Bioorganic & Medicinal Chemistry Letters 20 (2010) 1763–1766

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Preparation and in vitro screening of symmetrical bispyridinium cholinesterase inhibitors bearing different connecting linkage—initial study for Myasthenia gravis implications

Kamil Musilek [a,d,*], Marketa Komloova [b], Vlasta Zavadova [b], Ondrej Holas [b], Martina Hrabinova [c], Miroslav Pohanka [c], Vlastimil Dohnal [d], Florian Nachon [e], Martin Dolezal [b], Kamil Kuca [c,d], Young-Sik Jung [f,*]

[a] Department of Toxicology, Faculty of Military Health Sciences, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
[b] Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic
[c] Centre of Advanced Studies, Faculty of Military Health Sciences, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
[d] Department of Chemistry, Faculty of Science, University of Jan Evangelista Purkyne, Ceske mladeze 8, 400 96 Usti nad Labem, Czech Republic
[e] Toxicology Department, Enzymology Unit, Centre de Recherches du Service de Sante des Armees, BP 87, 38702 La Tronche Cedex, France
[f] Bioorganic Science Division, Korea Research Institute of Chemical Technology, PO Box 107, Yuseong, Daejeon 305-606, Republic of Korea

ARTICLE INFO

Article history:
Received 6 November 2009
Revised 4 January 2010
Accepted 6 January 2010
Available online 20 January 2010

Keywords:
Cholinesterase
Inhibitor
Myasthenia gravis
Quaternary
In vitro
Docking

ABSTRACT

Reversible inhibitors (e.g., pyridostigmine bromide, neostigmine bromide) of carbamate origin are used in the early treatment of Myasthenia gravis (MG) to block acetylcholinesterase (AChE) native function and conserve efficient amount of acetylcholine for decreasing number of nicotinic receptors. Carbamate inhibitors are known for many undesirable side effects related to the reversible inhibition of AChE. In contrast, this paper describes 20 newly prepared bispyridinium inhibitors of potential concern for MG. Although some compounds from this series have been known before, they were not assayed for cholinesterase inhibition yet.

The newly prepared compounds were evaluated in vitro on human erythrocyte AChE and human plasmatic butyrylcholinesterase (BChE). Their inhibitory ability was expressed as IC_{50} and compared to standard carbamate drugs. Three compounds presented promising inhibition (in μM range) of both enzymes in vitro similar to the used standards. The novel inhibitors did not present selectivity between AChE and BChE. Two newly prepared compounds were chosen for docking studies and confirmed apparent π–π or π–cationic interactions aside enzyme's catalytic sites. The kinetics assay confirmed non-competitive inhibition of AChE by two best newly prepared compounds.

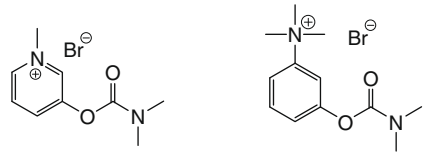
Inhibitors of acetylcholinesterase (EC 3.1.1.7; AChE) are widely scoped for various purposes such as Alzheimer disease (AD).[1] Differently, quaternary AChE inhibitors (e.g., pyridostigmine bromide **1**, neostigmine bromide **2**; Fig. 1) are used for the treatment of Myasthenia gravis (MG).[2] Namely, quaternary AChE inhibitors **(1–2)** take part as a symptomatic treatment in the mild stage of MG. In fact, MG is autoimmunity disease with progressive decrease of peripheral AChE nicotinic receptors dealing with muscle weakness and painless.[3] Quaternary AChE inhibitors are targeted to competitively block AChE vital function and consequently enable the excess of acetylcholine on decreasing amount of nicotinic receptors.[4]

Although **1–2** are widely used in the treatment of MG, they are known for many side effects including gastrointestinal effects (nausea, intestinal obstruction), increased bronchial secretion, cardiac arrhythmia or cholinergic crisis.[5] Mentioned side effects of **1–2** are related to the reversible inhibition of AChE. This reversible inhibition is common for carbamate inhibitors (e.g., **1–2**) that are binding to serine oxygen in the AChE active site.[6] The resulting carbamylated enzyme intermediate inhibits AChE activity until a water molecule attacks the carbonyl to reactivate enzyme and produces a carbamic acid derivative. The spontaneous regeneration of carbamylated enzyme proceeds in the range of minutes.

Furthermore, the blood–brain barrier penetration is not required for MG treatment to decrease central side effects and ensure necessary peripheral activity.[7] For this purpose, **1–2** contain quaternary nitrogen. This structural feature is important for increased peripheral effect of mentioned drugs, where the charged compounds are penetrating in minor ratio.[8] In fact, the BBB crossing of **1–2** is limited, but may proceed through disruptive mechanisms.[9] Thus, **1–2** may still exhibit strong central side effects related to carbamylation of brain AChE that results in cholinergic crisis.[6]

* Corresponding authors. Tel.: +420 973 251 523; fax: +420 495 518 094 (K.M.); tel.: +82 42 860 7135; fax: +82 42 860 7348 (Y.-S.J.).
E-mail addresses: musilek@pmfhk.cz (K. Musilek), ysjung@krict.re.kr (Y.-S. Jung).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.01.034



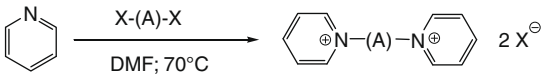
pyridostigmine bromide (**1**) neostigmine bromide (**2**)

Figure 1. Carbamate compounds are already used for early Myasthenia gravis treatment.

On the other hand, there is huge variety of compounds reversibly inhibiting AChE that might be used in the MG treatment.[10] In contrast to carbamates, their effect should originate for competitive reversible inhibition of AChE aside the active serine.[11] Moreover, their increased peripheral effect and decreased BBB crossing should be ensured. More valuably, the selective AChE inhibition instead of dual AChE/BChE (butyrylcholinesterase; EC 3.1.1.8) inhibition might keep the non-specific esterase (BChE) active for other toxic substrates.[12] Our approach originates from design, synthesis, in vitro screening and docking studies of selected bisquaternary pyridinium compounds connected by various linkers.

In this Letter, the preparation of 20 symmetrical bisquaternary AChE inhibitors (**3–22**) is reported (Fig. 2). Although the synthesis of such compounds is trivial, their structural differences are important for comprehension of key factors influencing inhibition of AChE and/or BChE (Table 1).[13] Moreover, these compounds were previously not evaluated as AChE or BChE inhibitors.[14–20]

Namely, presented compounds differ in the structure of the connecting linker. The key length and/or spatial orientation of connecting linker are highly important factors for molecular interactions among the enzyme active sites. Subsequently, the optimal structure of the linker depends not only on the length, but is usually related to presented π-electrons (double bond or aromatic residues) or heteroatom (hydrogen-bonding interactions), too.[21]



X = Cl, Br

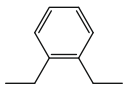
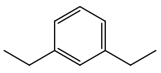
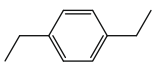
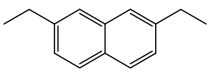
(A)	**Compound** (anion)
$(CH_2)_{1\text{-}12}$	**3-14** (Br)
CH_2OCH_2	**15** (Cl)
$(CH_2)_2O(CH_2)_2$	**16** (Br)
(*E*)-$CH_2CH{=}CHCH_2$	**17** (Br)
(*Z*)-$CH_2CH{=}CHCH_2$	**18** (Cl)
	**19** (Br)
	**20** (Br)
	**21** (Br)
	**22** (Br)

Figure 2. Prepared bisquaternary pyridinium salts bearing different linkers.

Table 1
Inhibitory results of tested compounds (a—no significant inhibition in the selected concentration scale)

Compound	AChE IC_{50} ± SD (μM)	BuChE IC_{50} ± SD (μM)	AChE K_{i1}/K_{i2} (μM/l)
Pyridostigmine (**1**)	40 ± 7.8	16000 ± 260.8	—
Neostigmine (**2**)	0.1 ± 0.02	0.8 ± 0.13	—
3	a	a	—
4	a	a	—
5	a	a	—
6	505 ± 98.5	9800 ± 159.7	—
7	1270 ± 247.6	120 ± 19.7	—
8	63 ± 12.3	130 ± 21.2	—
9	241 ± 47.0	78 ± 12.7	—
10	31 ± 6.0	29 ± 4.7	—
11	2 ± 0.4	6 ± 0.9	—
12	0.4 ± 0.08	5 ± 0.8	0.02/0.03
13	0.7 ± 0.14	7 ± 1.1	—
14	a	a	—
15	1770 ± 345.2	583 ± 95.0	—
16	2010 ± 631.2	2360 ± 384.7	—
17	2290 ± 392.0	541 ± 88.2	—
18	1990 ± 388.1	1270 ± 207.0	—
19	636 ± 124.0	345 ± 56.2	—
20	2360 ± 460.2	1380 ± 224.9	—
21	1540 ± 300.3	529 ± 86.2	—
22	0.2 ± 0.04	0.8 ± 0.13	0.14/0.14

The pyridinium part of the molecule was chosen among the other heteroaromatic rings due to its small size and universality. Moreover, the pyridinium ring is plain structure with π-electrons that may interact with many amino-acid residues (Phe, His, Trp, Tyr). These amino-acid residues are well known for their principal function in the enzyme active sites via non-covalent interactions (AChE or BChE).[22]

Additionally, the peripheral effect of AChE inhibitors valuable for MG treatment is preferred, because the reduced amount of such inhibitor in the central nervous system decreases side effects via minor interactions with the brain AChE. For this reason, the charged molecules were chosen. Moreover, the monoquaternary compounds are penetrating BBB at least in 10%, whereas the bisquaternary compounds were previously found to penetrate the blood–brain barrier (BBB) in less than 5%.[8,23] Hence, the bisquaternary molecules were designed to maintain the peripheral inhibitory activity against AChE.

The new inhibitors (**3–22**; Fig. 2) were prepared via standard synthetic strategy.[24] The solution of pyridine (1 ml, 12.4 mmol) and corresponding alkylating agent (5.6 mmol) in DMF (10 ml) was stirred at 70 °C. The reaction mixture was cooled to the room temperature, portioned with acetone (50 ml) and cooled in refrigerator (5 °C) overnight. The crystalline or amorphous crude product was collected by filtration, washed with acetone (3 × 20 ml) and recrystallized from MeCN. NMR, ESI-MS and elemental analysis determined the purity of all compounds.

The bisquaternary pyridinium compounds were assayed for their inhibitory ability in standard inhibition test using human erythrocyte AChE (hAChE) and human plasmatic BChE (hBChE).[25] The IC_{50} values of all compounds are listed in Table 1.

The commercial compound **1** presented satisfactory inhibition of hAChE in μM range. On the other hand, compound **1** showed no inhibition of hBChE. Differently, compound **2** resulted as strong inhibitor both of hAChE and hBChE and had approximately threefold lower IC_{50} compared to **1** for hAChE. From these in vitro results, compound **2** seems to be more valuable for MG treatment among two tested commercial reversible inhibitors.

Concerning the inhibition of the prepared compounds towards AChE, there may be seen at least three trends of inhibitory ability within the whole series. Firstly, compounds **3–5** and **14** did not

inhibit hAChE at selected concentration scale. Second variety of compounds (**6–9, 15–21**) displayed only poor inhibition (in mM range) without further concern. More interestingly, third group (**10–13** and **22**) presented inhibition in μM range. Among third group of compounds, **12–13** and **22** (0.7–0.2 μM) kept the best inhibitory ability towards hAChE in the whole series. Their inhibition of hAChE was similar, although the structure of the connecting linker was completely different. Apparently, the different weak (π–π or π–cationic) interactions of **12–13** and **22** with the AChE's active sites are the most important for the inhibitory ability of newly prepared compounds. Additionally, compound **22** exhibited similar inhibition of hAChE such as commercial compound **2**. Moreover, the inhibitory ability of compounds **10–13** and **22** exceeded the most used commercial compound **1**.

Concerning the inhibition of the prepared compounds towards BChE, some trends in the inhibitory ability of newly prepared compounds may be recognized. Firstly, compounds **3–5** and **14** presented no inhibition towards hBChE. Second variety of compounds (**6–8, 15–21**) displayed only poor inhibition of BChE (in mM range). Third variety of compounds (**9–13** and **22**) displayed inhibition in μM range of potential interest. Among these inhibitors, compound **22** displayed the best inhibitory ability towards BChE comparable to inhibitory ability of standard compound **2**. Moreover, compound **22** exceeded the inhibitory ability of commercial standard **1**.

Unfortunately, the new compounds **10–13** and **22** displayed only poor selectivity between hAChE and hBChE, if they were compared to **1**. This lack of selectivity may limit their further in vivo evaluation and possible use due to interference with BChE that is responsible for unspecific esterase activity in the organism.[12] Though BChE has not direct effect on physiological functions, its pharmacological and toxicological importance has been formerly recognised. Namely, BChE is involved in degradation of numerous drug and poisons.[12] While many drugs are mixed inhibitors of AChE and BChE, truly selective AChE inhibitors are compounds of interest to not affect non-specific function of BChE.

The dissociation constants (K_{i1} and K_{i2}) were calculated for two most promising compounds (**12, 22**) interacting with AChE.[26] In both cases, non-competitive mechanism of inhibition was confirmed by enzyme kinetics assay. Both inhibition constants found for compound **22** were very similar ($K_{i1} \sim K_{i2} = 0.14$ μmol/l). It seemed that inhibitor **22** was able to provide quite stable complex with enzyme as well as with enzyme and substrate. The inhibitor interaction was not influenced by the molecule of substrate located in catalytic unit. On the other side, compound **12** was found stronger inhibitor of AChE ($K_{i1} = 0.02$ μmol/l; $K_{i2} = 0.03$ μmol/l). The dissociation constant was slightly higher for complex enzyme–substrate–inhibitor (K_{i2}) compared to the constant of enzyme–inhibitor complex (K_{i1}). Regarding the obtained data, molecule **12** bound stronger to erythrocyte hAChE than compound **22**.

The docking studies were performed on 2 promising compounds after in vitro screening (**12, 22**) in order to rationalize possible interactions within AChE and BChE.[27–31] Regarding AChE, top-scored docking pose of **12** (−7.61 kcal/mol) showed apparent interactions with aromatic residues of internal anionic site (IAS) and peripheral anionic site (PAS) (Fig. 3). Namely, strong π–π or cation–π interaction between one pyridinium moiety and Trp86 (3.9 Å) from IAS occurred. The second pyridinium moiety was stacked to PAS residues resulting strong π–π or cation–π interaction Trp286 (3.7 Å). Similarly, compound **22** (−9.67 kcal/mol) presented double T-stacking with Trp86 (3.5 Å) and Trp286 (3.4 Å). The interactions seemed to be very similar and consequently compounds **12** and **22** resulted in the in vitro screening with very similar IC_{50} (0.4 and 0.2 μM).

For BChE, top-scored docking pose of **12** (−6.91 kcal/mol) showed interactions with aromatic Trp residues (Fig. 4). The π–π or cation–π interaction with Trp82 (3.7 Å) and the T-stacking with Trp231 (3.7 Å) occurred. Similarly, compound **22** (−7.45 kcal/mol) presented the π–π or cation–π interaction with Trp82 (4.1 Å) and T-stacking with Trp231 (3.4 Å). Moreover, naphtylene linker displayed one more T-stacking with Phe329 (3.7 Å) that closes the active site of the BChE. This interaction may explain, why the less flexible linkage (naphtylene) in compound **22** resulted as approximately onefold better inhibitor of BChE (0.8 μM vs 5 μM of **12**) in in vitro screening.

The SAR results originated from the docking studies and in vitro data.[32] Firstly, the pyridinium moiety showed its importance from point of view of π–π and π–cationic interactions with aromatic

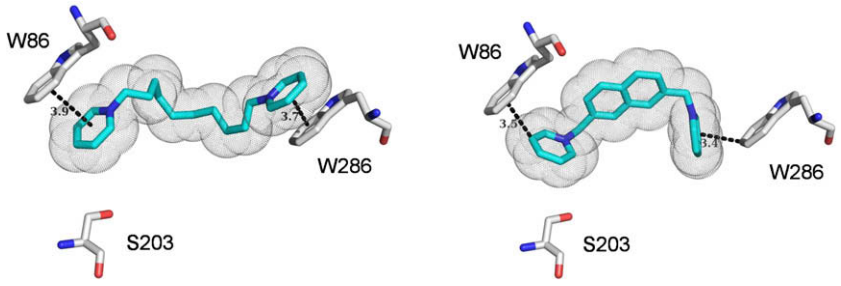


Figure 3. Docking results with compounds **12** and **22** for AChE.

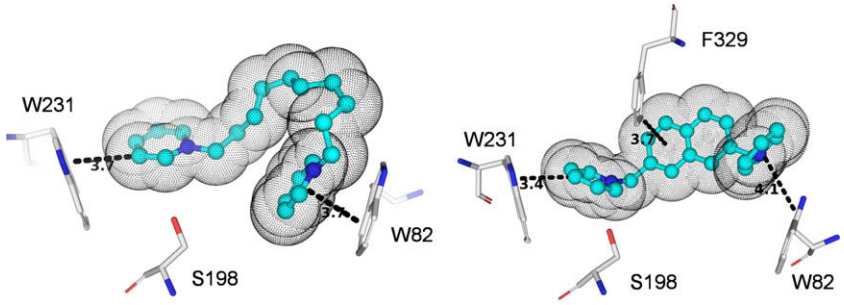


Figure 4. Docking results with compounds **12** and **22** for BChE.

residues of internal anionic and peripheral anionic site. Though the pyridinium moiety was used within this series of compounds, the other heteroaromatic moieties (e.g., quinolinium, *iso*-quinolinium) might be probably used and will be object of further interest.

Secondly, the used linkage highly influenced the inhibitory ability of prepared compounds. The length of the connecting linkage remained the most important factor within whole series of compounds. The compounds with 8–12 methylene units (**10–13**) resulted the as the best among tested inhibitors for hAChE. These results apparently correlate with the distance of IAS and PAS in hAChE and thus with interactions of pyridinium moiety. The situation changed for hBChE, where the PAS is not presented. Compounds **10–13** were stacked between two Tyr residues and consequently resulted as potent inhibitors. Compounds bearing shorter (**3–8**) or longer methylene linkers (**14**) were found inefficient for both enzymes.

Differently, heteroatom (**15–16**), double bond (**17–18**), xylene (**19–21**) and naphtylene linkage (**22**) were introduced to find possible interactions.[33] Not surprisingly, most of these compounds (**15–21**) displayed only minor inhibitory activity of both enzymes (mM range). The length of the linkage in mentioned compounds varied from 4 to 6 analogues of methylene units that were insufficient to interact similarly as the compounds **10–13**. Due to the poor in vitro results of **15–21**, the plausible interactions with both enzymes were not further studied. Most interestingly, compound **22** with naphtylene linkage exhibited the best inhibitory ability for both used enzymes. Although its linker was shorter compared to **10–13**, it displayed the same binding between the AChE active sites. Moreover, compound **22** showed one more T-stacking in BChE directly released by naphtylene linker and consequently resulted as the best inhibitor of hBChE.

In summary, 20 symmetrical bispyridinium compounds were prepared. Their ability to inhibit hAChE or hBChE was tested in vitro and expressed as IC_{50}. The inhibitory results were compared to standard compounds for early MG treatment (pyridostigmine bromide, neostigmine bromide). Three newly prepared compounds showed IC_{50} comparable to neostigmine bromide and better than pyridostigmine bromide. Mentioned promising compounds did not present selectivity between AChE and BChE. Consequently, two prepared compounds with promising inhibitory ability were determined via docking study with AChE and BChE. The apparent molecular interactions of π–π or π–cationic origin were described, binding aside enzyme's catalytic sites found out and subsequently the in vitro data resolved. The kinetic studies of two most promising compounds confirmed non-competitive inhibition of AChE.

Acknowledgements

The authors express their appreciation to Mrs. M. Hrabinova and Mr. Petr Stodulka for their technical assistance. This work was supported by the Grant Agency of the Czech Republic No. 203/09/P130 and by the Grant Agency of the Charles University No. 117909/2009/B-CH/FaF.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.034.

References and notes

1. Giacobini, E. In *Cholinesterases and Cholinesterase Inhibitors*; Giacobini, E., Ed.; Martin Dunitz: London, 2000; pp 1–226.
2. Garcia-Carrasco, M.; Escarcega, R.; Fuentes-Alexandro, S.; Riebeling, C.; Cervera, R. *Autoimmun. Rev.* **2007**, *6*, 373.
3. Vernino, S.; Cheshire, V. P.; Lennon, V. A. *Auton. Neurosci. Basic.* **2001**, *88*, 187.
4. Newsom-Davis, J. *Semin. Neurol.* **2003**, *23*, 191.
5. Vincent, A.; Palace, J.; Hilton-Jones, D. *Lancet* **2001**, *357*, 2122.
6. Gupta, R. In *Toxicology of Organophosphate and Carbamate Compounds*; Elsevier Academic Press: London, 2006; pp 209–218.
7. Sinton, C. M.; Fitch, T. E.; Petty, F.; Haley, R. W. *Toxicol. Appl. Pharmacol.* **2000**, *165*, 99.
8. Sakurada, K.; Matsubara, K.; Shimizu, K.; Shiono, H.; Seto, Y.; Tsuge, K.; Yoshino, M.; Sakai, I.; Mukoyama, H.; Takatori, T. *Neurochem. Res.* **2003**, *28*, 1401.
9. Pavlovsky, L.; Browne, R. O.; Friedman, A. *Exp. Neurol.* **2003**, *179*, 181.
10. Kaur, J.; Zhang, M. Q. *Curr. Med. Chem.* **2000**, *7*, 273.
11. Barril, X.; Kalko, S. G.; Orozco, M.; Luque, F. J. *Mini-Rev. Med. Chem.* **2002**, *2*, 27.
12. Masson, P.; Carletti, E.; Nachon, F. *Protein Pept. Lett.* **2009**, *16*, 1215.
13. Musilek, K.; Holas, O.; Jun, D.; Dohnal, V.; Gunn-Moore, F.; Opletalova, V.; Dolezal, M.; Kuca, K. *Bioorg. Med. Chem.* **2007**, *15*, 6733.
14. Muramatsu, T.; Hanaya, K.; Ikegami, Y. *Chem. Lett.* **1986**, *15*, 2139.
15. Muramatsu, T.; Hanaya, K.; Onodera, S.; Ikegami, Y. *Chem. Lett.* **1987**, *16*, 1683.
16. Ayers, J. T.; Dwoskin, L. P.; Deaciuc, A. G.; Grinevich, V. P.; Zhu, J.; Crooks, P. A. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3067.
17. Lyle, R. E.; Gardikes, J. J. *J. Am. Chem. Soc.* **1955**, *77*, 1291.
18. Ng, C. K. L.; Singhal, V.; Widmer, F.; Wright, L. C.; Sorrell, T. C.; Jolliffea, K. A. *Bioorg. Med. Chem. Lett.* **2007**, *15*, 3422.
19. Oshikiri, T.; Takashima, Y.; Yamaguchi, H.; Harada, A. *J. Am. Chem. Soc.* **2005**, *127*, 12186.
20. Crooks, P. A.; Ayers, J. T.; Xu, R.; Sumithran, S. P.; Grinevich, V. P.; Wilkins, L. H.; Deaciuc, A. G.; Allen, D. D.; Dwoskin, L. P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1869.
21. Kuca, K.; Jun, D.; Musilek, K. *Mini-Rev. Med. Chem.* **2006**, *6*, 269.
22. Saxena, A.; Fedorko, J. M.; Vinayaka, C. R.; Medhekar, R.; Radic, Z.; Taylor, P.; Lockridge, O.; Doctor, B. P. *Eur. J. Biochem.* **2003**, *270*, 4447.
23. Lorke, D. E.; Kalasz, H.; Petroianu, G. A.; Tekes, K. *Curr. Med. Chem.* **2008**, *15*, 743.
24. Musilek, K.; Lipka, L.; Racakova, V.; Kuca, K.; Jun, D.; Dohnal, V.; Dolezal, M. *Chem. Pap.* **2006**, *60*, 48.
25. Pohanka, M.; Jun, D.; Kuca, K. *Talanta* **2008**, *77*, 451.
26. Pohanka, M.; Hrabinova, M.; Kuca, K. *Sensors* **2008**, *8*, 5229.
27. Sauvaitre, T.; Barlier, M.; Herlem, D.; Gresh, N.; Chiaroni, A.; Guenard, D.; Guillou, C. *J. Med. Chem.* **2007**, *50*, 5311.
28. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639.
29. Available on the World Wide Web http://www.pdb.org.
30. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.
31. DeLano, W.L. The PyMOL Molecular Graphics System (2002) on the World Wide Web http://www.pymol.org.
32. Kuca, K.; Jun, D.; Musilek, K.; Bajgar, J. *Front. Drug Des. Disc.* **2007**, *3*, 381.
33. Musilek, K.; Kuca, K.; Jun, D.; Dohnal, V.; Dolezal, M. *J. Enzyme Inhib. Med. Chem.* **2005**, *20*, 409.