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THE DESIGN AND SYNTHESIS OF THE FOUR 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. Use of nerve agents is strictly limited to research purposes only and it is under control of Organisation for the Prohibition of Chemical Weapons, OPCW. Despite all efforts to limit the use of nerve agents, unfortunately the danger of nerve agents being used in war aggression and terrorist attacks is still present. 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 four novel compounds of tacrine and aroylacrylic acid phenylamide moieties, connected via a long methylene chain to target two distinct topologically separated anionic areas on the AChE. The inhibitory activity of the compounds toward the Electric eel AChE's as well as the horse serum BChE was determined by Ellman assay. The designed compounds may represent a new class of promising leads for developing more effective pre-treatment options.*

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

1. INTRODUCTION

The use of chemical and biological weapons in war was firstly prohibited by Geneva Protocol in 1925 as a part of Geneva Conventions. Geneva Conventions are international humanitarian laws that establish international legal standards for humanitarian treatment in war and each country that has signed it is obligated, among other, to prevent employment of chemical weapons in war. Today, the use of organophosphorous chemical warfare agents (i. e. nerve agents, OP) is restricted to research purposes only and it is monitored by Organization for the Prohibition of Chemical Weapons, OPCW. Despite all efforts, cases of use of nerve agents are still recorded in terroristic attacks and military aggressions.

Cholinergic nerve system is the target of nerve agents. They exert their toxic effects by covalent binding to the catalytic Ser203 of acetylcholinesterase (AChE, EC 3.1.1.7.), enzyme responsible for cease of neurotransmission in cholinergic nervous system. AChE is a carboxylesterase which terminates cholinergic neurotransmission by hydrolyzing the neurotransmitter acetylcholine (ACh) in a synaptic cleft of nerve- and nerve-muscle junctions [1]. In the peripheral nervous system, acetylcholine accumulation leads to persistent muscarinic receptor overstimulation that triggers various symptoms, including miosis, profuse secretions, bradycardia, bronchoconstriction, 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.

Mechanism of nerve agent biding to catalytic Ser203 residue of AChE is presented in Scheme1. Covalent complex that is formed between AChE and nerve agent is similar to the acetylated enzyme which is formed during hydrolysis of the ACh. Hydrolysis of the OP-AChE complex is an extremely slow process. 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 [2]. This process is called aging and therapeutic intervention by an oxime (pralidoxime or obidoxime) is only possible before the onset of this process [3]. Also, oximes are permanently positively charged which prevents them to cross blood-brain barrier, which means if applied on time,

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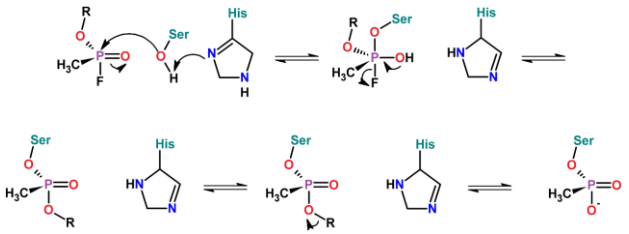
they can only reactivate AChE in peripheral nervous system, while central nervous system AChE still remains inhibited.



Scheme 1. Mechanism of AChE phosphorylation by nerve agents.

Depending of the bulkiness of alkoxy group of nerve agent, aging process occurs in different time interval. In case of soman this interval is very short and AChE is irreversibly inhibited in just a few minutes [4]. Because of time sensitive application of standard treatment, which is not possible in battlefield conditions, and lack of the ability of oximes to reactivate AChE in central nerve system, new approach was suggested. The so called pre-treatment consists of application of pseudo-irrreversible or reversible inhibitors of AChE to healthy individuals when there is a reasonable suspicion of increased danger of nerve agent poisoning. Reversible inhibitor would occupy active site of AChE during the exposure to nerve agents. In this way, part of AChE would be protected against irreversible inhibition by nerve agent which would enable easier recovery and greater chances of survival of affected individuals.

In the past several decades, protective effects toward nerve agent poisoning of many reversible inhibitors AChE were examined [5]. Initially, the emphasis of these tests was on already available anticholinergic drugs. One of the first compounds investigated as a pre-treatment option was pyridostigmine bromide, which is used for treatment of *Myasthenia gravis*. Protective effects of Huperzine A, natural product of plant *Huperzia Serrata*, was thoroughly examined against nerve agent poisoning [6]. The advantage of Huperzine A is reflected in its ability to pass through a blood-brain barrier, which means that AChE in central nerve system can be reached and protected during nerve agent poisoning. [7] Protective effects of FDA-approved drugs for the treatment of Alzheimer's disease, donepezil and galantamine, were also investigated [8-12]. [8]-[12]

In this work we present the design, synthesis, NMR characterization, and anticholinesterase activity of four novel nanomolar dual-binding reversible inhibitors of cholinesterases.

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 d_6-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 and 9-chloro-1,2,3,4-tetrahydroacridine were previously described in the literature [13-14].

Synthetic procedure for **N1-(1,2,3,4-Tetrahydro—9-acridinyl)-heptane-1,7-diamine(4)**: 1,7-diamino octane (0.89 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* column chromatography on silica gel that was previously thermally treated at 100 ºC for 30 min and cooled at room temperature before use. Solvent system used for purification was $CHCl_3$/MeOH/Et_3N=7/3/0.07. Pure compound was obtained as yellow semi-solid with reaction yield of 42%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (*d*, *J* = 8.4 Hz, 1H, tacrine-C**H**), 7.89 (*d*, *J* = 8.4 Hz, 1H, tacrine-C**H**), 7.54 (*t*, *J* = 7.6 Hz, 1H, tacrine-C**H**), 7.33 (*t*, *J* = 7.6 Hz, 1H, tacrine-C**H**), 3.47 (*t*, *J* = 7.2 Hz, 2H, linker-NH-C**H2**-), 3.05 (*s*, 2H, tacrine-C**H2**), 2.74 – 2.62 (*overlapped m*, *J* = 14.9, 7.9 Hz, 4H, linker-NH-C**H2**- and tacrine-C**H2**), 1.96 – 1.85 (*overlapped m*, 6H, tacrine-C**H2**, linker-N**H**- and linker-N**H2**), 1.65 (*quin*, *J* = 14.4, 7.2 Hz, 2H, linker-NH-CH2-C**H2**-), 1.46 – 1.26 (*m*, 8H, linker-C**H2**-). ^{13}C NMR (101 MHz, CDCl3) δ 158.55, 150.90, 147.58, 128.82, 128.37, 123.68, 122.94, 120.36, 115.99, 49.60, 42.18, 34.12, 33.64, 31.84, 29.32, 27.02, 26.88, 24.91, 23.18, 22.90.

General synthetic procedure for compounds **9-12:** N1-(1,2,3,4-Tetrahydro-9-acridinyl)-octane-1,7-diamine was dissolved in DCM, catalytic amount of Et3N (15%) was added and reaction mixture was stirred for 15 min. Then the chosen aroylacrylic acid phenylamide was added to reaction mixture in molar ratio of N1-(1,2,3,4-Tetrahydro-acridin-9-yl)-octane-1,7-diamine /aroylacrylic acid phenylamide=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 on rotary vacuum evaporator and purified by gradient *dry-flash* column chromatography on Florisil using the solvent systems hexane/ethyl acetate/ NH_4OH=1/1/0.02, hexane/ethyl acetate/methanol/NH_4OH=6/2/1/0.18, hexane/ethyl acetate/methanol/NH_4OH=1/1/1/0.03.

Synthetic procedure for ***N*-(phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (9):**

In addition to general procedure, the white solid was formed and filtered from reaction mixture before solvent

evaporation. Compound was obtained as yellow semi-solid with the reaction yield of 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.58 (*s*, 1H, amido-N**H**-), 7.95 (*d*, *J* = 8.5 Hz, 1H, tacrine-C**H**), 7.92 (*d*, *J* = 8.5 Hz, 1H, tacrine -C**H**), 7.74 (*s*, 1H, aroyl-*o*-C**H**), 7.71 (*d*, *J* = 7.8 Hz, 1H, aroyl-*o*-C**H**), 7.59 (*d*, *J* = 7.8 Hz, 2H, phenyl-*o*-C**H**), 7.57 – 7.51 (*m*, 1H, tacrine-C**H**), 7.36 – 7.29 (*overlapped m*, 3H, tacrin-C**H** and phenyl-*m*-C**H**), 7.21 (*d*, *J* = 7.8 Hz, 1H, aroyl-*m*-C**H**), 7.09 (*t*, *J* = 7.4 Hz, 1H, phenyl-*p*-C**H**), 3.68 (*dd*, *J* = 8.7, 3.1 Hz, 1H, ABX), 3.62 (*dd*, *J* = 17.4, 3.1 Hz, 1H, ABX), 3.47 (*t*, *J* = 7.1 Hz, 2H, linker-NH-C**H**$_2$-), 3.26 (*dd*, *J* = 17.4, 8.7 Hz, 1H, ABX), 3.06 (*s*, 2H, tacrine-C**H**$_2$-), 2.75 – 2.66 (*overlapped m*, 3H, tacrine-C**H**$_2$ and linker-NH-C**H**$_2$-), 2.57 (*dt*, *J* = 11.5, 7.1 Hz, 1H, linker-NH-C**H**$_2$-), 2.34 – 2.27 (*overlapped m*, 6H, aroyl-C**H**$_3$), 1.96 – 1.87 (*m*, 4H, tacrine-C**H**$_2$-), 1.64 (*quin*, *J* = 14.1, 7.2 Hz, 2H, linker-NH-C**H**$_2$-C**H**$_2$-), 1.54 – 1.46 (*m*, 2H, linker-NH-C**H**$_2$-C**H**$_2$-), 1.43 – 1.30 (*m*, 6H, linker-C**H**$_2$-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.58, 172.10, 150.99, 143.46, 137.92, 137.24, 134.35, 130.10, 129.43, 129.13, 128.70, 128.49, 128.34, 126.06, 124.22, 123.76, 122.98, 120.27, 119.40, 115.90, 77.16, 59.69, 49.59, 48.54, 40.32, 34.04, 31.85, 30.28, 29.33, 27.26, 27.02, 24.89, 23.16, 22.87, 20.18, 19.89.

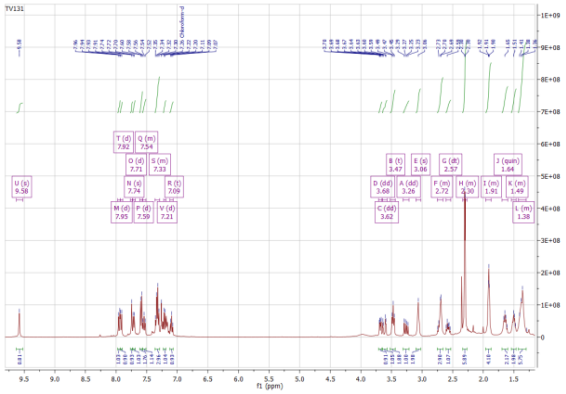


Figure 1. 1H NMR spectrum of *N*-(phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (9).

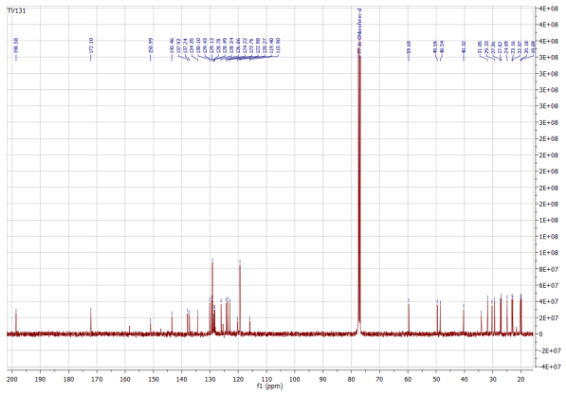


Figure 2. 13C NMR spectrum of N-(phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (9).

Synthetic procedure for ***N*-[4-(propan-2-yl)-phenyl]-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (10):**

Following the general procedure compound was obtained as yellow semi-solid with the reaction yield of 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.46 (*s*, 1H, amido-N**H**), 7.95 (*d*, *J* = 8.5 Hz, 1H, tacrine-C**H**), 7.91 (*d*, *J* = 8.5 Hz, 1H, tacrine-C**H**), 7.58 – 7.47 (*overlapped m*, 4H, tacrine-C**H**, aroyl-*o*-C**H**), 7.34 (*t*, *J* = 7.6 Hz, 1H, tacrine-C**H**), 7.28 – 7.22 (*overlapped m*, 2H, phenyl-*o*-C**H** and solvent), 7.21 – 7.15 (*m*, 2H, phenyl-*m*-C**H**), 7.12 (*d*, *J* = 7.8 Hz, 1H, aroyl-*m*-C**H**), 3.67 (*dd*, *J* = 8.6, 3.2 Hz, 1H, ABX), 3.54 (*dd*, *J* = 17.5, 3.2 Hz, 1H, ABX), 3.48 (*t*, *J* = 7.2 Hz, 2H, linker-NH-C**H**$_2$-), 3.22 (*dd*, *J* = 17.5, 8.6 Hz, 1H, ABX), 3.06 (*s*, 2H, takrine-C**H**$_2$-), 2.87 (*sept*, *J* = 6.9 Hz, 1H, phenyl-*p*-C**H**(CH_3)$_2$), 2.77 – 2.66 (*overlapped m*, 3H, linker-NH-C**H**$_2$- and takrine-C**H**$_2$-), 2.58 (*dt*, *J* = 11.5, 7.1 Hz, 1H, linker-NH-C**H**$_2$-), 2.35 (*overlapped m*, 4H, aroyl-*m*-C**H**$_3$ and aroyl-*p*-C**H**$_3$), 1.96 – 1.88 (*m*, 4H, tacrine-C**H**$_2$-), 1.66 (dt, *J* = 14.4, 7.1 Hz, 2H, linker-NH-CH_2-C**H**$_2$-), 1.56 – 1.47 (*m*, 2H, linker-NH-CH_2-C**H**$_2$-), 1.45 – 1.33 (*m*, 6H, linker-C**H**$_2$-), 1.22 (*d*, *J* = 6.9 Hz, 6H, phenyl-*p*-CH(C**H**$_3$)$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.54, 171.83, 158.47, 150.98, 145.00, 136.89, 135.63, 135.57, 135.52, 132.78, 132.19, 129.74, 129.17, 128.75, 128.49, 128.36, 127.01, 125.43, 123.77, 122.98, 120.31, 119.54, 115.94, 59.82, 49.61, 48.51, 42.98, 34.07, 33.74, 31.87, 30.34, 29.37, 27.31, 27.05, 24.91, 24.17, 23.18, 22.90, 21.58, 21.21, 21.01.

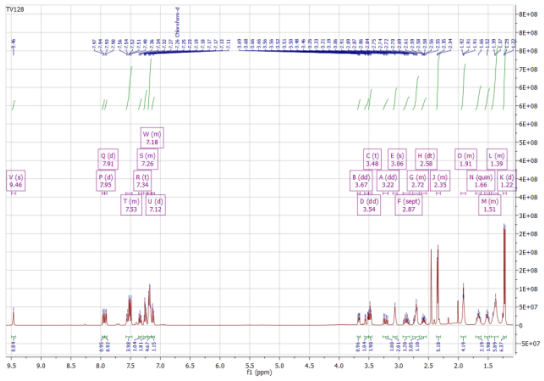


Figure 3. 1H NMR spectrum of N-[4-(propan-2-yl)-phenyl]-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (10).

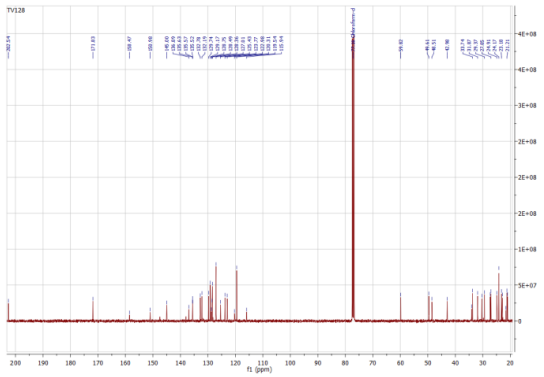


Figure 4. 13C NMR spectrum of N-[4-(propan-2-yl)-phenyl]-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (10).

Synthetic procedure for ***N*-(3,5-methoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (11):**

Following the general procedure compound was obtained as yellow semi-solid with the reaction yield of 16%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (s, 1H, amido-N**H**), 7.99 – 7.92 (*overllapped m*, 2H, tacrine-*o*-C**H**), 7.74 (s, 1H, aroyl-*o*-C**H**), 7.71 (d, *J* = 7.8 Hz, 1H, aroyl-*o*-C**H**), 7.55 (t, *J* = 7.5 Hz, 1H, tacrine-*m*-C**H**), 7.34 (t, *J* = 7.5 Hz, 1H, tacrine-*m*-C**H**), 7.21 (d, *J* = 7.8 Hz, 1H, aroyl-*m*-C**H**), 6.84 (d, *J* = 2.1 Hz, 2H, phenyl-*o*-C**H**), 6.23 (t, *J* = 2.1 Hz, phenyl-*m*-C**H** 1H), 3.77 (s, 6H, phenyl-OC**H₃**), 3.68 – 3.64 (m, 1H, ABX), 3.63 – 3.56 (m, 1H, ABX), 3.50 (t, *J* = 7.1 Hz, 2H, linker -NH-C**H₂**-), 3.25 (dd, *J* = 17.3, 8.7 Hz, 1H, ABX), 3.07 (s, 2H, tacrine -C**H₂**), 2.73 – 2.65 (overlapped m, 3H, tacrine -C**H₂** and linker NH_2-C**H₂**-), 2.59 – 2.51 (m, 1H, linker NH_2-C**H₂**-), 2.34 – 2.25 (m, 6H, aroyl-m-C**H₃** and aroyl-p-C**H₃**), 1.91 (s, 4H, tacrine -C**H₃**), 1.65 (quin, *J* = 14.2, 7.3 Hz, 2H, linker -NH-CH_2-C**H₂**-), 1.56 – 1.46 (m, 2H, linker -NH-CH_2-C**H₂**-), 1.42 – 1.28 (m, 6H, linker -C**H₂**-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.52, 172.19, 161.27, 143.52, 139.68, 137.27, 134.31, 130.12, 129.45, 128.74, 128.36, 126.07, 123.86, 123.06, 97.69, 96.68, 77.16, 59.72, 55.53, 49.55, 48.48, 40.24, 31.83, 30.26, 29.32, 27.27, 27.03, 24.82, 23.10, 22.76, 20.19, 19.89.

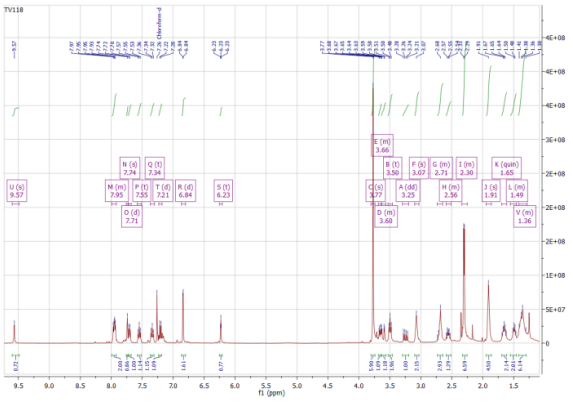


Figure 5. 1H NMR spectrum of N-(3,5-methoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (11).

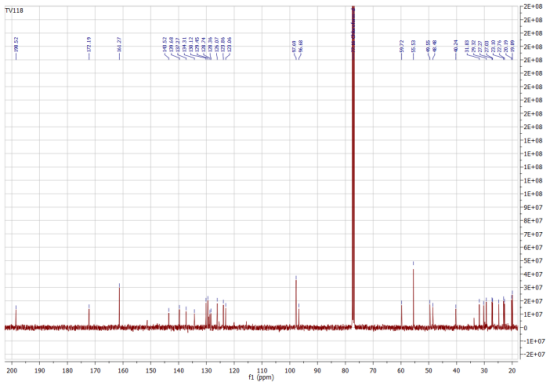


Figure 6. 13C NMR spectrum of N-(3,5-methoxy-phenyl)-4-(3,4-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (11).

Synthetic procedure for ***N*-(3,5-methoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (12):**

In addition to general procedure, the white solid formed and filtered from reaction mixture before solvent evaporation. Compound was obtained as yellow semi-solid with the reaction yield of 79%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.54 (*s*, 1H, amido-N**H**-), 7.95 (*d*, *J* = 8.5 Hz, 1H, tacrine-C**H**-), 7.90 (*d*, *J* = 8.5 Hz, 1H, tacrine-C**H**-), 7.57 – 7.50 (*m*, 2H, tacrine-C**H**- and aroyl-*o*-C**H**), 7.33 (*t*, *J* = 7.6 Hz, 1H, tacrine-C**H**-), 7.28 – 7.10 (m, 2H, aroyl-*m*-C**H** and aroyl-*p*-C**H**), 6.84 (d, *J* = 2.0 Hz, 2H, phenyl-*o*-C**H**), 6.23 (*s*, 1H, phenyl-*p*-C**H**), 3.77 (*s*, 6H, phenyl-3,5-diOC**H₃**), 3.66 (*dd*, *J* = 8.5, 3.2 Hz, 1H, ABX), 3.53 (*dd*, *J* = 17.6, 3.2 Hz, 1H, ABX), 3.48 (*t*, *J* = 7.2 Hz, 2H, linker-NH-C**H₂**-), 3.24 (*dd*, *J* = 17.6, 8.5 Hz, 1H, ABX), 3.06 (*s*, 2H, tacrine-C**H₂**-), 2.75 – 2.65 (*overlapped m*, 3H, linker-NH-C**H₂**- and tacrine-C**H₂**-), 2.61 – 2.52 (*m*, 1H, linker-NH-C**H₂**-), 2.46 (*s*, 3H, aroyl-*o*-C**H₃**), 2.34 (*s*, 3H, aroyl-*m*-C**H₃**), 1.95 – 1.87 (*m*, 4H, tacrine-C**H₂**-), 1.70 – 1.60 (*m*, 2H, linker-NH-CH_2-C**H₂**-), 1.56 – 1.46 (*m*, 2H, linker-NH-CH_2-C**H₂**-), 1.44 – 1.30 (*m*, 6H, linker-C**H₂**-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.38, 172.10, 161.26, 158.48, 150.97, 139.66, 136.78, 135.59, 135.57, 132.84, 132.22, 129.75, 129.16, 128.71, 128.47, 128.35, 125.42, 123.75, 122.98, 120.32, 115.95, 97.70, 96.65, 59.83, 55.52, 49.60, 48.43, 42.72, 34.05, 31.88, 30.30, 29.35, 27.31, 27.07, 24.90, 23.17, 22.89, 21.57, 21.23, 21.01.

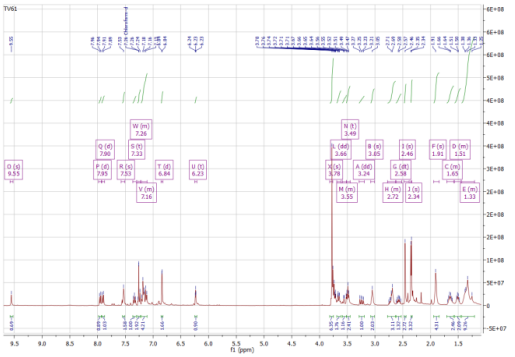


Figure 7. 1H NMR spectrum of N-(3,5-methoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (12).

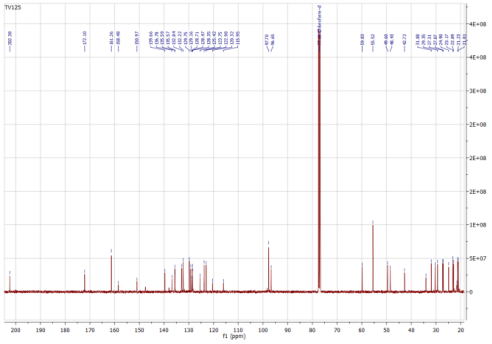


Figure 8. 13C NMR spectrum of N-(3,5-methoxy-phenyl)-4-(2,5-methyl-phenyl)-4-oxo-2-[7-(1,2,3,4-tetrahydro-9-acridinylamino)-heptylamino]-butyramide (12).

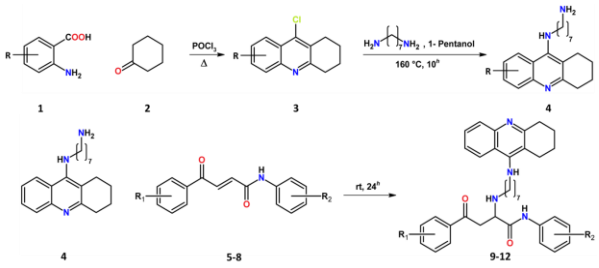
2.2. Biology

The inhibition potency of the compounds **9**–**12** toward E. Eel AChE and horse serum BChE (*Eq*BChE) was evaluated by Ellman assay, 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/mL 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), 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 **9-12** is given in **Scheme2.**



Scheme 2. Synthetic path for compounds 9-12.

The Niementowski reaction between 2-aminobenzoic acid **1** and cyclohexanone **2** proceeded smoothly to give **3**. 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** as described in synthetic procedure. Michael's addition of **4** and substituted aroylacrylic phenylamides **5-8** was used to obtain the final products, compounds **9-12**. Procedures for the synthesis of aroylacrylic phenylamides are described in the previous work [16]. General structure of synthesized compounds was given on **Figure9**, while substitution (R1, R2) and coresponding reaction yields are presented in **Table 1**.

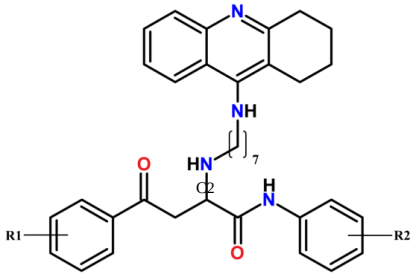


Figure 9. General structure of compounds 9-12. R1 is representing substitution of aroyl- and R2 substitution of phenylamide segment.

Table 1. Reaction yield for compounds **9-12**.

No.	R1	R2	Yield (%)
9	3,4-CH_3	-H	61
10	3,4-CH_3	4-$CH(CH_3)_2$	63
11	3,4-CH_3	3,4-OCH_3	16
12	2,5-CH_3	3,4-OCH_3	79

The final synthetic step for all presented compounds went smoothly with adequate reaction yield. In case of compound **11,** reaction yield is notably lower than that of the other three compounds. Comparing the reaction yields of compounds **9** and **10**, it can be observed that R2 substitution does not have any significant influence on reaction yield since **10** has a very bulky isopropyl group, and 9 lacks R2 substitution, but reaction yields of these compounds are very similar. The reaction yields of compounds **11** and **12** drastically differ. This may suggest that substitution pattern at aroyl ring (R1) has greater impact on the overall reaction yield than substitution at phenylamide ring (R2).

This can be ascribed to the greater influence of R1 substituents on the overall conformation of the aroylacrylic phenylamide and its subsequent influence on the electrophillic properties of C2-carbon which may be, depending on the conformation, more or less susceptible to nuclepohilic attack of the Michael's donor. Further study is needed in order to accurately determine the way in which R1 has the influence on reaction yield.

3.2. Biology

Inhibition potency of compounds **9**–**12** was determined toward *Ee*AChE and *Eq*BChE. The results are shown in **Table 2**. All compounds are highly potent nanomolar inhibitors of *Ee*AChE and *Eq*BChE.

Table 2. IC50 values of compounds 9-12.

No.	R1	R2	IC_{50} (nM±SD)	
			***Ee*AChE**	***Eq*BChE**
9	3,4-CH_3	-H	33.56±1.24	2.11±0.27
10	3,4-CH_3	4-$CH(CH_3)_2$	64.91±1.75	2.32±0.12
11	3,4-CH_3	3,4-OCH_3	42.71±1.39	2.13±0.46
12	2,5-CH_3	3,4-OCH_3	45.03±0.61	1.97±0.09

All compounds exhibited low nanomolar activity toward *Eq*BChE. Having in mind that all IC_{50} values are very similar and about 2 nM, it is evident that a great variety of aroylacrylyc fragment substituents may be accommodated

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

in the upper part of *Eq*BChE active site gorge.

The variation in AChE inhibition potency may be ascribed to the variation in the nature, position and bulkiness of substituents at phenylamide moiety of molecules. The lowest activity of 10 can be ascribed to the bulky isopropyl substituent in *p*-position of phenylamide fragment (R2), indicating possible steric hindrance between this moiety of **10** and PAS aminoacid residues of AChE. Somewhat higher potency have derivatives **11** and **12**, with less bulky dimetoxy substituents, and finaly, the most active derivative 9, lacks any substituents at phenylamide ring. All this indicates that any kind of phenylamide substitution has negative effects on AChE inhibition potency. Also, the position of -CH_3 group at aroyl fragment (R1) is not the determining factor for compounds inhibition potency toward *Ee*AChE.

Comparing the results of our previous work [14] with inhibition potency of compounds **11** and **12**, the greatest impact on activity toward *Ee*AChE has the linker length. Briefly, in previous work compounds similar in structure to **11** and **12** were synthesized. The only difference was the length of linker: 1,8-diaminooctane was used in previous work, while here we used 1,7-diaminoseptane-linker, shorter for one methylene group. The compounds with longer linker showed greater activity toward *Ee*AChE. The possible explanation for this effect can be ascribed to the inability of compounds with shorter linker to properly accommodate in the AChE gorge and subsequently the interaction in PAS may not be optimal or are weaker than those of compounds with longer linker. To confirm this theory, further study of AChE-compound interactions is needed.

The greater inhibition potency of 9-12 toward *Eq*BChE is expected since the anchoring moiety of the derivatives, i.e. tacrine fragment, has a greater potency toward BChE than AChE.

5. CONCLUSION

Four new reversible, dual-binding AChE inhibitors were synthesized and their inhibitory activity toward *Ee*AChE and *Eq*BChE was examined. Path for synthesis consisted of five reaction, including reaction of Niementowski, nuclephilic aromatic substitution, and Michaels addition as last step. Compound **9**, **10** and **12** gave adequate reaction yields, while reaction of compound **11** resulted in lower reaction yield. Compound **9** exhibited lowest IC_{50} toward *Ee*AChE and the least active was compound **10.** All compounds exhibited similar activity toward *Eq*BChE. All compounds exhibited low nanomolar activity toward both cholinesterase and are considered good leads for further research.

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