Supporting Information

Macrocyclic derivatives of 6-methyluracil as ligands of the peripheral anionic site of acetylcholinesterase

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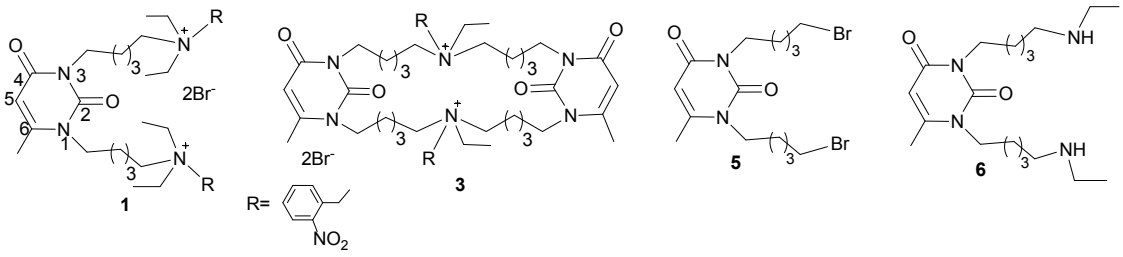
Contents

Experimental Section

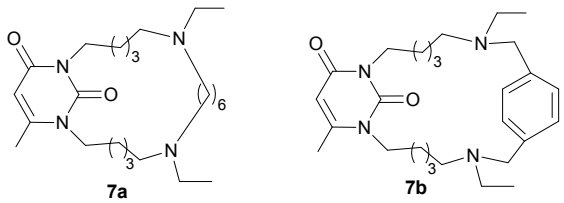
Chemistry

General Methods: The NMR experiments were carried out on Bruker spectrometers AVANCE-400 (400.1 MHz (^{1}H). Electron impact mass spectra were recorded on a Finnigan MAT-212 mass spectrometer (70 eV), MALDI-TOF mass spectra were recorded on a Bruker ULTRAFLEX mass spectrometer in *p*-nitroaniline matrix. The IR spectra of compounds were recorded on a Vector 22 FTIR Spectrometer (Bruker) in the 4000-400 cm^{-1} range at a resolution of 1 cm^{-1}. Microelemental analyses data were obtained on a CHN-3 analyzer, they were within ±0.3% of theoretical values for C, H, and N. The melting points were measured on a Boetius hot-stage apparatus. Thin layer chromatography was performed on Silufol-254 plates; visualization of spots was carried out under UV light (λ=254 nm). For column chromatography silica gel of 60 mesh from Fluka was used. All solvents were dried according to standard protocols.

Initial compounds 5,6, acyclic compound 1 and pyrimidinophanes 3,9: Synthesis of initial compounds 1,3-bis(5-ethylaminopentyl)-6-methyluracil (**5**) and 1,3-bis(5-ethylaminopentyl)-6-methyluracil (**6**), pyrimidinophanes **3** and **9** was reported previously.[1,2]



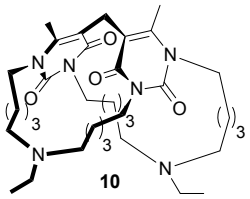
Synthesis of new pyrimidinophanes 7a,b. General procedure: A mixture of diamine **5** (6.5 mmol), 1,6-dibromohexane or *p*-bis(bromomethyl)benzene (7.2 mmol) and potassium carbonate (18.1 mmol) was stirred in CH_3CN (150 mL) at 50-55 °C for 8 h. The precipitate was filtered off. The solution was concentrated to 10-15 mL and transferred to a column with SiO_2. The column was successively washed with diethyl ether, and a 10:10:1 diethyl ether-ethyl acetate-diethyl amine mixture. The target pyrimidinophanes **2a,b** were isolated from the ethyl ether-ethyl acetate-diethyl amine fractions.



Pyrimidinophane 7a: Yield 10%; oil; IR (neat, cm^{-1}) ν_{max}: 2932, 1701, 1662, 1467, 1431, 1156; ^1H NMR ($CDCl_3$, 400 MHz) δ: 5.55 (s, 1H, $C^5_{uracil}H$), 3.94 (t, 2H, $N^3_{uracil}CH_2$, J = 7.0 Hz), 3.83 (t, 2H, $N^1_{uracil}CH_2$, J = 7.3 Hz), 2.50-2.48 (m, 4H, $2NCH_2$), 2.41-2.39 (m, 8H, $4NCH_2$), 2.22 (s, 3H, $C^6_{uracil}CH_3$), 1.66-1.64 (m, 4H, $2CH_2$), 1.52-1.44 (m, 8H, $4CH_2$), 1.38-1.32 (m, 8H, $4CH_2$), 1.02-1.00 (m, 6H, $2CH_3$). HRMS (EI) 434.3621 (M^+). Anal Calcd for $C_{25}H_{46}N_4O_2$: C, 69.08; H, 10.67; N, 12.89. Found: C, 69.00; H, 10.75; N, 12.85.

Pyrimidinophane 7b: Yield 22%; oil; IR (neat, cm^{-1}) ν_{max}: 2932, 1702, 1662, 1467, 1431, 1156; ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.26-7.22 (m, 4H, 4ArH), 5.53 (s, 1H, $C^5_{uracil}H$), 3.89 (t, 2H, $N^3_{uracil}CH_2$, J = 6.8 Hz), 3.75 (t, 2H, $N^1_{uracil}CH_2$, J = 7.3 Hz), 3.54 (s, 2H, NCH_2), 3.52 (s, 2H, NCH_2), 2.59-2.56 (m, 4H, $2NCH_2$), 2.28-2.25 (m, 4H, $2NCH_2$), 2.20 (s, 3H, $C^6_{uracil}CH_3$), 1.55-1.48 (m, 4H, $2CH_2$), 1.41-1.38 (m, 4H, $2CH_2$), 1.27-1.22 (m, 4H, $2CH_2$), 1.10-1.06 (m, 6H, $2CH_3$). HRMS(EI) 454.3307 (M^+). Anal Calcd for $C_{27}H_{42}N_4O_2$: C, 71.33; H, 9.31; N, 12.32. Found: C, 71.47; H, 9.22; N, 12.21.

Synthesis of pyrimidinophane 10: A mixture of *cis*-isomer **9** (400 mg, 0.65 mmol) and paraformaldehyde (30 mg, 1 mmol) in aqueous 1.0 N HCl (50 mL) was sealed in a glass ampule. The ampule was heated in an oil bath at 140-145 °C for 70 h. The content of the ampule was neutralized before pH 7.0-7.5 with aqueous 10% NaOH solution, evaporated, and extracted by $CHCl_3$ (2×100 ml). The extract was concentrated to 10-15 mL and transferred to a column with SiO_2. The column was successively washed with EtOAc and ethyl acetate-diethyl amine 30:1 and 20:1mixtures. Target macrocyclic compound obtained from the fractions of the EtOAc-$NHEt_2$ 20:1 mixture was washed with diethyl ether and dried under vacuum. Yield of pyrimidinophane **10** 325 mg (80%); oil; IR (neat, cm^{-1}) ν_{max}: 2937, 1700, 1657, 1454, 1365, 732; ^1H NMR ($CDCl_3$, 400 MHz) δ: 4.54-4.47 (m, 2H, $N_{uracil}CH_2$), 4.27 (d, 1H, $C^5_{uracil}CHC^5_{uracil'}$, J = 15.7 Hz), 4.09-4.03 (m, 2H, $N_{uracil}CH_2$), 3.92-3.85 (m, 2H, $N_{uracil}CH_2$), 3.50-3.44 (m, 2H, $N_{uracil}CH_2$), 3.04 (d, 1H, $C^5_{uracil}CHC^5_{uracil'}$, J = 15.7 Hz), 2.57-2.28 (m, 12H, $6NCH_2$), 2.23 (s, 6H, $C^6_{uracil}CH_3$), 1.70-1.50 (m, 16H, $8CH_2$), 1.38-1.28 (m, 8H, $4CH_2$), 1.06-1.03 (m, 6H, $2CH_3$). MALDI-MS (*m/z*): calcd for $C_{35}H_{58}N_6O_4$ $[M]^+$ 626.5, found: 626.7. Anal. Calcd for $C_{35}H_{58}N_6O_4$: C, 67.06; H, 9.33; N, 13.41. Found: C, 67.04; H, 9.38; N, 13.42.



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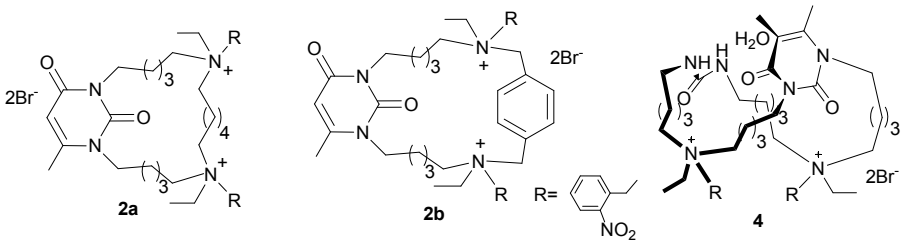
Quaternization of N atoms in the bridges of pyrimidinophanes 7a,b and 10. General procedure: A solution of pyrimidinophane **7a,b**, **10** (0.50 mmol) and 2.2-fold excess of *o*-nitrobenzyl bromide **8** in CH_3CN (50 mL) was refluxed for 30 h. The solvent was distilled off. The residue was thoroughly triturated in diethyl ether (5×30 mL), each time decanted after settling of the compound, and finally the solvent was evaporated.



Pyrimidinophane 2a: Yield 90%; yellow solid; mp 120 °C; IR (KBr, cm^{-1}) ν_{max}: 2945, 1694, 1655, 1470, 1432, 1350, 726; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.05-7.49 (m, 8H, 8ArH), 5.58 (s, 1H, $C^5_{uracil}H$), 4.84 (br. s, 4H, $2CH_2Ph$), 3.97-3.95 (m, 2H, $N^3_{uracil}CH_2$), 3.80-3.78 (m, 2H, $N^1_{uracil}CH_2$), 3.50-3.27 (m, 12H, $6NCH_2$), 2.25 (s, 3H, $C^6_{uracil}CH_3$), 1.85-1.36 (m, 20H, $10CH_2$), 1.22-1.18 (m, 6H, $2CH_3$). MALDI-MS (*m/z*): calcd for $C_{39}H_{58}Br_2N_6O_6$ [*M*-2Br]$^+$, [*M*-2Br-$C_7H_6NO_2$]$^+$ 706.4, 570.4, respectively, found: 706.6, 570.4. Anal. Calcd for $C_{39}H_{58}Br_2N_6O_6$: C, 54.04; H, 6.75; Br, 18.44; N, 9.70. Found: C, 54.10; H, 6.82; Br, 18.54; N, 9.81.

Pyrimidinophane 2b: Yield 74%; yellow solid; mp 250-251 °C; IR (KBr, cm^{-1}) ν_{max}: 2943, 1696, 1654, 1470, 1435, 1351, 727; ^{1}H NMR (D_2O, 400 MHz) δ: 8.04-7.40 (m, 12H, 12ArH), 5.58 (s, 1H, $C^5_{uracil}H$), 4.90 (br. s, 8H, $4CH_2Ph$), 3.95-3.75 (m, 4H, $N^3_{uracil}CH_2$, $N^1_{uracil}CH_2$), 3.45-3.25 (m, 8H, $4NCH_2$), 2.28 (s, 3H, $C^6_{uracil}CH_3$), 1.60-1.25 (m, 12H, $6CH_2$), 1.25-1.20 (m, 6H, $2CH_3$). MALDI-MS (*m/z*): calcd for $C_{41}H_{54}Br_2N_6O_6$ [*M*-Br]$^+$, [*M*-2Br-$C_7H_6NO_2$]$^+$ 805.3, 590.4, respectively, found: 805.2, 590.3. Anal. Calcd for $C_{41}H_{54}Br_2N_6O_6$: C, 55.53; H, 6.14; Br, 18.02; N, 9.48. Found: C, 55.45; H, 6.06; Br, 18.11; N, 9.46.

Pyrimidinophane 4: Yield 80%; yellow solid; mp 125-127 °C; IR (KBr, cm^{-1}) ν_{max}: 2953, 1694, 1653, 1470, 1432, 1350, 725; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.06-7.78 (m, 8H, 8ArH), 4.94 (s, 2H, CH_2Ph), 4.89 (s, 2H, CH_2Ph), 4.22 (d, 1H, $C^5_{uracil}CHC^5_{uracil'}$, J = 15.4 Hz), 4.05-4.00 (m, 4H, $2N_{uracil}CH_2$), 3.97-3.90 (m, 4H, $2N_{uracil}CH_2$), 3.10-2.95 (9H, $4NCH_2$, $C^5_{uracil}CHC^5_{uracil'}$), 2.27 (s, 3H, $C^6_{uracil}CH_3$), 1.80-1.55 (m, 16H, $8CH_2$), 1.45-1.25 (m, 14H, $4CH_2$, $2CH_3$). MALDI-MS (*m/z*): calcd for $C_{49}H_{70}Br_2N_8O_8$ [*M*-Br]$^+$, [*M*-2Br-$C_7H_6NO_2$]$^+$ 977.5, 762.5, respectively, found: 978.4, 762.3. Anal. Calcd for $C_{41}H_{54}Br_2N_6O_6$: C, 55.58; H, 6.66; Br, 15.09; N, 10.58. Found: C, 55.61; H, 6.60; Br, 15.14; N, 10.56.

Biological assay

In vitro cholinesterase inhibition assay

Reagents and chemicals—acetylthiocholine iodide, butyrylthiocholine iodide, AChE from human erythrocytes, BuChE from human plasma and 5,5'-dithio-bis(2-nitrobenzoic) acid (DNTB)—were purchased from Sigma–Aldrich. All assays were performed using Perkin Elmer λ25 spectrophotometer with detection at 412 nm. The enzyme-catalyzed hydrolysis reaction was carried out in 0.1 M phosphate buffer, pH 8.0 containing 0.25 units of AChE or BuChE, and 2 mM acetylthiocholine or butyrylthiocholine as substrates. Concentration range for the tested compounds was from 10^{-10} to 10^{-5} M (10 concentration points with increments of about a half). The tested compounds were incubated with the enzyme for 20 min at 25 °C prior to starting the reaction by adding the substrate. After 10, 20 or 30 min with substrate reaction was stopped by adding neostigmine (0.1 mM). Then DTNB (0.1 mM) was added to the reaction solution, and production of a yellow 5-thio-2-nitro-benzoate anion was measured spectrophotometrically. The rate of thiocholine production during hydrolysis reaction (measurements at 10, 20, 30 min) was calculated. Sample without inhibitor was used as a control (100% of cholinesterase activity). Sample without substrate was used as a blank. IC_{50} (concentration of drug producing 50% of enzyme activity inhibition) was determined using Origin 7.5. Percentage of inhibition was calculated by Hill plot.[3]

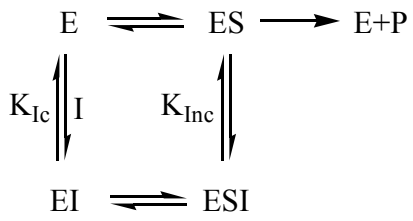
Studies of AChE inhibition mechanism

For inhibition constant (K_I) determination, two concentrations of pyrimidinophane **2b** or compound **1** were pre-incubated with AChE in 0.1 M phosphate buffer, pH 8.0, for 20 min. The acetylthiocholine iodide hydrolysis rate was then measured at 37 °C at 412 nm using a Perkin Elmer λ25 spectrophotometer after addition of DTNB (finally 0.1 mM) and acetylthiocholine iodide as substrate. The initial velocity (V) of substrate hydrolysis was measured at different substrate (S) concentrations in the range 0.05-1 mM. Lineweaver-Burk plot, i.e. the reciprocal of initial velocity (1/V) versus the reciprocal of substrate concentration (1/S) was obtained. Inhibition constant (K_I) was determined by secondary replot of Lineweaver-Burk slope versus inhibitor concentration.

Enzyme inhibition data vs IC_{50} were analyzed according to Cheng and Prusoff.[4] For mixed-type inhibition, the relationship between IC_{50} and K_i is described by equation (1):

$$\mathrm{IC}_{50} = \frac{\left(K_M + [S]\right)}{\left(\frac{K_M}{K_{Ic}} + \frac{[S]}{K_{Inc}}\right)} \qquad (1)$$

In equation (1), K_M is the Michaelis constant, K_{Ic} is the inhibition constant of the free enzyme (competitive inhibition) and K_{Inc} is inhibition constant for the enzyme-substrate-complex (non-competitive inhibition) in Scheme 3.



Scheme 3 Competitive and non-competitive inhibition of enzyme

Since experiments were performed at [S]>>K_M, equation (1) becomes equation (2) with $\alpha = K_{Ic}/K_{Inc} > 1$:

$$IC_{50} = \frac{(K_I)}{\left(\frac{K_M}{[S]} + \frac{K_{Ic}}{K_{Inc}}\right)} \quad (2)$$

Molecular docking

Molecular docking with a Lamarckian Genetic Algorithm[21] was performed with Autodock 4.2.5.1[5] software. For AChE model *Mus musculus* AChE X-ray structure (PDB ID 2HA2 [11], resolution 2.05Å, was used. Grid box for docking included the whole gorge from the mouth to the active site, including PAS. The grid box size was 22.5Å×22.5Å×22.5Å for AChE. The main of selected Lamarckian Genetic Algorithm[6] parameters were 256 runs, 25×10^6 evaluations, 27×10^4 generations and population size 300. For the best docked positions additional 256 runs of local search were performed.

Acute toxicity evaluation

Experiments involving animals were performed in accordance with the guidelines set forth by the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the protocol of experiments approved by the Animal Care and Use Committee of Kazan State Medical University.

Toxicological estimation was performed for IP injection of aqueous solution of the different compounds in rats weighting 250-300 g. Wistar rats were isolated in individual cages before manipulation and treated with compound **1**, pyrimidinophane **2b** or pyridostigmine bromide. Five different doses (determined during preliminary tests) were used with six animals per dose. Rats were observed during 72 hrs after injection, and symptoms of intoxication were registered. LD_{50},

dose (in mg/kg) causing lethal effects in 50% of animals was taken as a criterion of toxicity. LD_{50} were determined by the method of Weiss.[7]

Animal model of EAMG

Experimental autoimmune model of myasthenia gravis (EAMG) was produced according to the following protocol.[8] Rats (females, 6-8 weeks old) were immunized twice (1 month interval) by subcutaneous administration of the peptide (DGDFAIVKFTKVLLDYTGHI) mixed in Freund's adjuvant complete (first injection) and incomplete (second injection). The development of muscle weakness was diagnosed *in vivo* by the marked decrement of surface electromyogram amplitude (i.e. integral AP) of hind limb muscles. Under urethane anesthesia (1.2 g/kg, intraperitoneally) the sciatic nerve was stimulated (40 Hz, train of 200 stimuli) in the femoral part, and the integral muscle action potential from posterior surface of lower leg was recorded using skin electrodes. The integral muscle action potential was recorded using an FE132 amplifier (AD Instruments), digitized with PowerLab4/35 system (AD Instruments). Averaged values of decrement of AP amplitude in control group (five animals) and in groups with EAMG (five animals per group) were compared. Pyridostigmine bromide, compound **1** and pyrimidinophane **2b** were delivered intraperitoneally, dissolved in H_2O.

References

1 K.A. Anikienko, E.A. Bychikhin, V.S. Reznik, V.D. Akamsin and I.V. Galyametdinova, *Chem. Biol. Interact.*, 2008, **175**, 286-292.

2 V.E. Semenov, A.D. Voloshina, E.M. Toroptzova, N.V. Kulik, V.V. Zobov, R.Kh. Giniyatullin, A.S. Mikhailov, A.E. Nikolaev, V.D. Akamsin and V.S. Reznik, *Eur. J. Med. Chem.*, 2006, **41**, 1093-1101.

3 G.L. Ellman, K.D. Courtney, V. Andres and R.M. Feather-Stone, *Biochem. Pharmacology*, 1961, **7**, 88-95.

4 Y. Cheng and W.H. Prusoff, *Biochem. Pharmacology*, 1973, **22**, 3099-3108.

5 G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell and A.J. Olson, *J. Comput. Chem.*, 2009, **30**, 2785-2791.

6 G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew and A.J. Olson, *J. Am. Chem. Soc.*, 1998, **19**, 1639-1662.

7 E. S. Weiss, *Am. J. Public Health*, 1948, **38**, 22-24.

8 F. Baggi, A. Annoni, F. Ubiali, M. Milani, R. Longhi, W. Scaioli, F. Cornelio and R. Mantegazza and C. Antozzi, *J. Immunology*, 2004, **172**, 2697-703.