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THE DESIGN AND SYNTHESIS OF THE THREE NOVEL DUAL REVERSIBLE INHIBITORS OF ACETYLCHOLINESTERASE BASED ON THE TACRINE AND AROYLACRYLIC ACID PHENYLAMIDE SUBSTRUCTURES

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Abstract: *Organophosphorous chemical warfare agents (i.e., nerve agents) exhibit toxic effects mainly through covalent, irreversible inhibition of acetylcholinesterase (EC 3.1.1.7), an enzyme that terminates cholinergic neurotransmission, by hydrolyzing acetylcholine at nerve and nerve-muscle junctions. The reversible inhibition of AChE was suggested as the pre-treatment option against nerve agents' intoxications. Aiming to investigate novel pre-treatment options, we designed and synthesized the three novel compounds consisting of tacrine and aroylacrylic acid phenylamide moieties, connected via a long methylene chain to target two distinct topologically separated anionic sites on the AChE. The inhibitory activity of the compounds toward the Electric eel AChE's was determined by the Ellman assay. The designed compounds may represent a new class of promising leads for developing more effective pre-treatment options.*

Keywords: *Acetylcholinesterase, dual-binding inhibitors, nerve agents, pre-treatment, tacrine, aroylacrylic acid derivatives.*

1. INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7.) is a carboxylesterase which terminates cholinergic neurotransmission by hydrolyzing the neurotransmitter acetylcholine (ACh) in a synaptic cleft of nerve- and nerve-muscle junctions [1]. Organophosphorus compounds are serine esterase and protease inhibitors widely used in agriculture as insecticides and acaricides, in industry and technology as softening agents and additives to lubricants. Some of them are declared chemical warfare agents. Organophosphorous pesticides are considered trialkyl esters of orthophosphoric acid, *i.e.* organophosphates. Nerve agents have a structure which can be derived from phosphonic acid, i.e. they are organophosphonates. They are divided according to the nature of the leaving group directly attached to the phosphorous atom into two groups: G-agents (alkyl methylphosphonofluoridates) and V-agents (alkyl dialkylaminophosphonotiolates) [2-3]. They rapidly inactivate AChE by binding to the catalytic Ser203 leading to the accumulation of ACh in the synaptic cleft. In the peripheral nervous system, acetylcholine accumulation leads to persistent muscarinic receptor overstimulation that triggers various symptoms, including miosis, profuse secretions, bradycardia, broncho-constriction, hypotension, and diarrhea. It also leads to overstimulation of nicotinic receptors, causing severe skeletal muscle fasciculation and subsequent weakness. Central nervous system-related effects include anxiety, restlessness, confusion, ataxia, tremors, seizures, cardiorespiratory paralysis, and coma. Organophosphate binds to catalytic Ser 203 residue, making a covalent complex similar to the acetylated enzyme formed during hydrolysis of the ACh. Hydrolysis of the formed complex is an extremely slow process, and in some instances, depending on the structure of the nerve agent bound to AChE, dealkylation, i.e., leaving of alkoxy group from AChE-nerve agent phosphate, occurs, resulting in a negatively charged dealkylated AChE-OP complex [4]. This process is called 'aging'. Before aging, therapeutic intervention is possible, and the organophosphate can be removed from the active AChE center by an oxime (pralidoxime or obidoxime). Still, AChE is permanently inhibited if aging occurs and cannot be recovered by any means [5].

Treatment for OP intoxication consists of an anticholinergic drug, such as atropine, which relieves the muscarinic symptoms, an oxime, which is able, to some extent, to reactivate the irreversibly inhibited AChE and restore its activity, and an anticonvulsant drug (diazepam or its pro-drug avizafone) which can block nerve agent-induced seizures. This treatment is not fully effective under life-threatening conditions. Oximes are considered

ineffective in the case of soman poisoning, mainly due to the rapid aging process, which prevents enzyme reactivation. The current therapy does not block efficiently nerve-agent induced seizures, which may lead to severe and prolonged brain injury. This treatment must be administered as soon as possible after exposure has happened, which can be complicated in battlefield conditions or any case of massive casualties (accidents, terrorist attacks) [6-7]. Given the drawbacks of the above described standard treatment, the so-called 'pre-treatment' option was proposed. The pre-treatment is given to healthy individuals when there is a higher probability of a chemical attack. The role of reversible AChE inhibitor in the pre-treatment mixture is to temporarily inhibit the fraction of the enzyme and protect it from irreversible, permanent inhibition by OP's. This might be especially important in the case of soman intoxications, where reactivation of AChE is impossible due to a rapid aging reaction.

The efficiency of reversible inhibitors against nerve agent toxicity was examined in several *in vitro* studies. Green was the first to propose that kinetic factors of the reactions between AChE with pre-treatment drug and the nerve agent govern the protective action of carbamates against the toxic action of the nerve agents. He found good qualitative agreement between the proposed theoretical model and *in vivo* experimental results [8].

Petroianu et al. investigated the effects of several moderately potent reversible AChE inhibitors on the rates of irreversible inhibition by organophosphate pesticides paraoxon and mipafox [9]. *In vitro* studies have shown that rantidin can grant some protection against inhibition of cholinesterases by paraoxon. The results of *in vivo* studies were consistent with *in vitro* results. Administration of ranitidine before exposure to paraoxon increases the number of rats surviving an acute paraoxon exposure and protects the cholinesterases from organophosphate inhibition [10].

Eckert et al. developed a dynamically working *in vitro* model to estimate the protective effects of reversible inhibitors. They proved that pre-treatment with reversible inhibitors results in higher residual AChE activity during the presence of an irreversible inhibitor. Also, they showed that different kinetic behavior of reversible inhibitors determines the level of AChE residual activity and, therefore, the protection [11,12].

Few studies designed novel reversible, highly potent AChE inhibitors and tested *in vitro* their protective potential. Lenina et al. proposed that slow-binding reversible AChE inhibitors, with the slow dissociation from the enzyme and longer residence times in the micro-anatomical compartments such as neuromuscular junctions, may display better protective action compared to other reversible AChE inhibitors [13]. They examined *in vitro* and *in vivo* effects of compound C547, which was found to be a nanomolar reversible inhibitor in *in vitro* studies. Mice treated with C547 before exposure to paraoxon had a higher survival rate than untreated mice. The authors concluded that long-lasting slow-binding reversible AChE inhibitors could be considered new potential drugs to increase the duration of pre-exposure treatment of OP poisoning.

Based on the previously derived 3D-QSAR model, in our previous work we designed and synthesized three novel dual binding AChE inhibitors. All three compounds were highly potent low nanomolar inhibitors of three cholinesterases (*Hu*AChE, *Hu*BChE, and *Ee*AChE). The experiments revealed that the compounds were able to protect AChE from inhibition by nerve agents partially at compound concentrations higher than their IC_{50} values [14-15].

In this work, we continued the previously described studies and present synthesis, NMR characterization, and anticholinesterase activity of three novel nanomolar dual-binding reversible inhibitors of AChE, differently substituted at aroylphenyl and amidophenyl rings.

2. MATERIALS AND METHODS

2.1. Chemistry

All chemicals were purchased from Sigma Aldrich or Merck, and were used as received. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or d6-DMSO on Bruker AVANCE400/101 MHz instrument. Chemical shifts are reported in parts per million (*ppm*) relative to solvent shift. Spin multiplicities are given as follows: *s* (singlet), *d* (doublet), *t* (triplet), *m* (multiplet). Synthetic procedures for aroylacrylic acid amides were previously described in the literature [16].

Synthetic procedure for **9-chloro-1,2,3,4-tetrahydroacridine (3):** 2-aminobenzoic acid (2.1 g, 15.3 mmol) and cyclohexanone (1.86 mL, 18 mmol) were added to the round bottom flask and placed in ice-bath, then phosphoryl chloride (16 mL) was added drop wise to the reaction mixture. After adding phosphoryl chloride, the reaction mixture was stirred and heated at the reflux temperature for 2,5^{h}. The reaction solution was then concentrated under reduced pressure (30 ºC/560 mmHg, about half of the solution, was removed) and poured into a mixture of ice, deionized water, and acetone. The pH of the solution was set between 8 and 9 by adding potassium carbonate. Crude, solid compound was obtained from solution by filtration and purified by crystallization from acetone. Pure compound was obtained as a yellow solid substance with a reaction yield of 82%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (*d*, *J* = 8.4 Hz, 1H, *o*-C**H**), 8.00 (*d*, *J* = 8.4 Hz, 1H, *o*-C**H**), 7.66 (*t*, *J* = 7.7 Hz, 1H, *m*-C**H**), 7.53 (*t*, *J* = 7.6 Hz, 1H, *m*-C**H**), 3.13 (*t*, *J* = 6.2 Hz, 2H, *o*-C**H2**), 3.01 (*t*, *J* = 5.9 Hz, 2H, *o*-C**H2**), 2.04 – 1.87 (*overlapped m*, 4H, *m*-C**H2**). ^{13}C NMR (101 MHz, CDCl3) δ 159.60, 146.62, 141.82, 129.50, 129.06, 128.64, 126.70, 125.56, 123.85, 77.16, 34.22, 27.64, 22.76.

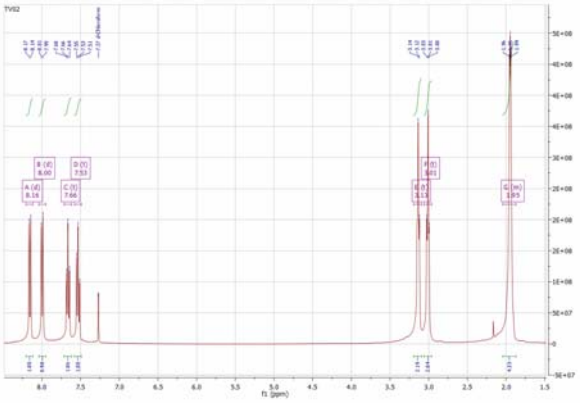


Figure 1. ^{1}H NMR spectrum of 9-chloro-1,2,3,4-tetrahydroacridine.

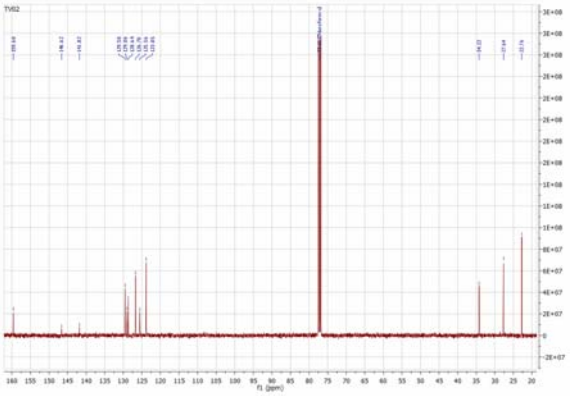


Figure 2. ^{13}C NMR spectrum of 9-chloro-1,2,3,4-tetrahydroacridine.

Synthetic procedure for **N1-(1,2,3,4-Tetrahydro—9-acridinyl)-octane-1,8-diamine(4)**: 1,8-diamino octane (0.99 g, 6.9 mmol), 9-chloro-1,2,3,4-tetrahydroacridine (0.5 g, 2.3 mmol) and 2 mL of 1-pentanol were added to the stainless-steel reactor. Reactor was sealed and heated to 160-165 ºC for 8^{h}. After that, reaction mixture was transferred into separation funnel, diluted with ethyl acetate and washed with 10% aqueous solution of sodium hydroxide, deionized water and saturated aqueous solution of sodium chloride. Organic layer was dried over anhydrous magnesium sulphate and filtered. Solvents were removed under reduced pressure. Crude compound was purified using *dry-flash* chromatography with solvent system $CHCl_3$/MeOH/Et_3N=7/3/0.07. Pure compound was obtained as yellow semi-solid with reaction yield of 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (*d*, *J* = 8.5 Hz, 1H, *tacrine-o*-C**H**), 7.89 (*d*, *J* = 8.5 Hz, 1H, *tacrine-o*-C**H**), 7.53 (*t*, *J* = 7.6 Hz, 1H, *tacrine-m*-C**H**), 7.32 (*t*, *J* = 7.6 Hz, 1H, *tacrine-m*-C**H**), 3.46 (*t*, *J* = 7.1 Hz, 2H, linker NH-C**H2**), 3.04 (*s*, 2H, *tacrine*-C**H2**), 2.72 – 2.62 (*overlapped m*, 4H, *tacrine*-C**H2**, linker NH2-C**H2**), 2.17 (*br*, 3H, linker N**H** and N**H2**), 1.90 (*s*, 4H, *tacrine*-C**H2**), 1.63 (*quint*, *J* = 14.4, 7.1 Hz, 2H, linker NH-CH2-C**H2**), 1.50 – 1.23 (*overlapped m*, 10H, linker CH2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.42, 150.98, 147.44, 128.67, 128.42, 123.68, 122.97, 120.26, 115.85, 49.58, 42.15, 34.02, 33.55, 31.85, 29.43, 29.39, 26.96, 26.84, 24.87, 23.14, 22.86.

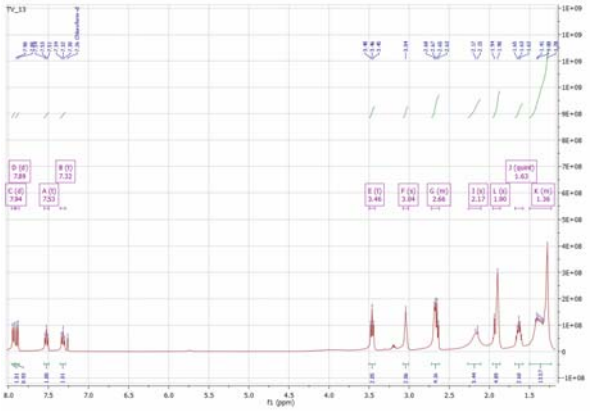


Figure 3. ^{1}H NMR spectrum N*1*-(1,2,3,4-Tetrahydro-acridin-9-yl)-octane-1,8-diamine.

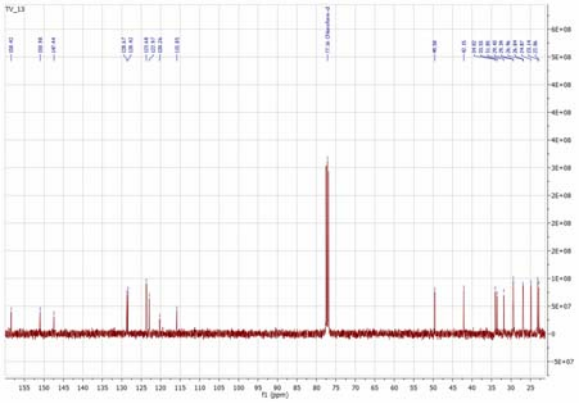


Figure 4. ^{13}C NMR spectrum N1-(1,2,3,4-Tetrahydro-acridin-9-yl)-octane-1,8-diamine.

General synthetic procedure for compounds **8-10:** N1-(1,2,3,4-Tetrahydro-9-acridinyl)-octane-1,8-diamine was dissolved in DCM and then chosen aroylacrylic acid phenylamide was added. Molar ratio of N1-(1,2,3,4-Tetrahydro-acridin-9-yl)-octane-1,8-diamine /aroylacrylic acid phenylamide used for synthesis was 1/1. The reaction mixture was stirred at room temperature for 24^{h}. Reaction progress was monitored by TLC (solvent system Tol/EA/MeOH/Et_3N=2/2/1/0.05). Crude compounds were obtained by solvent evaporation and purified by *dry-flash* column chromatography on silica gel using the solvent system Tol/EA/MeOH/Et3N=2/2/1/0.05.

Synthetic procedure for ***N*-(3,5-Dimethoxyphenyl)-4-(4-methoxyphenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-9-acridinylamino)-octylamino]-butyramide(8)**: Following the described general synthetic procedure, compound was obtained as yellow semi-solid with the reaction yield of 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.58 (*s*, 1H, amido-N**H**), 8.07 – 7.92 (*overlapped m*, 4H, aryol-*o*-phenyl and *tacrine-o*-C**H**), 7.57 (*t*, *J* = 7.6 Hz, 1H, *tacrine-m*-C**H**), 7.35 (*t*, *J* = 7.6 Hz, 1H, *tacrine-m*-C**H**), 6.93 (*d*, *J* = 7.6 Hz, 2H, aroyl-*m*-phenyl), 6.85 (*d*, *J* = 2.1 Hz, 2H,amido-*o*-phenyl-C**H**), 6.25 (*t-like*, 1H, amido-*p*-phenyl), 4.16 (*br*, 1H, linker N**H**), 3.86 (*s*, 3H, aroyl-*p*-OC**H3**), 3.78 (*d*, *J* = 1.1 Hz, 6H, amido-3,5-phenyl-diOC**H3**), 3.65 (*dd*, *J* = 8.6,2.4 Hz, 1H, ABX), 3.59 (*dd*, *J* = 17.3, 2.4 Hz, 1H, ABX), 3.53 (*t*, *J* = 6.8 Hz, 2H, linker NHC**H2**), 3.24 (*dd*, *J* = 17.3, 8.6 Hz, 1H, ABX), 3.10 (*s*, 2H, *tacrine*-C**H2**),

2.75 – 2.65 (*overlapped m*, 3H, *tacrine*-C**H2** and linker C**H2**), 2.61 – 2.52 (*m*, 1H, linker-C**H2**), 2.17 (*s*, 1H, linker-N**H**) 1.92 (*s*, 4H, tacrine-C**H2**), 1.67 (*quintet*, *J* = 14.0, 6.9 Hz, 2H, linker-C**H2**), 1.50 (quintet, *J* = 12.3, 6.4 Hz, 2H, linker-C**H2**), 1.44 – 1.25 (*m*, *J* = 33.7, 16.3 Hz, 8H, linker-C**H2**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.12, 172.20, 164.09, 164.04, 161.28, 139.70, 130.67, 129.49, 128.94, 127,89, 123.89, 123.12, 114.05, 97.73, 96.71, 59.83, 55.64, 55.52, 49.53, 48.55, 39.98, 33.41, 31.79, 31.00, 30.30, 29.42, 27.25, 26.98, 24.73, 23.04, 22.64.

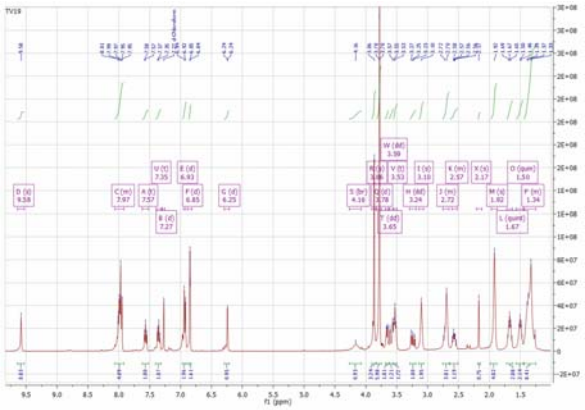


Figure 5. ^{1}H NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(4-methoxyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

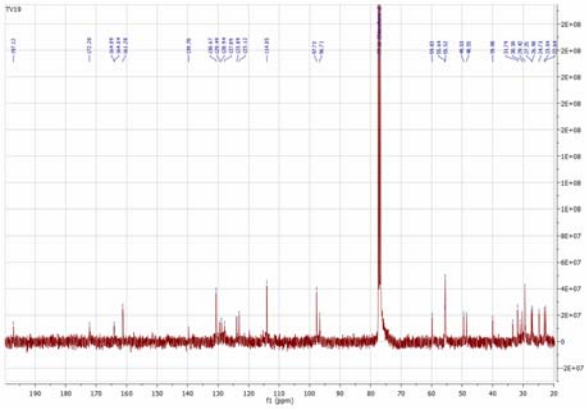


Figure 6. ^{13}C NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(4-methoxyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

Synthetic procedure for **N-(3,5-Dimethoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide(9):** In addition to described general synthetic procedure reaction mixture was heated to reflux and catalytic amount of Et_3N was added. Compound was obtained as yellow semi-solid with the reaction yield of 17%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.59 (*s*, 1H, amido-N**H**), 7.95 (*d*, *J* = 8.3 Hz, 1H, *tacrine*-C**H**), 7.89 (*d*, *J* = 8.4 Hz, 1H, *tacrine*-C**H**), 7.74 (*s*, 1H, aroyl-*o*-phenylC**H**), 7.70 (*d*, *J* = 7.7 Hz, 1H, aroyl-*o*-phenylC**H**), 7.53 (*t*, *J* = 7.5 Hz, 1H, tacrine-C**H**), 7.33 (*t*, *J* = 7.6 Hz, 1H, tacrine-C**H**), 7.25-7.15 (*m*, 2H, aroyl-*m*-phenyl-C**H**), 6.84 (*d*, *J* = 2.1 Hz, 2H, amido-*o*-phenyl-C**H**), 6.23 (*t*, *J* = 2.0 Hz, 1H, amido-*p*-phenyl-C**H**), 3.77 (*s*, 6H, amido-*m*-phenyl-OC**H3**), 3.72 – 3.55 (*overlapped m*, 2H, ABX), 3.47 (*t*, *J* = 7.2 Hz, 2H, linker-NH-C**H2**-), 3.25 (*dd*, *J* = 17.3, 8.7 Hz, 1H, ABX), 3.05 (*s*, 2H, *tacrine*-C**H2**-), 2.74 – 2.63 (*m*, 3H, *tacrine*-C**H2**- and linker-NH-C**H2**-), 2.59 – 2.51 (*m*, 1H, linker-NH-C**H2**-), 2.30 (*overlapped m*, 6H, aroyl-C**H3**), 1.90 (*s*, 4H, *tacrine*-C**H2**-), 1.68 – 1.60 (*m*, 2H, linker-NH-CH2-C**H2**-), 1.53 – 1.44 (*m*, 2H, linker-NH-CH2-C**H2**-), 1.42 – 1.25 (*m*, 4H, linker-C**H2**-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.50, 172.21, 161.25, 158.38, 151.04, 143.46, 139.67, 137.24, 134.32, 130.09, 129.43, 129.15, 128.50, 128.34, 126.06, 123.72, 123.01, 120.24, 115.83, 97.68, 96.70, 59.71, 55.50, 49.60, 48.51, 40.20, 33.95, 31.86, 30.32, 29.45, 27.27, 27.01, 24.87, 23.14, 22.85, 20.16, 19.86.

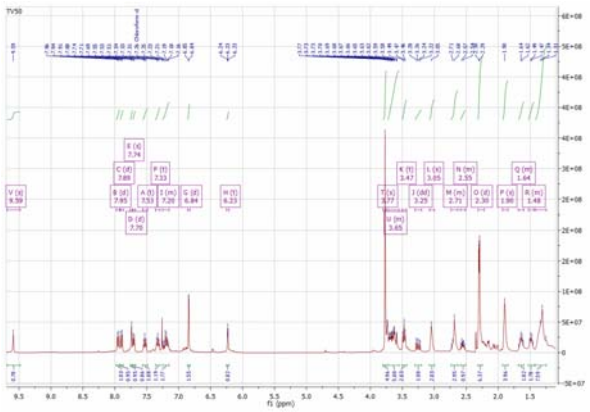


Figure 7. ^{1}H NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

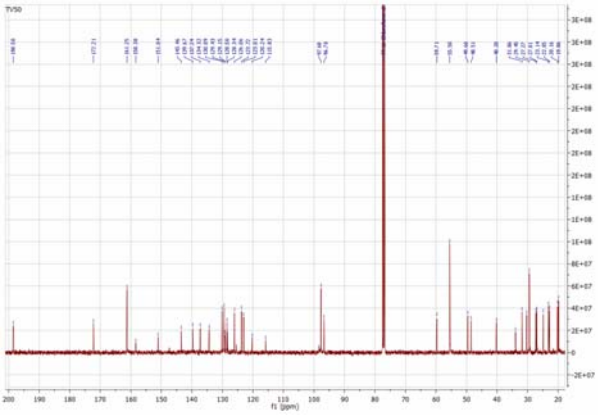


Figure 8. ^{13}C NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

Synthetic procedure for **N-(3,5-Dimethoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide(10):** In addition to described general synthetic procedure reaction mixture was heated to reflux and catalytic amount of Et_3N was added. Compound was obtained as yellow semi-solid with the reaction yield of 26%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.55 (*s*, 1H, amido-N**H**), 7.95 (*d*, *J* = 8.3 Hz, 1H, *tacrine-o*-C**H**), 7.90 (*d*, *J* = 8.5 Hz, 1H, *tacrine-o*-C**H**), 7.53 (*s*, 1H, aroyl-*o*-phenylC**H**), 7.33 (*t*, *J* = 7.6 Hz, 1H, *tacrine-m*-C**H**), 7.28 – 7.23 (*overlapped m*, 2H, tacrine-*m*-C**H** and solvent), 7.21 – 7.11 (*overlapped m*, 2H, aroyl-*m* and aroyl-*p*-C**H**) , 6.84 (*d*, *J* = 2.2 Hz, 2H, amido-*p*-phenyl-C**H**), 6.23 (*t*, *J* = 2.1 Hz, 1H, amido-*p*-

phenyl-C**H**), 3.78 (*s*, 6H, amido-*m*-phenyl-diOC**H3**), 3.66 (*dd*, J = 8.6, 3.1 Hz, 1H, ABX), 3.59 – 3.51 (*m*, 1H, ABX), 3.49 (*t*, *J* = 7.3 Hz, 2H, linker-NH-C**H2**-), 3.24 (*dd*, *J* = 17.5, 8.6 Hz, 1H, ABX), 3.05 (*s*, 2H, tacrine-C**H2**-), 2.77 – 2.63 (*overlapped m*, 3H, tacrine-C**H2**- and linker NH-C**H2**-), 2.58 (*dt*, *J* = 18.4, 6.1 Hz, 1H, linker NH-C**H2**-), 2.46 (*s*, 1H, linker-N**H**-), 2.35 (*s*, 3H, aroyl-C**H3**), 2.34 (*s*, 3H, aroyl-C**H3**), 1.91 (*s*, 4H, *tacrine*-C**H2**-), 1.70 – 1.58 (*m*, 2H, linker-NH-CH2C**H2**-), 1.56 – 1.46 (*m*, 2H, linker-NH-CH2C**H2**-), 1.44 – 1.21 (*m*, 4H, linker-C**H2**-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.39, 172.12, 161.26, 139.68, 136.79, 135.58, 132.84, 132.22, 129.77, 129.17, 128.59, 128.36, 125.43, 123.77, 123.03, 97.69, 96.67, 59.84, 55.53, 49.62, 48.47, 42.73, 31.89, 31.05, 30.37, 29.49, 27.31, 27.03, 24.87, 23.14, 22.84, 21.58, 21.25, 21.02.

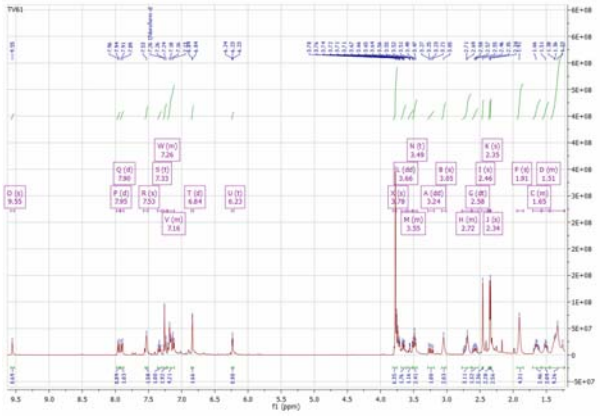


Figure 9. ^{1}H NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

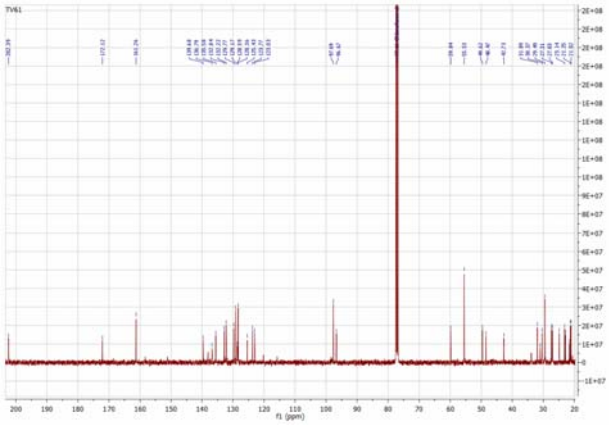


Figure 10. ^{13}C NMR spectrum of N-(3,5-Dimethoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[8-(1,2,3,4-tetrahydro-acridin-9-ylamino)-octylamino]-butyramide.

2.2. Biology

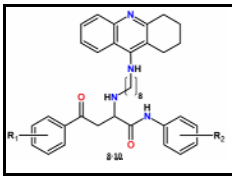
The inhibition potency of the compounds **8**–**10** toward E. Eel AChE was evaluated by Ellman assay [20], using the type VI-S enzyme (Sigma) and acetylthiocholine iodide (in final concentration 0.28 mM) as a substrate. The measurements were done on Epoch Microplate Spectrophotometer (Biotek Instruments, USA). A broad range of concentrations, which produce 20–80% of enzyme activity inhibition, were used for each compound. The reaction took place in the final volume of 0.2 mL of 0.1 M potassium phosphate buffer, pH 8.0, containing 0.02 U of AChE and 0.3 mM of 5,5-dithio-bis(2-nitrobenzoic)acid (DTNB), used to produce yellow anion of 5-thio-2-nitrobenzoic acid in reaction with thiocholine released by AChE. The tested compound (5 μL) was added to the enzyme solution (95 μL) and preincubated at 25 °C for 10 min, followed by the addition of DTNB (95 μL) and substrate (5 μL). The reaction was monitored for 3 min (absorbance was measured every 30 s), and the color production was measured at 412 nm. Determination of inhibition curves was performed at least in triplicate. One triplicate sample without a test compound was always present to yield 100% of AChE activity.

3. RESULTS AND DISCUSSION

3.1. Chemistry

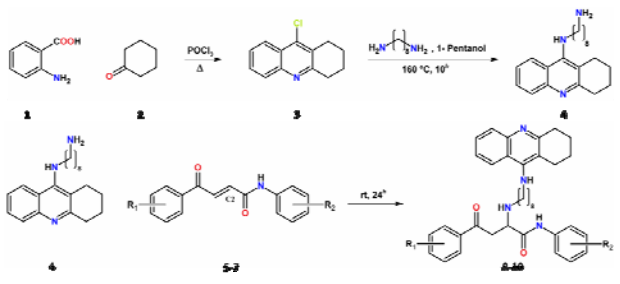
The synthetic path for compounds **8-10** is given in **Scheme1**, and structures of synthesized compounds and corresponding reaction yields are presented in **Table 1**.

Table 1. Reaction yield of compounds 8-10.

	Com. No.	R1	R2	Yield (%)
	**8**	p-OMe	3,5-OMe	74
	9	3,4-diMe	3,5-OMe	17
	10	2,5-diMe	3,5-OMe	27

The Niementowski reaction between 2-aminobenzoic acid **1** and cyclohexanone **2** proceeded smoothly to give **3**. The procedure was slightly changed in accordance with the previously used procedure. During the process of purification, acetone was added to the mixture of water and ice, which made it possible for the compound to better distribute through the solution and facilitated neutralization. Compound **4** was obtained in the nucleophilic aromatic substitution (S_NAr) reaction of compounds **3** and 1,8-diaminooctane (linker). The reaction took place in a sealed stainless steel reactor. Although a triple amount of linker was used, a considerable amount of tacrine homodimer[1] was formed as a by-product, and it was separated from the targeted compound **4** by *dry-flash* column chromatography.

Michael's addition of **4** and substituted aroylacrylic phenylamides **5-7** was used to obtain the final products, compounds **8-10**. Procedures for the synthesis of aroylacrylic phenylamides are described in the previous work [16].



Scheme 1. Synthetic path for compounds 8-10.

[1] N,N'-Bis-(1,2,3,4-tetrahydro-acridin-9-yl)-octane-1,8-diamine.

The final synthetic step for compound **8** went smoothly with adequate reaction yield. However, compounds **9** and **10** were obtained in significantly lower reaction yields of 17% and 27%, respectively. In the case of compounds **9** and **10**, 24^h from the beginning of Michael's addition, we still noticed the presence of reactants on the TLC plates. To move the reaction equilibrium on the product's side, reaction mixtures were heated for a few hours, and then a catalytic amount of Et_3N was added. This did not increase the reaction yields. The effect of solvent on Micahel's addition was examined by replacing DCM with MeOH. The decision to take this step was based on previous work that studied the kinetics of the reaction of Michael's addition using methanol as solvent [17]. However, this also did not increase the reaction yields nor reduce the reaction time needed to obtain the products.

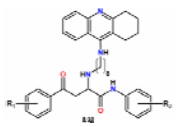
Structures of the compounds differ only in substitution at aroyl ring (R1). In the case of compound **8**, corresponding aroylacrylic phenylamide **5** contains an electronegative atom in the *para* position (R1= *p*-**O**-CH_3) which can withdraw electron density from the conjugated system, making C2 more partially positive and promoting its electrophilic character. In opposition, corresponding aroylacrylic phenylamides **6** and **7** of compounds **9** and **10** are substituted with –**CH_3** group as R1 in positions 3,4- and 2,5- respectively. The methyl group has an electron-donating inductive effect, which increases electron density on the aroyl ring.

Because the electronegative atom is present as an R1 substituent in the structure of compound **5**, the electron density is shifted toward the –**O**Me group, which results in an easier nucleophilic attack of compound **4**.
This effect may explain the faster reaction rate in the case of compound **8** compared to compounds **9**-**10**.

3.2. Biology

Inhibition potency of compounds **8**–**10** was determined toward *Ee*AChE. The results are shown in **Table 2**. All three compounds are highly potent low nanomolar inhibitors of *Ee*AChE. The most potent compound is **8**, and the compound with the lowest inhibition potency is compound **10**.

Table 2. Inhibitory activity of compounds 9-10 toward AChEexpressed as IC_{50} value.

	Com. No.	R1	R2	AChE IC_{50}±SEM (nM)
	**8**	*p*-OMe	3,5-OMe	5.56±0.34
	9	3,4-diMe	3,5-OMe	6.97±0.50
	10	2,5-diMe	3,5-OMe	25.13±0.27

It has been proven in several previous studies that dual AChE inhibitors bind in the way in which the tacrine substructure is oriented toward the bottom of the active site gorge, interacting with Trp86 and Tyr337 residues. In contrast, the other, a usually aromatic and polycyclic fragment of the molecule, is oriented toward the entrance of the active site gorge and interacts with amino acid residues that belong to the peripheral anionic site (PAS), namely Trp286, among others. PAS area of AChE is very wide and can accommodate a variety of highly voluminous molecular fragments.

All three compounds are low nanomolar inhibitors of AChE. The difference in inhibitory activity of compounds **8** and **9** toward AChE is negligible, and it seems that methyl substitution at positions 3 and 4 of the aroylphenyl ring does not influence the IC_{50} values. However, in compound **10**, the methyl substitution at positions 2 and 5 on the aroylphenyl ring notably increased the IC_{50} values, i.e., reduced inhibitory activity. This may be due to the possible steric hindrance occurring at the PAS site. Still, it is also possible that conformational changes induced by R1 and R2 are responsible for different inhibitory activity toward AChE. To confirm this theory, further examination is needed.

4. CONCLUSION

Three novel dual-binding reversible inhibitors of AChE were synthesized, and their inhibitory activity toward *Ee*AChE was examined. The synthesis path included the reaction of Niementowski, nucleophilic aromatic substitution, and Michaelis' addition. Compound **8** had the greatest overall reaction yield and the lowest IC_{50} of 5.56 nM. Compounds **9** and **10** were obtained in lower yields, and compound **10** was the least active with an IC_{50} value of 25 nM. All three compounds exhibited low nanomolar activity toward *Ee*AChE and are considered good leads for further research.

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