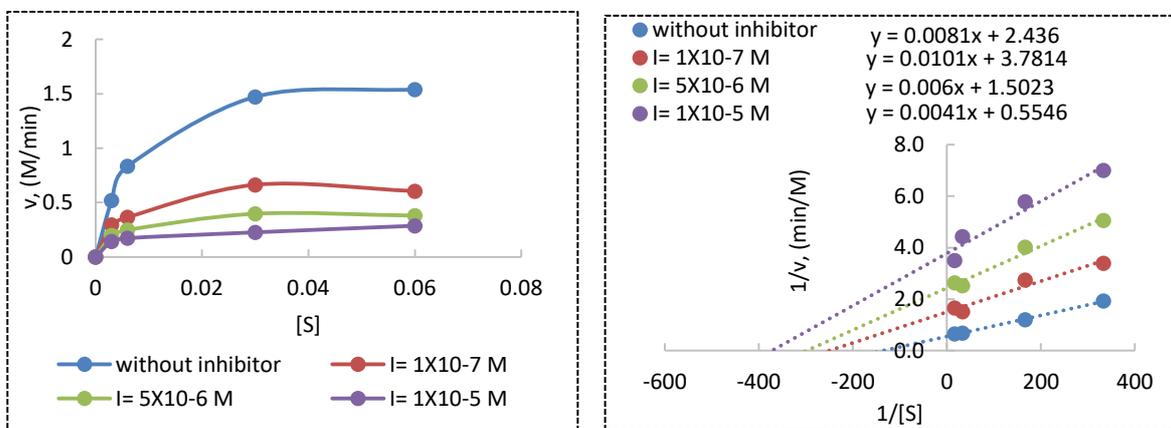
Michaelis-Menten and Lineweaver-Burke plots for 4b



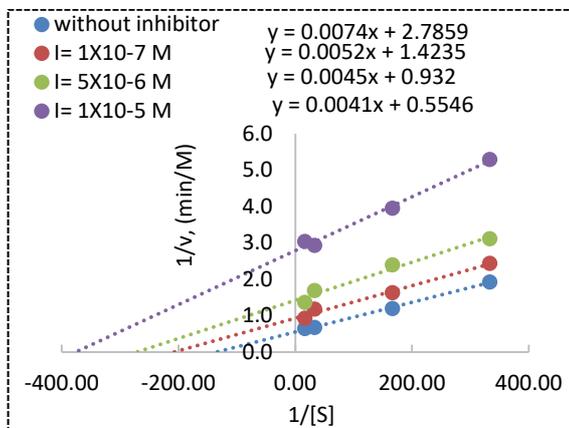
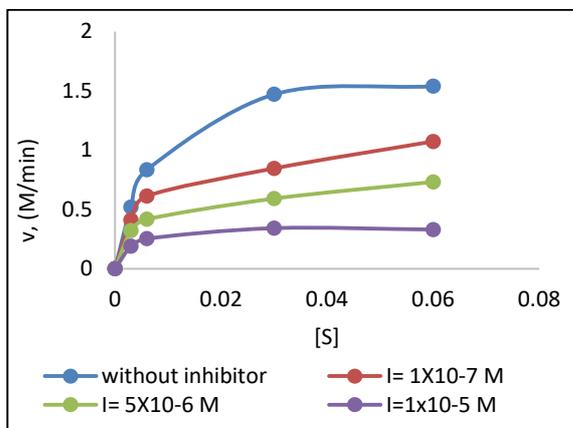
Michaelis-Menten and Lineweaver-Burke plots for 5b



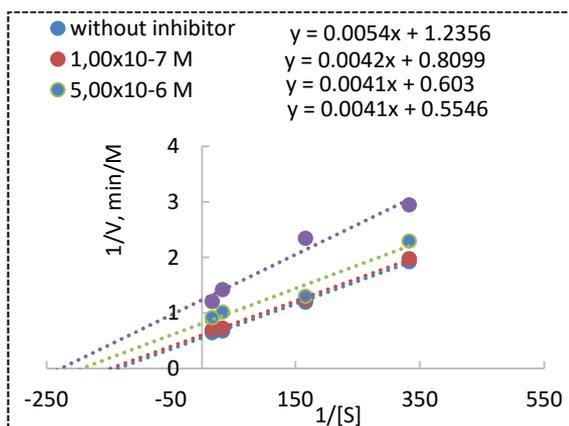
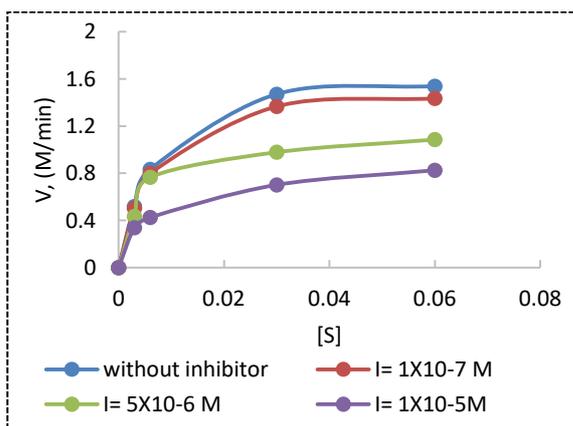
Michaelis-Menten and Lineweaver-Burke plots for 7b



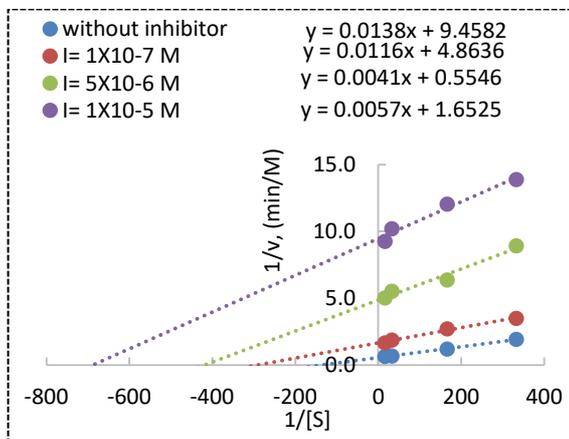
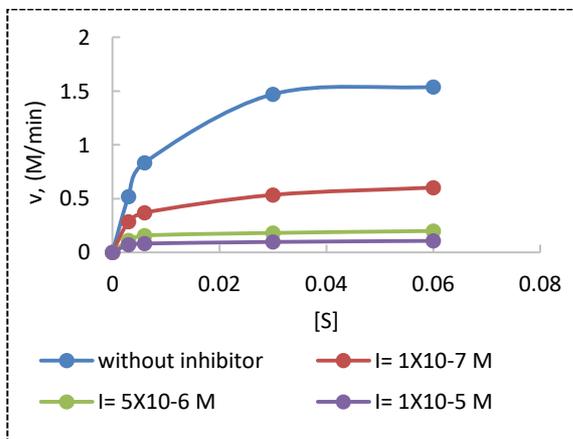
Michaelis-Menten and Lineweaver-Burke plots for 8b



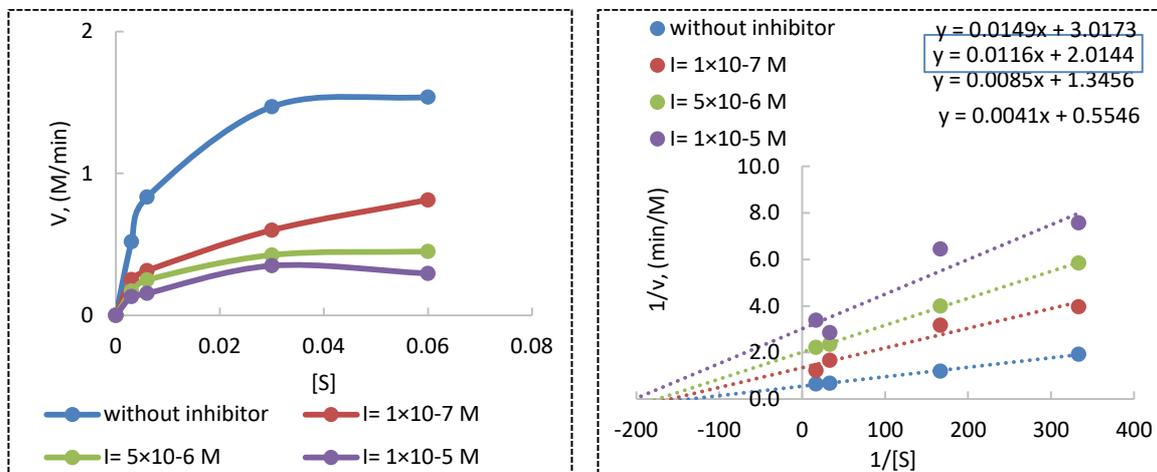
Michaelis-Menten and Lineweaver-Burke plots for 9b



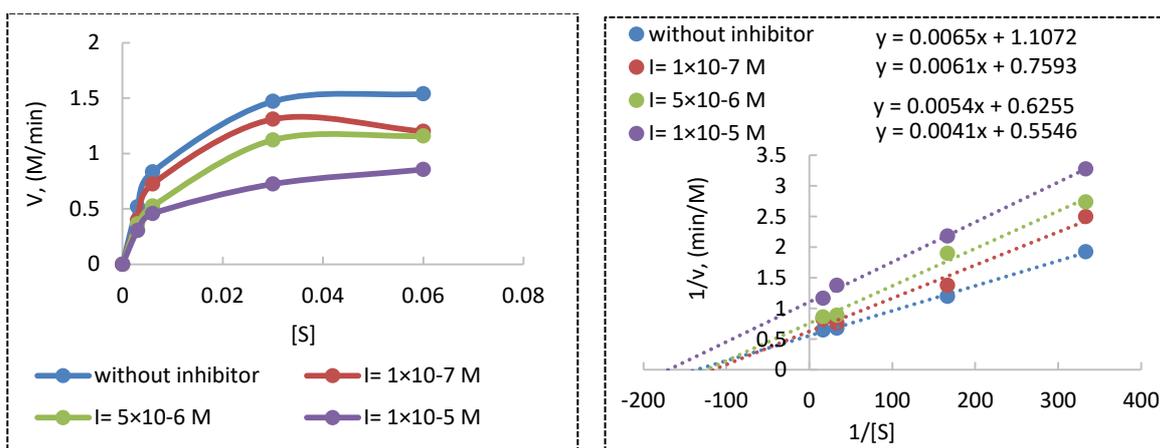
Michaelis-Menten and Lineweaver-Burke plots for 10b



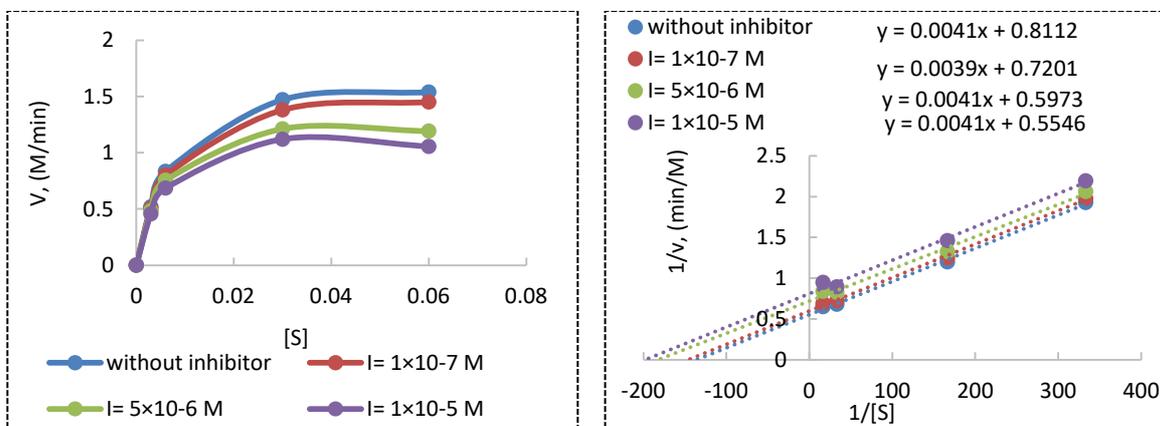
Michaelis-Menten and Lineweaver-Burke plots for 11b



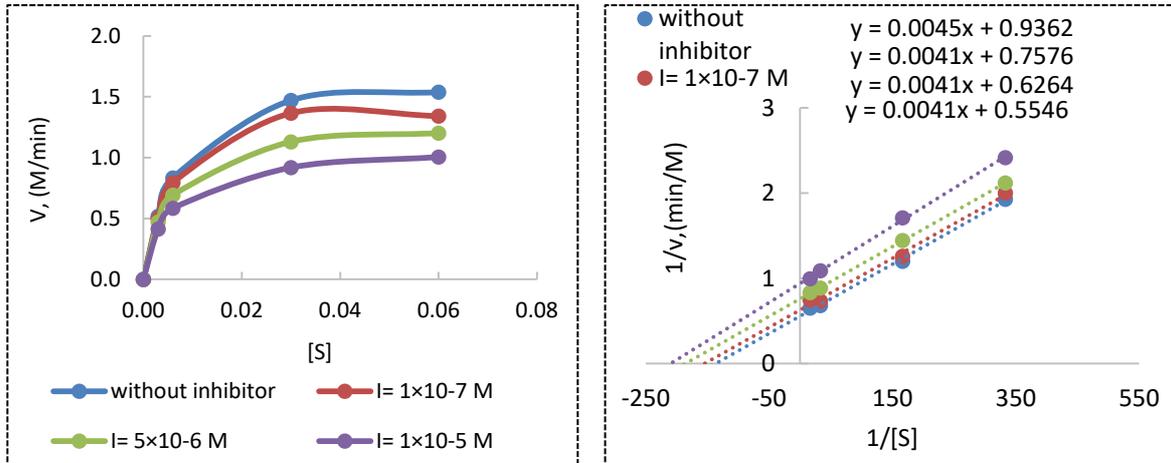
Michaelis-Menten and Lineweaver-Burke plots for 12b



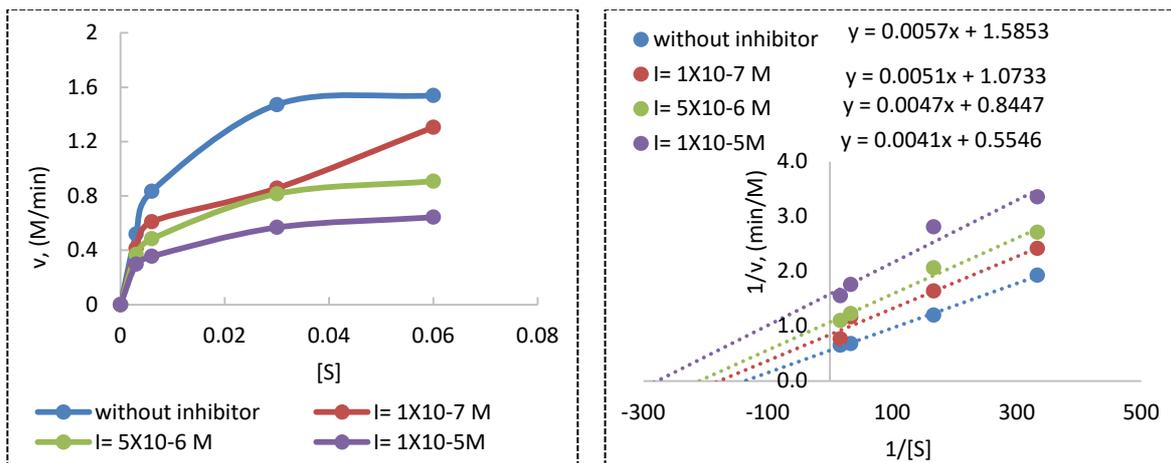
Michaelis-Menten and Lineweaver-Burke plots for 13b



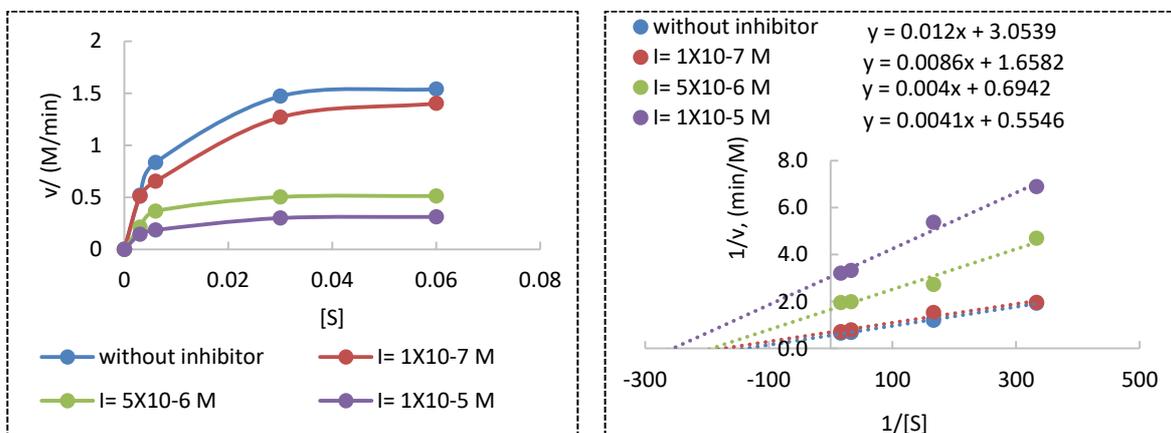
Michaelis-Menten and Lineweaver-Burke plots for 14b



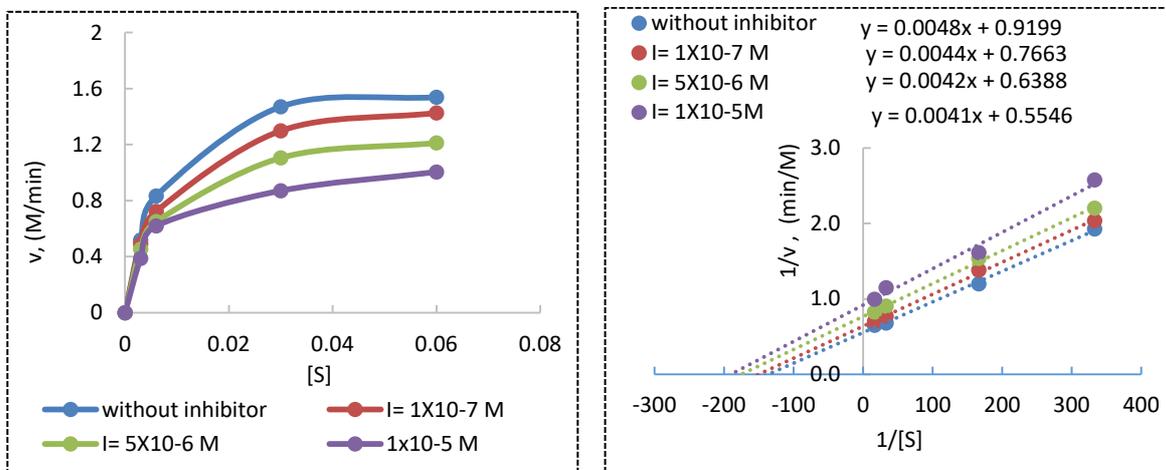
Michaelis-Menten and Lineweaver-Burke plots for 15b



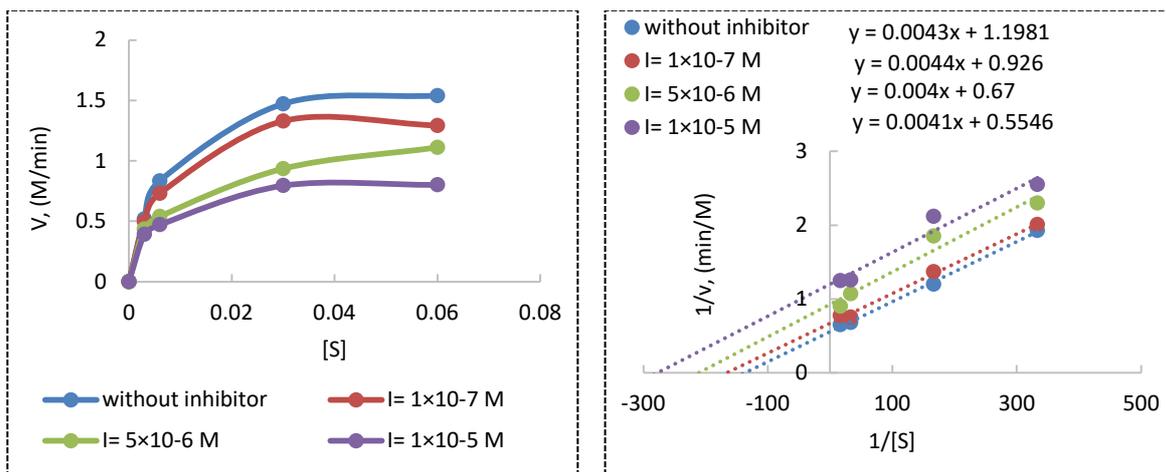
Michaelis-Menten and Lineweaver-Burke plots for 16b



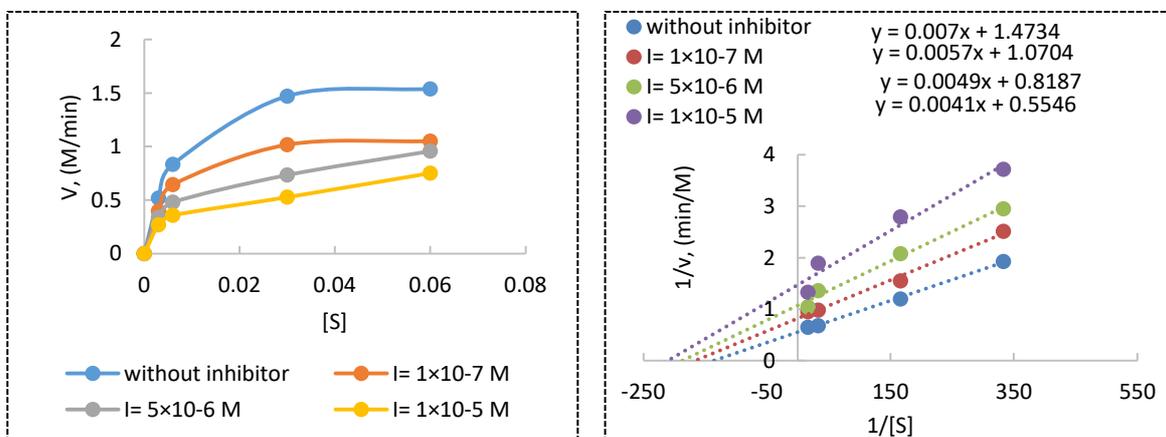
Michaelis-Menten and Lineweaver-Burke plots for 17b



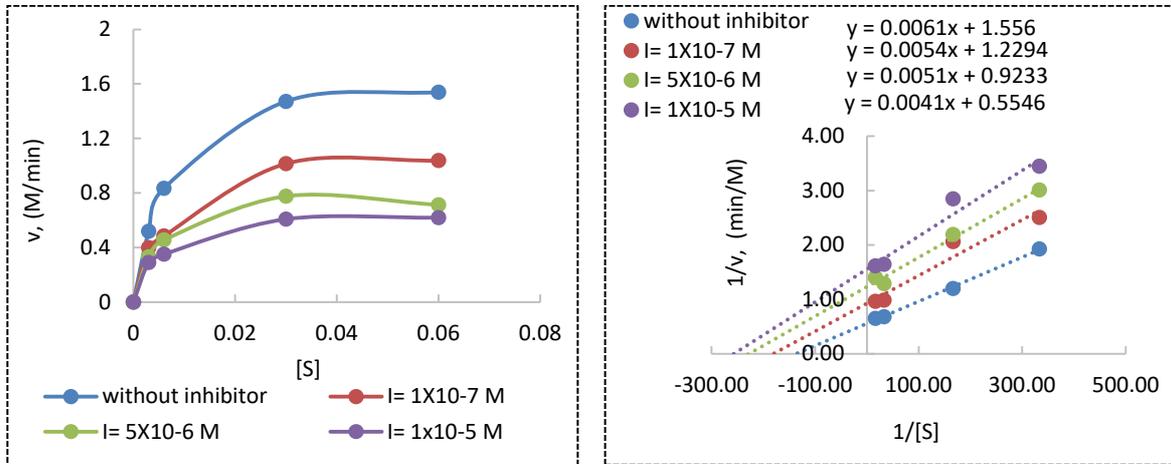
Michaelis-Menten and Lineweaver-Burke plots for 18b



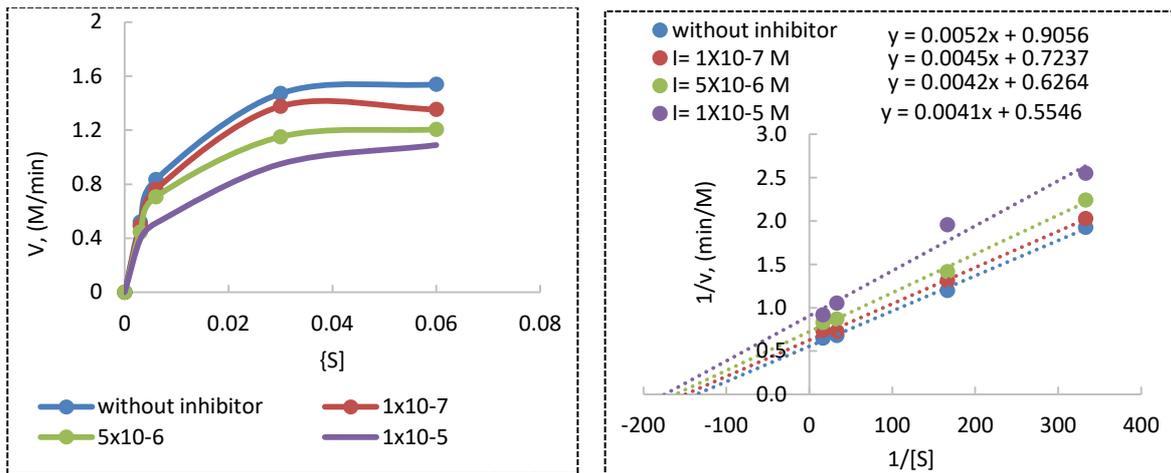
Michaelis-Menten and Lineweaver-Burke plots for 19b



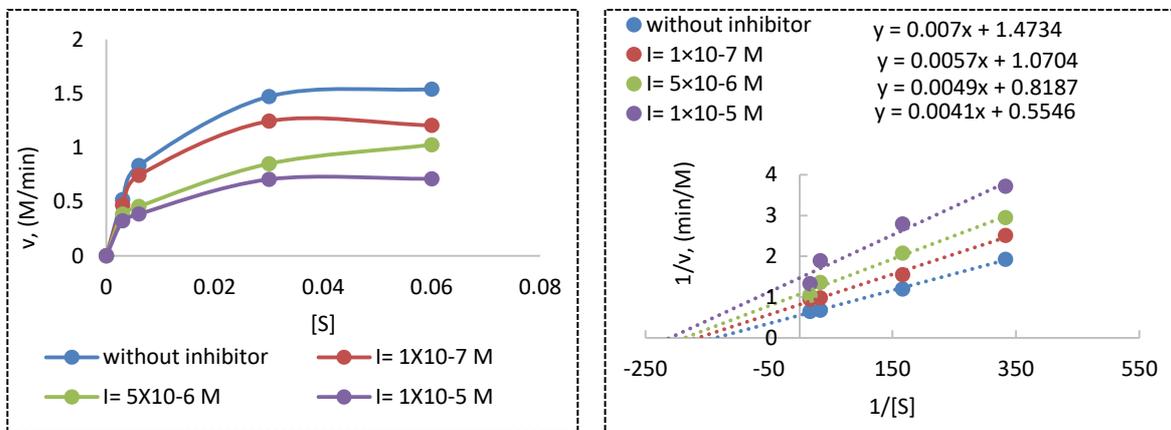
Michaelis-Menten and Lineweaver-Burke plots for 20b



Michaelis-Menten and Lineweaver-Burke plots for 21b



Michaelis-Menten and Lineweaver-Burke plots for 22b



## Michaelis-Menten and Lineweaver-Burke plots for donepezil

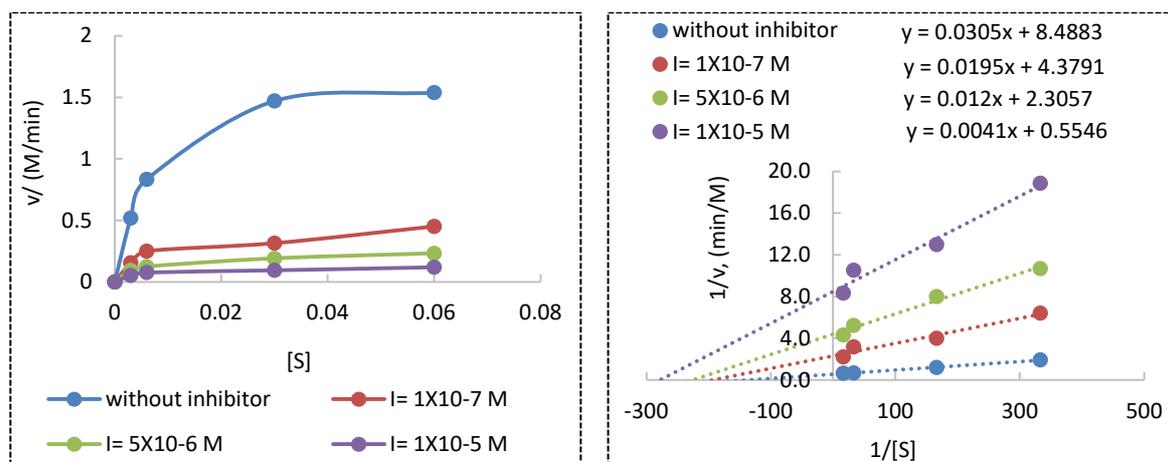


Fig. 3. Michaelis-Menten and Lineweaver-Burk plots for all compounds and Donepezil (except compound 6)

The compounds that involved electron-withdrawing and electron-donating groups exhibited higher inhibitory activities against AChE than the precursor compound **1b**. Among the series of 22 compounds, **11b** ( $IC_{50} = 1.75 \mu\text{M}$ ) was the most active. Compound **9b** ( $IC_{50} = 1.84 \mu\text{M}$ ) was the second most active compound of the series (Fig. 4). Compound **13b** ( $IC_{50} = 7.56 \mu\text{M}$ ), which is an iodine substituent, showed less activity compared to **22b**.

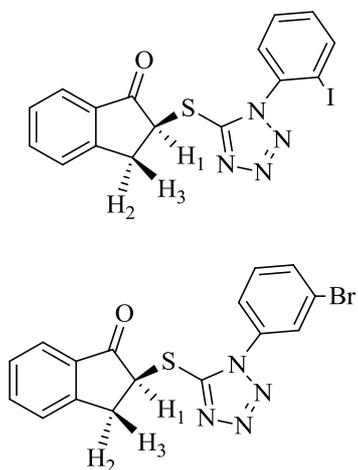


Fig. 4. Structure of compounds with the highest inhibition (11b, 9b)

According to structure-activity relationships (SAR) studies [26], hydrophobic binding is important for inhibitors targeting the amino acid residues found in the structure of AChE (Trp 83, Gly 149, Tyr 162, Glu 237, and His 480). The hydrophobic

effect plays an important role in the formation of hydrogen bonds between AChE and its inhibitors [27–29]. We know that halogen substituents (especially –Br and –I) increase the lipophilicity (or hydrophobic effect) of the molecules due to their London dispersion forces. Iodine and bromine atoms exert a greater hydrophobic effect than the studied halogens (–F and –Cl). Thus, the hydrophobic effect may account for the inhibitory activities of compounds **11b** and **9b** against AChE. The inhibitory activities for the compounds with –Br, –F, and –CH<sub>3</sub> substituents can be ranked as  $o- > p- > m-$ . The lowest and highest  $IC_{50}$  values were observed for compounds **11b** and **13b**, respectively.

### 3.2.1. Electron-withdrawing groups

The  $IC_{50}$  values of the compounds involving –F and –Br increase as follows:  $o- < p- < m-$ . Despite that, values of structures involving –I and –NO<sub>2</sub> increase as follows:  $p- < o- < m-$ . Due to the insolubility of **6b**, a comparison was made between **5b** and **7b**. Compound **5b** exhibited higher inhibitory activities against AChE than **7b**. Among the halogen groups having a  $p-$  position, the inhibition increased with increasing electronegativity.

### 3.2.2. Electron donating groups

The inhibition effect of the compounds with a methyl substituent increases as follows:  $o- < p- < m-$ . The  $IC_{50}$  values of compounds involving –OCH<sub>3</sub> increased as follows:  $m- < p- < o-$  (Table 1). The inhibitory activity against AChE for  $m-$

OCH<sub>3</sub> was higher than that of the *m*-CH<sub>3</sub> containing compound.

Table 1

*IC*<sub>50</sub>, *K*<sub>i</sub> constants and the inhibition type of synthesized compounds and donepezil.HCl

Compounds	R	Inhibition Type	<i>IC</i> <sub>50</sub> [x10 <sup>-6</sup> M]	<i>K</i> <sub>i</sub>
1b	-H	Mixed	8.28	24.11
2b	<i>o</i> -[F]	Mixed	4.04	0.65
3b	<i>m</i> -[F]	Mixed	2.63	24.25
4b	<i>p</i> -[F]	Mixed	3.04	14.22
5b	<i>o</i> -[Cl]	Mixed	2.79	14.30
*6b	<i>m</i> -[Cl]	–	–	–
7b	<i>p</i> -[Cl]	Mixed	3.89	3.80
8b	<i>o</i> -[Br]	Mixed	5.23	9.69
9b	<i>m</i> -[Br]	Mixed	1.84	34.65
10b	<i>p</i> -[Br]	Mixed	4.07	3.49
11b	<i>o</i> -[I]	Mixed	1.75	51.70
12b	<i>m</i> -[I]	Mixed	3.58	73.80
13b	<i>p</i> -[I]	Mixed	10.96	132.90
14b	<i>o</i> -[NO <sub>2</sub> ]	Mixed	8.10	12.87
15b	<i>m</i> -[NO <sub>2</sub> ]	Mixed	2.86	15.83
16b	<i>p</i> -[NO <sub>2</sub> ]	Mixed	6.67	6.14
17b	<i>o</i> -[OCH <sub>3</sub> ]	Mixed	2.21	108.70
18b	<i>m</i> -[OCH <sub>3</sub> ]	Mixed	7.37	52.87
19b	<i>p</i> -[OCH <sub>3</sub> ]	Mixed	3.41	75.81
20b	<i>o</i> -[CH <sub>3</sub> ]	Mixed	7.56	13.35
21b	<i>m</i> -[CH <sub>3</sub> ]	Mixed	2.34	46.53
22b	<i>p</i> -[CH <sub>3</sub> ]	Mixed	3.72	19.73
Donepezil.HCl		Mixed	7.78x10 <sup>-3</sup>	151.92

\*: insoluble

#### 4. CONCLUSION

Here, we report the synthesis and AChE inhibitory activity of tetrazole derivatives. We synthesized 21 novel compounds (**2b–22b**), all of which have an inhibitory effect on the AChE enzyme. The structures of these compounds were elucidated using spectroscopic methods. Compound **11b** had the highest inhibitory effect (lowest *IC*<sub>50</sub> value) and compound **13b** had the lowest inhibition effect (highest *IC*<sub>50</sub> value). The inhibitory effect is improved in substituted phenyl compounds (**13b**) in which the phenyl is connected to the tetrazole ring. For the halogen-containing compounds, the inhibitory effect, excluding **11b** and **9b**, was increased by increasing the electronegativity of the halogen. Furthermore, the inhibitory effect increased with the presence of electron-withdrawing groups (halogen and nitro groups) at the *m*- position, while the inhibitory effect for electron-donating groups increased at the *o*- position and was observed to not have a correlation in

the *m*- and *p*- positions for electron-donating groups.

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