

Design, synthesis and biological evaluation of new 2-benzoxazolinone derivatives as potential cholinesterase inhibitors for therapy of Alzheimer's disease

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Received November 19, 2010, accepted August 8, 2010

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Pharmazie 66: 399–403 (2011)

doi: 10.1691/ph.2011.0815

Currently acetylcholinesterase inhibitor (AChEI) therapy is one of the most frequently used methods in the treatment of Alzheimer's disease; tacrine, donepezil, rivastigmine and galantamine are applied in different stages of AD. In the present study, we propose a new series of 2-benzoxazolinone derivatives as potential cholinesterase inhibitors. These compounds were synthesized by condensation of 6-chloro acetyl-2-benzoxazolinone with the corresponding amine and evaluated as acetylcholinesterase inhibitors using the colorimetric Ellman's method. Selectivity and the IC₅₀ values were determined for the received derivatives. All tested compounds exhibited the inhibitory activity towards acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Compound **3e** showed stronger activity than the standard tacrine, and compound **3a** showed activity similar to that of tacrine for AChE. Compounds **3a**, **3b**, **3c**, and **3e** showed stronger activity than the standard donepezil towards the inhibition of BChE, and the compound **3e** showed stronger activity than donepezil towards AChE.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is a neurodegenerative disorder characterized by progressive degeneration of some parts of the brain such as: hippocampus and associative regions of cerebral cortex. Irreversible degeneration of cholinergic neurons and synapses leads to disturbances in memory, cognitive functions, learning, speech, and emotional behaviour, causing full dependency to a caregiver and finally death. Alzheimer's disease affects at present about 5–10% of the population aged 65 and nearly 50% over the age of 80 (Magierski et al. 2004; Blennow et al. 2006; Ellis 2005).

Senile dementia of the Alzheimer type is characterized by lesions at the micro- and macroscopic level. Macroscopic lesions are manifested by dramatic atrophy of certain parts of the brain like frontal, parietal and temporal lobes. At the microscopic level the most characteristic changes are: the presence of neuritic or senile plaques and neurofibrillary tangles. The plaques are composed of the insoluble form of beta-amyloid which are deposited around nerves and cells. Neurofibrillary tangles, deposited within the cells, are mostly made up of hyperphosphorylated tau protein (Blennow et al. 2006; Bolognesi et al. 2005; Musial et al. 2007). The third characteristic lesion is the loss of number of cholinergic neurons and as a result the decrease in cholinergic neurotransmission (Bolognesi et al. 2005). On the basis of many observations two hypotheses have been formulated which try to explain the etiopathogenesis of Alzheimer's disease. They are called the amyloid cascade hypothesis and the cholinergic hypothesis (Magierski et al. 2004; Racchi and Govoni 2003; Bartus et al. 1982; Sugimoti 2008). On the

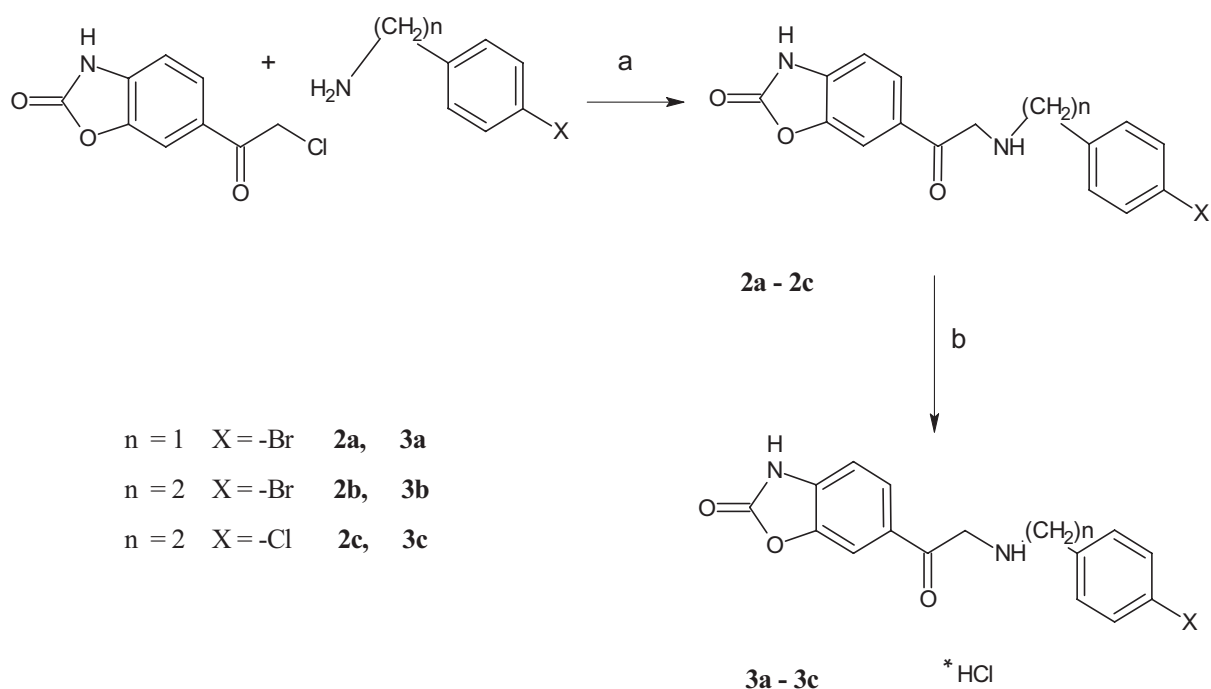
basis of the cholinergic hypothesis the first group of drugs – acetylcholinesterase inhibitors (AChEI) – were introduced for AD treatment. The first one approved in the United States for the treatment of Alzheimer's disease was tacrine, at present there are four inhibitors registered by the Food and Drug Administration (FDA): tacrine, rivastigmine, galantamine and donepezil (Musial et al. 2007; Shah et al. 2008; Sonkusare et al. 2005; Calabria et al. 2009).

In the present study, synthesis and biological evaluation of a series of 2-benzoxazolinone derivatives coupled with the N-substituted piperazines and primary aryl-aliphatic amines are described. All obtained compounds were tested for their selectivity and activity towards the inhibition of acetylcholinesterase and butyrylcholinesterase with the colorimetric Ellman's method (Szymanski et al. 2006; Calis et al. 2001).

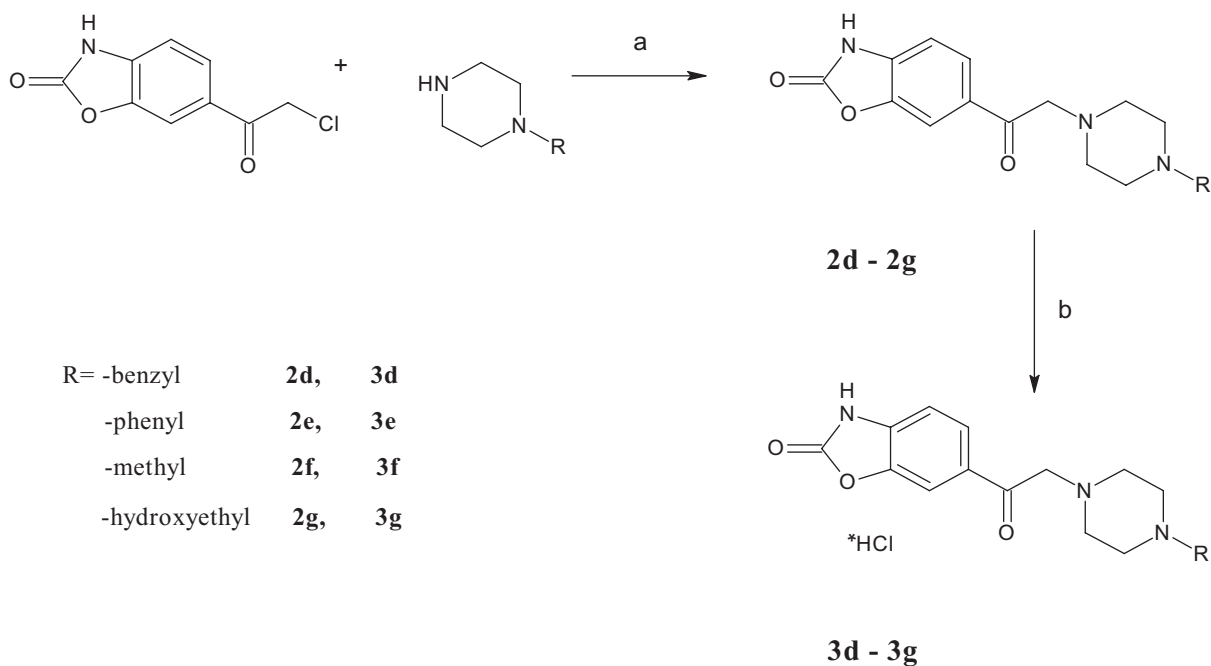
2. Investigations and results

Compounds **2a–2c** were obtained by the reaction of 6-chloroacetyl-2-benzoxazolinone with the corresponding 4-substituted aryl-aliphatic amines: 4-bromobenzylamine (**2a**), 4-bromophenethylamine (**2b**), and 4-chlorophenethylamine (**2c**) in acetonitrile (reflux at 80 °C/2 h; Scheme 1). The reaction was conducted in the presence of argone.

Compounds **2d–2g** were obtained by the reaction of 6-chloroacetyl-2-benzoxazolinone with the corresponding amines: *N*-benzylpiperazine (**2d**), *N*-phenylpiperazine (**2e**), *N*-methylpiperazine (**2f**) and *N*-hydroxyethylpiperazine (**2g**) in acetone (reflux at 60 °C, 2 or 4 h; Scheme 2). The reaction was conducted in the presence of argone. Our synthesis



Scheme 1: Scheme of synthesis of 6-chloroacetyl-2-benzoxazolinone with 4-substituted aryl-aliphatic amine derivatives (a) acetonitrile/80 °C/2 h/reflux; (b) HCl/methanol



Scheme 2: Scheme of synthesis of 6-chloroacetyl-2-benzoxazolinone with N-substituted piperazine derivatives (a) acetone/60 °C/2-4 h/reflux; (b) HCl/methanol

methodology was based on the modification of methods devised by Calis and Gorkan (2001) and Mentrup et al. (1976). The crude product was obtained by pouring the warm reaction mixture into the ice water. Monitoring the reaction by TLC showed that the reaction was usually completed within 1 h (for compounds **2g-2h**). Purified by the crystallization from methanol, the compounds were transformed to hydrochlorides (**3a-3f**) and examined for their activity towards the inhibition of AChE and BChE using tacrine and donepezil as standards.

To determine the type of inhibition we used the linear transformation of the Michaelis-Menten equation: the Lineweaver-Burk plot ($1/v$ vs. $1/[S]$). Michaelis-Menten constants: K_m and V_{max} were also obtained from the Lineweaver-Burk plot by the linear regression of the reaction rate in relation to the substrate concentration. Values of K_i constants were calculated by using

nonlinear regression. Statistical parameters and values of K_m and V_{max} for AChE and BChE are shown in Table 1. In accordance with the standard methodology, we obtained AChE and BChE inhibition activity and selectivity of the received derivatives

Table 1: Statistical parameters and values of K_m and V_{max} for AChE and BChE

Parameters	AChE	BChE
K_m	$3.71 \times 10^{-8} \text{ M}$	$9.93 \times 10^{-8} \text{ M}$
V_{max}	$5.79 \times 10^{-8} \text{ M/ml/min}$	$8.95 \times 10^{-8} \text{ M/ml/min}$
r^2	0.97	0.99
Standard error	0.00619	0.00076

Table 2: IC₅₀ values for activities towards acetylcholinesterase and butyrylcholinesterase

Compd.	AChE inhibition (IC ₅₀ , M)	BChE inhibition (IC ₅₀ , M)	Selectivity for AChE ^a	Selectivity for BChE ^b
3a	1.82×10^{-11}	9.08×10^{-13}	0.05	20.08
3b	1.03×10^{-9}	3.84×10^{-12}	3.74×10^{-3}	2.67×10^2
3c	2.16×10^{-9}	1.64×10^{-12}	7.57×10^{-3}	1.32×10^3
3d	8.04×10^{-9}	2.30×10^{-8}	2.87	0.35
3e	5.69×10^{-13}	3.18×10^{-12}	5.59	0.18
3f	3.11×10^{-8}	1.34×10^{-8}	0.04	2.32
3g	3.98×10^{-8}	2.53×10^{-8}	0.64	1.57
Tacrine	1.97×10^{-11}	5.99×10^{-13}	0.03	32.93
Donepezil	5.76×10^{-11}	3.08×10^{-10}	5.34×10^2	1.87×10^{-3}

^a Selectivity for AChE is defined as IC₅₀(BChE)/IC₅₀(AChE)^b Selectivity for BChE is defined as IC₅₀(AChE)/IC₅₀(BChE)

towards cholinesterases (Table 2). One can find that **3e** is more active compound towards the inhibition of AChE than tacrine, while compound **3a** has similar activity to tacrine. Compound **3e** has better activity than donepezil. Considering the inhibition of butyrylcholinesterase we can see that **3a**, **3b**, **3c** and **3e** are more active compounds compared with donepezil. All tested compounds exhibit lower inhibition of butyrylcholinesterase than tacrine. Out of all described derivatives, compound **3e** has the highest selectivity towards the inhibition of acetylcholinesterase and derivative **3c** has the strongest selectivity towards the inhibition of butyrylcholinesterase. All obtained compounds show lower selectivity towards the inhibition of acetylcholinesterase than donepezil and higher (except **3b** and **3c**) than tacrine. All compounds are characterized by better selectivity towards the inhibition of butyrylcholinesterase than donepezil and derivatives **3b** and **3c** have higher selectivity than tacrine.

3. Discussion

The synthesis and biological evaluation of series of 6-acetyl-2-benzoxazolinone analogs led to the design of new potential cholinesterase inhibitors. These compounds were prepared from 6-chloroacetyl-2-benzoxazolinone in the reaction with the corresponding amine. Obtained derivatives were evaluated as cholinesterase inhibitors by using the colorimetric method of Ellman. Then the IC₅₀ values and selectivity for AChE and BChE were calculated and compared with standards (tacrine, donepezil). We have found four derivatives that show higher activity towards the inhibition of BChE than donepezil, and one derivative better than tacrine and donepezil as AChE inhibitor. The most promising compound is **3e**. This derivative possesses higher inhibitory activity for AChE than tacrine and donepezil, and is a better butyrylcholinesterase inhibitor than donepezil. The results suggest that the new derivatives may be potential drugs for treatment of Alzheimer's disease. The structure of 6-acetyl-2-benzoxazolinone is an innovation and it opens the way for the modification of these molecules in searching compounds with better activities and selectivity for the inhibition of cholinesterases.

4. Experimental

4.1. Chemistry

Reactions were monitored by TLC using 25 DC-Alufolien Kieselgel 60F254 plates (Merck). Melting points were measured on the Electrothermal apparatus in open capillaries and were uncorrected. IR spectra were recorded in KBr using a Mattson Infinity Series FT-IR spectrophotometer. ¹H NMR spectra were recorded with a Varian Mercury 300 MHz spectrometer, using tetramethylsilane as an internal standard. Mass spectra were performed by the Centre of Molecular and Macromolecular Studies in Lodz (Polish Academy of Sciences) on an apparatus Finnigan Mat 95. All the results of elemental analyses were in an acceptable range.

4.1.1. 6-[(4-Bromobenzyl)amino]acetyl]-1,3-benzoxazol-2(3H)-one (**2a**)

6-Chloroacetyl-2-benzoxazolinone (0.40 g, 1.89 mmol) was dissolved in acetonitrile (40 ml, 766 mmol), then 4-bromobenzylamine (0.70 g, 3.78 mmol) was added and the mixture was heated in 80 °C under reflux for 75 min. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2a**: m.p.: 160–162 °C; yield: 53%; ¹H NMR (DMSO) (δ ppm): 4.23 (2H, d, J=1.0, CH₂), 4.56 (2H, s, CH₂), 7.17–7.19 (1H, d, J=7.6, ArH), 7.45–7.49 (2H, d, J=8.2, ArH), 7.64–7.66 (2H, d, J=7.9, ArH); 7.85–7.91 (2H, m, ArH), 9.77 (2H, 2xNH); MS(FAB) m/z (M+1): 361.1, 170.8; MS-HR calcd: 361.018224, found: 361.018790 C₁₆H₁₃BrN₂O₃.

4.1.2. 6-([2-(4-Bromophenyl)ethyl]amino)acetyl]-1,3-benzoxazol-2(3H)-one (**2b**)

6-Chloroacetyl-2-benzoxazolinone (0.40 g, 1.89 mmol) was dissolved in acetonitrile (40 ml, 766 mmol), then 4-bromophenethylamine (0.59 g, 3.78 mmol) was added and the mixture was heated in 80 °C under reflux for 135 min. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2b**: m.p.: 122–125 °C; yield: 49%; ¹H NMR (DMSO) (δ ppm): 3.07–3.12 (2H, m, CH₂CH₂), 3.16–3.25 (2H, m, CH₂CH₂), 4.90 (2H, s, CH₂), 7.35–7.42 (3H, m, ArH), 7.56–7.62 (2H, m, ArH), 7.91–7.97 (2H, m, ArH), 11.30 (2H, 2xNH); MS(FAB) m/z (M+1): 375.3, 184.8; MS-HR calcd: 375.033874; found: 375.034440 C₁₇H₁₅BrN₂O₃.

4.1.3. 6-([2-(4-Chlorophenyl)ethyl]amino)acetyl]-1,3-benzoxazol-2(3H)-one (**2c**)

6-Chloroacetyl-2-benzoxazolinone (0.40 g, 1.89 mmol) was dissolved in acetonitrile (40 ml, 766 mmol), then 4-bromophenethylamine (0.59 g, 3.78 mmol) was added and the mixture was heated in 80 °C under reflux for 1 h. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2c**: m.p.: 117–120 °C; yield 54%; ¹H NMR (DMSO) (δ ppm): 3.02–3.08 (2H, m, CH₂CH₂), 3.08–3.20 (2H, m, CH₂CH₂), 4.80 (2H, s, CH₂), 7.27–7.32 (3H, m, ArH), 7.40–7.42 (2H, d, ArH), 7.86–7.91 (2H, m, ArH), 9.37 (2H, 2xNH); MS(FAB) m/z (M+1): 331.1, 138.9, 156.8; MS-HR calcd: 330.084396; found (M+1): 331.084945 C₁₇H₁₅ClN₂O₃.

4.1.4. 6-[(4-Benzylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one (**2d**)

6-Chloroacetyl-2-benzoxazolinone (0.20 g, 0.94 mmol) was dissolved in acetone (20 ml, 272 mmol), then *N*-benzylpiperazine (0.33 g, 1.89 mmol) was added and the mixture was heated in 60 °C under reflux for 2 h. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a yellow solid. Compound **2d**: m.p. 140–142 °C; yield 50%; ¹H NMR (DMSO) (δ ppm): 3.35–3.69 (8H, m, piperaz.), 4.42 (2H, s, ArCH₂), 5.02 (2H, s, CH₂), 7.31–7.34 (1H, m, ArH), 7.52–7.68 (5H, m, ArH), 7.87–7.96 (2H, m, ArH), 12.11 (1H, NH); Anal. calcd for C: 68.36, N: 11.95, H: 6.02; found: C: 68.29, N: 11.74, H: 6.30 C₂₀H₂₁N₃O₃.

4.1.5. 6-[(4-Phenylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one (2e)

6-Chloroacetyl-2-benzoxazolinone (0.20 g, 0.94 mmol) was dissolved in acetone (20 ml, 272 mmol), then *N*-phenylpiperazine (0.31 g, 1.86 mmol) was added and the mixture was heated in 60 °C under reflux for 2 h. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2e**: m.p. 182–185 °C; yield 17%; ¹H NMR (DMSO) (δ ppm): 2.49–2.68 (4H, m, piperaz.), 3.10–3.37 (4H, m, piperaz.), 3.88 (2H, s, CH₂), 6.74–6.79 (1H, m, ArH), 6.91–6.94 (2H, d, J = 7.7, ArH), 7.17–7.23 (3H, m, ArH), 7.89–7.93 (2H, m, ArH), 12.57 (1H, NH); MS(FAB) m/z (M+1): 338.2, 175.0; MS-HR calcd: 338.149918; found: (M+1): 338.150466 C₁₉H₁₉N₃O₃.

4.1.6. 6-[(4-Methylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one (2f)

6-Chloroacetyl-2-benzoxazolinone (0.20 g, 0.94 mmol) was dissolved in acetone (20 ml, 272 mmol), then *N*-methylpiperazine (0.19 g, 1.86 mmol) was added and the mixture was heated in 60 °C under reflux for 2 h. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2f**: m.p. 130–134 °C; yield: 23%; ¹H NMR (DMSO) (δ ppm): 2.80 (3H, s, CH₃), 2.82–3.45 (8H, m, piperaz.), 4.91 (2H, s, CH₂), 7.26–7.29 (1H, m, ArH), 7.85–7.89 (2H, m, ArH), 12.36 (1H, s, NH); MS(FAB) m/z (M+1): 276.1, MS-HR calcd: 276.134268; found: (M+1): 276.134816. C₁₄H₁₇N₃O₃.

4.1.7. 6-[(4-(2-Hydroxyethyl)piperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one (2g)

6-Chloroacetyl-2-benzoxazolinone (0.30 g, 1.42 mmol) was dissolved in acetone (20 ml, 272 mmol), then *N*-hydroxyethylpiperazine (0.37 g, 1.86 mmol) was added and the mixture was heated in 60 °C under reflux for 4 h. The warm reaction mixture was poured into ice-water. The precipitate was filtered and dried in vacuum. Recrystallization from methanol gave the product as a white solid. Compound **2g**: m.p.: 182–184 °C; yield: 26%; ¹H NMR (DMSO) (δ ppm): 2.36–2.49 (4H, m, CH₂CH₂), 3.30–3.46 (8H, m, piperaz.), 5.11 (2H, s, CH₂), 7.18–7.22 (1H, m, ArH), 7.87–7.93 (2H, m, ArH). C₁₅H₁₉N₃O₄.

4.1.8. 6-[(4-Bromobenzyl)amino]acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3a)

Compound **2a** (0.20 g, 0.55 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3a**: m.p.: 262–266 °C; yield: 51%; IR(KBr) ν(cm⁻¹): 1561.6, 1619.5, 1679.5, 1755.2, 2928.5, 3123.6; ¹H NMR (DMSO) (δ ppm): 4.17 (2H, d, J = 0.4, CH₂), 4.73 (2H, s, CH₂), 7.25–7.28 (1H, d, J = 7.9, ArH), 7.52–7.55 (2H, d, J = 8.7, ArH), 7.64–7.66 (2H, d, J = 8.3, ArH), 7.83–7.89 (2H, m, ArH), 9.70 (2H, 2×NH). C₁₆H₁₄BrClN₂O₃.

4.1.9. 6-([2-(4-Bromophenyl)ethyl]amino)acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3b)

Compound **2b** (0.20 g, 0.53 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and the next recrystallised from methanol, isolated and dried in vacuum. Compound **3b**: m.p.: 270–271 °C; yield 56%; IR(KBr) ν(cm⁻¹): 1491.2, 1620.2, 1679.0, 1757.1, 2930.7, 3130.3; ¹H NMR (DMSO) (δ ppm): 3.02–3.07 (2H, m, CH₂CH₂), 3.18–1.23 (2H, m, CH₂CH₂), 4.80 (2H, s, CH₂), 7.23–7.30 (3H, m, ArH), 7.53–7.57 (2H, m, ArH), 7.86–7.92 (2H, m, ArH), 11.26 (2H, 2×NH). C₁₇H₁₆BrClN₂O₃.

4.1.10. 6-([2-(4-Chlorophenyl)ethyl]amino)acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3c)

Compound **2c** (0.20 g, 0.60 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3c**: m.p.: 262–264 °C; yield 56%; IR(KBr) ν(cm⁻¹): 1494.6, 1620.1, 1678.6, 1762.7, 2928.5, 3133.0; ¹H NMR (DMSO) (δ ppm): 3.03–3.08 (2H, m, CH₂CH₂), 3.18–3.20 (2H, m, CH₂CH₂), 4.80 (2H, s, CH₂), 7.27–7.32 (3H, m, ArH), 7.40–7.43 (2H, d, J = 8.3, ArH), 7.86–7.91 (2H, m, ArH), 9.14 (2H, 2×NH). C₁₇H₁₆Cl₂N₂O₃.

4.1.11. 6-[(4-Benzylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3d)

Compound **2d** (0.20 g, 0.57 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3d**: m.p.: 232–236 °C; yield: 55%; IR(KBr) ν(cm⁻¹): 1630.5, 1692.2, 1775.4, 3016.6, 3367.0; ¹H NMR (DMSO) (δ ppm): 3.30–3.60 (8H, m, piperaz.), 4.39 (2H, s, ArCH₂), 5.00 (2H, s, CH₂), 7.26–7.29 (1H, m, ArH), 7.45–7.65 (5H, m, ArH), 7.85–7.90 (2H, m, ArH), 12.39 (1H, NH); MS(FAB) m/z (M+1): 352.1, 189.0; MS-HR: calcd: 352.165568; found: (M+1): 352.166116. C₂₀H₂₂ClN₃O₃.

4.1.12. 6-[(4-Phenylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3e)

Compound **2e** (0.10 g, 0.30 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3e**: m.p.: 260–264 °C; yield: 50%; IR(KBr) ν(cm⁻¹): 1618.7, 1690.4, 1769.6, 2923.9, 3338.9; ¹H NMR (DMSO) (δ ppm): 3.25–3.83 (8H, m, piperaz.), 5.17 (2H, s, CH₂), 6.85–6.90 (1H, m, ArH), 7.01–7.10 (2H, d, J = 7.9, ArH), 7.25–7.33 (3H, m, ArH), 7.86–7.90 (2H, m, ArH), 12.52 (1H, NH). C₁₉H₂₀ClN₃O₃.

4.1.13. 6-[(4-Methylpiperazin-1-yl)acetyl]-1,3-benzoxazol-2(3H)-one hydrochloride (3f)

Compound **2f** (0.10 g, 0.36 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3f**: m.p.: 274–278 °C; yield: 18%; IR(KBr) ν(cm⁻¹): 1622.5, 1683.6, 1782.7, 2820.8, 2962.9, 3456.4; ¹H NMR (DMSO) (δ ppm): 2.73 (3H, s, CH₃), 2.82–3.46 (8H, m, piperaz.), 4.86 (2H, s, CH₂), 7.26–7.29 (1H, m, ArH), 7.85–7.90 (2H, m, ArH), 12.33 (1H, s, NH). C₁₄H₁₈ClN₃O₃.

4.1.14. 6-([4-(2-Hydroxyethyl)piperazin-1-yl]acetyl)-1,3-benzoxazol-2(3H)-one hydrochloride (3g)

Compound **2g** (0.10 g, 0.33 mmol) was dissolved in methanol (3 ml), HCl/methanol was added dropwise. The reaction mixture was stirred in crushed ice. The precipitate was isolated and next recrystallised from methanol, isolated and dried in vacuum. Compound **3g**: m.p.: 268–272 °C; yield: 32%; IR(KBr) ν(cm⁻¹): 1609.4, 1662.9, 1760.0, 2924.5, 3419.1; ¹H NMR (DMSO) (δ ppm): 2.41–2.51 (4H, m, CH₂CH₂), 3.42–3.50 (8H, m, piperaz.), 5.14 (2H, s, CH₂), 7.14–7.17 (1H, m, ArH), 7.84–7.87 (2H, m, ArH); MS(FAB) m/z (M+1): 306.2, 143.0; MS-HR: calcd: 306.144833; found: (M+1) 306.145381

4.2. Enzymatic assay

Cholinesterases and their potential inhibitor activities were determined by the spectrophotometric method of Ellman with some modifications. Hydrolysis rates (v) were measured at 8 various substrate concentrations [S] – acetylthiocholine iodide in 3 ml of the sample volume contained also phosphate-buffered solution (0.1 M, pH 8.0), a solution of 5.50-diethoxybisnitrobenzoic acid (DTNB, 0.05 ml, 0.5 M), enzyme (AChE or BChE, 0.05 ml, 5 U/ml) and the appropriate inhibitor. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB and spectrophotometric assay at 412 nm after 1 min. Determination samples without inhibitor gave 100% of AChE or BChE activity. The IC₅₀ values for inhibitor concentration was calculated by using linear transformation of the Michaelis-Menten equation: the Lineweaver-Burk plot.

Acknowledgement: This work was supported by the grant from Polish Ministry of Science and Higher Education (No NN 405 302 636).

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