DOI: 10.1002/cmdc.200900455

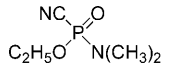
# Monooxime-monocarbamoyl Bispyridinium Xylene-Linked Reactivators of Acetylcholinesterase—Synthesis, In vitro and Toxicity Evaluation, and Docking Studies

Kamil Musilek,*[a, d] Ondrej Holas,[b] Jan Misik,[a] Miroslav Pohanka,[c] Ladislav Novotny,[c] Vlastimil Dohnal,[d] Veronika Opletalova,[b] and Kamil Kuca[a, c, d]

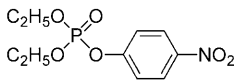
Acetylcholinesterase (AChE) reactivators are crucial antidotes to organophosphate intoxication. A new series of 26 monooxime-monocarbamoyl xylene-linked bispyridinium compounds was prepared and tested in vitro, along with known reactivators (pralidoxime, HI-6, obidoxime, trimedoxime, methoxime, K107, K108 and K203), on a model of tabun- and paraoxon-, methylparaoxon- and DFP-inhibited human erythrocyte AChE. Although their ability to reactivate tabun-inhibited AChE did not exceed that of the previously known compounds, some newly prepared compounds showed promising reactivation of pesticide-inhibited AChE. The acute toxicity of the novel compounds was also determined. Docking studies using tabun-inhibited AChE were performed for three compounds of interest. The structure–activity relationship (SAR) study confirmed the apparent influence of the xylene linkage and carbamoyl moiety on the reactivation ability and toxicity of the agents.

## Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) plays a very important role in human neurotransmission, degrading the neurotransmitter acetylcholine within the synaptic cleft. A considerable number of both natural and artificial AChE inhibitors have been produced, tested and used for various purposes.[1–2] The organophosphate inhibitors (OPIs) belong to the synthetic class of AChE inhibitors, and many are available worldwide (e.g., pesticides: chlorpyrifos, parathion, diazinon).[3] Some OPIs



tabun



paraoxon

are among the most toxic artificial compounds known and have been used as chemical warfare agents (e.g., sarin, soman, tabun (GA), VX).[3–4] Industrial OPIs (e.g., tri-*O*-cresylphosphate) are used as plasticizers or flame retardants, while these compounds are toxic, this effect does not originate from the inhibition of AChE.[5]

OPIs inhibit AChE via covalent binding to the serine hydroxy group within the active site.[3–4] When inhibited, AChE is not able to fulfil its essential role in neurotransmission—nondegraded acetylcholine accumulates within the synaptic cleft resulting in over-stimulation and a subsequent cholinergic crisis, which results in serious malfunction of the breathing centre followed by death.[3] Various therapies are used to counteract the toxic effects of OPIs. A pretreatment therapy is used in individuals primarily exposed to OPIs (e.g., soldiers); it contains weak AChE inhibitors (e.g., pyridostigmine) to sequester the enzyme and may additionally contain oxime reactivators (e.g., oxime HI-6), or other esterases (e.g., human butyrylcholinesterase) to scavenge the OPI.[6–8] The oxime reactivators are the drugs of choice for the post-treatment of OPI intoxication and are used worldwide.[5] They contain an oxime (hydroxyiminomethyl) group that cleaves the AchE–OPI covalent bond, restoring enzymatic function. Pralidoxime (**1**; 2-hydroxyiminomethyl-1-methylpyridinium chloride), oxime HI-6 (**2**; 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) obidoxime (**3**; 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride) trimedoxime (**4**; 1,3-bis(4-hydroxyiminomethylpyridinium)propane dibromide) or methoxime (**5**; 1,1-bis(4-hydroxyiminomethylpyridinium)methane dichloride) are commercially available oxime reactivators.[9–11] In addition to AChE reactivators, atropine therapy is

[a] *Dr. K. Musilek, J. Misik, Dr. K. Kuca*
*Department of Toxicology, Faculty of Military Health Sciences*
*University of Defence, Trebesska 1575, Hradec Kralove (Czech Republic)*
*Fax: (+42) 495-518-094*
*E-mail: musilek@pmfhk.cz*

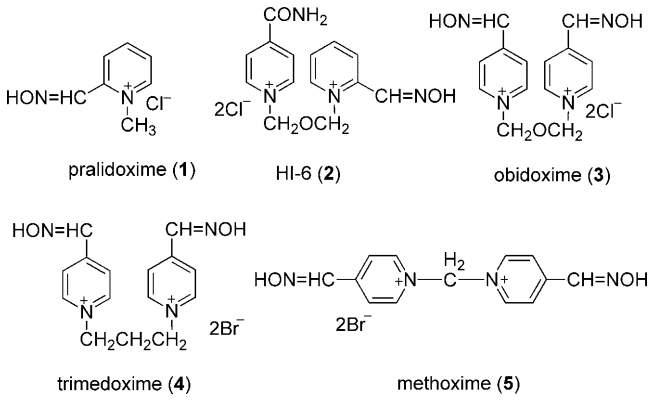
[b] *O. Holas, V. Opletalova*
*Department of Pharmaceutical Chemistry and Drug Control*
*Faculty of Pharmacy in Hradec Kralove, Charles University in Prague*
*Heyrovskeho 1203, 500 05 Hradec Kralove (Czech Republic)*

[c] *Dr. M. Pohanka, L. Novotny, Dr. K. Kuca*
*Centre of Advanced Studies, Faculty of Military Health Sciences*
*Trebesska 1575, Hradec Kralove (Czech Republic)*

[d] *Dr. K. Musilek, Dr. V. Dohnal, Dr. K. Kuca*
*Department of Chemistry, Faculty of Science, University of Jan Evangelista Purkyne, Ceske mladeze 8, 400 96 Usti nad Labem (Czech Republic)*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200900455.




also used to protect neurotransmission against a cholinergic crisis, and diazepam is used as an anticonvulsant.[3]

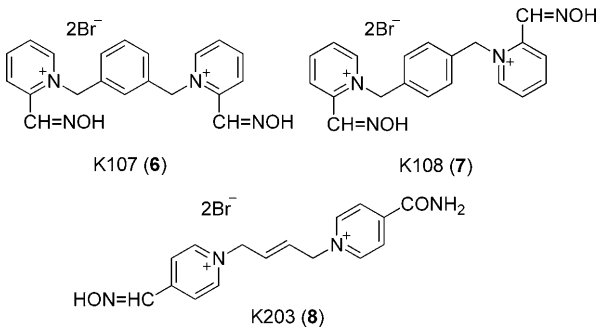
After AChE inhibition, a process called aging takes place. Further to the covalent binding of OPI in the active site, some important hydrogen bonds change, and a negative charge is formed due to partial degradation of the OPI–AChE complex. The aged OPI–AChE complex can not be reactivated by nucleophilic oxime.[12–13]

Herein, we present detailed results on the design, synthesis and in vitro evaluation of a new series of monooxime-monocarbamoyl xylene-linked bispyridinium reactivators. This series extends previous successful compounds against tabun (GA) and/or organophosphate pesticides, which are currently being tested worldwide.[14–18]

## Results and Discussion

### Design and synthesis of bispyridinium reactivators

Bispyridinium reactivators described herein were primarily based on promising known oximes. The molecular features of K107 (**6**), K108 (**7**) and K203 (**8**) were combined to design monooxime-monocarbamoyl xylene-linked compounds **9**–**34**.




Firstly, symmetrical xylene-linked bispyridinium bisoximes **6**–**7** showed promising reactivation of chlorpyrifos- and tabun-inhibited AChE in vitro.[19–20] Though they were selected by in vitro screening, they showed increased in vivo toxicity (lower $LD_{50}$ values) compared to all commercial reactivators.[21] However, the xylene linker was still considered to be a valuable molecular tool in the design of new AChE reactivators. The xylene linker appears to be involved in cation–π interactions within the enzyme active site. Moreover, nonsymmetrical xylene-linked bispyridinium bisoximes showed extended ability to reactivate paraoxon-inhibited enzymes in vitro, although there were inefficient against GA intoxication.[20]

Secondly, bispyridinium monooxime **8** showed the most effective reactivation of tabun-inhibited AChE in vitro among all commercial and previously prepared compounds, regarding activity–toxicity properties.[22] Its excellent reactivation of tabun-inhibited AChE was also confirmed in vivo.[23] Much like compounds **6**–**7**, **8** also contains source of π-electrons ((*E*)-butene linker), but only one oxime moiety. The second oxime group was replaced by carbamoyl functional group that decreases the toxicity of **8** compared with compounds **3**–**4**.[22] Different functional groups were tested to improve the properties of compound **8**, however, only the carbamoyl showed significant reactivation ability of tabun-inhibited AchE in vitro.[24–26]

Hence, the valuable molecular features from previous experiments (xylene linkage, carbamoyl functional group) were combined to afford monooxime-monocarbamoyl xylene-linked compounds.

The new reactivators (**9**–**34**; Figure 1, Table 1) were prepared via a general synthetic strategy (Scheme 1).[27–28] The monoquaternary monooxime salts were prepared according to a literature procedure.[20] The reaction yields varied greatly (3–95%), depending on the reactivity of the pyridine compounds with the carbamoyl moiety. Whereas the 3- and 4-carbamoylpyri-

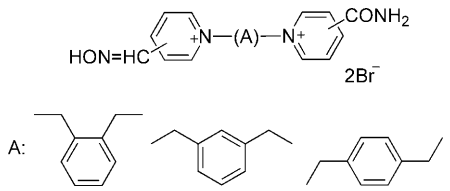
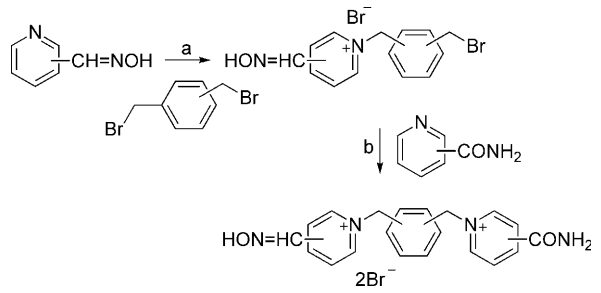



**Figure 1.** Structure of newly prepared monooxime-monocarbamoyl xylene linked compounds.

**Table 1.** Structure of newly prepared compounds.

| Compd | A | Oxime | Carbamoyl | Compd | A | Oxime | Carbamoyl |
|---|---|---|---|---|---|---|---|
| **9** | o | 2′ | 2 | **22** | m | 3′ | 4 |
| **10** | o | 2′ | 3 | **23** | m | 4′ | 2 |
| **11** | o | 2′ | 4 | **24** | m | 4′ | 3 |
| **12** | o | 3′ | 3 | **25** | m | 4′ | 4 |
| **13** | o | 3′ | 4 | **26** | p | 2′ | 2 |
| **14** | o | 4′ | 2 | **27** | p | 2′ | 3 |
| **15** | o | 4′ | 3 | **28** | p | 2′ | 4 |
| **16** | o | 4′ | 4 | **29** | p | 3′ | 2 |
| **17** | m | 2′ | 2 | **30** | p | 3′ | 3 |
| **18** | m | 2′ | 3 | **31** | p | 3′ | 4 |
| **19** | m | 2′ | 4 | **32** | p | 4′ | 2 |
| **20** | m | 3′ | 2 | **33** | p | 4′ | 3 |
| **21** | m | 3′ | 3 | **34** | p | 4′ | 4 |



**Scheme 1.** Synthesis of monooxime-monocarbamoyl xylene linked compounds. *Reagents and conditions*: a) acetone, reflux; b) DMF, 50–100°C.

dines presented very good reactivity, 2-carbamoyl pyridine reacted not sufficiently due to steric hindrance close to pyridine nitrogen. Nevertheless, the necessary amount of all compounds for in vitro evaluation was obtained.

One planned compound, (2-carbamoyl-3′-hydroxyiminomethyl-1,1′-(1,2-phenylenedi-methyl)bispyridinium dibromide), could not be made by the synthetic strategy described in Scheme 1, however, we propose its minor reactivation ability based on our observations below.

### OPI-inhibited AChE reactivation

Known compounds (**1**–**8**) and the prepared reactivators **9**–**34** were assayed for their reactivation potency using human erythrocyte AChE inhibited by tabun (GA) and organophosphate pesticides: paraoxon (POX), methylparaoxon (MePOX) and mimic agent diisopropylfluorophosphate (DFP).[29] The selected organophosphates were chosen for testing based on their molecular divergence after AChE inhibition: dimethyl- (MePOX), diethyl- (POX) or diisopropyl- (DFP) moiety. The screening concentrations were selected as in previous experiments, such that a concentration of 100 μM should be attainable after in vivo administration.[30] The reactivation results are shown in Table 2.

Reactivation in vitro should exceed 10% to suggest a promising compounds warranting further testing.[30] Concerning tabun-inhibited AChE, commercial compounds **1**–**5** presented the expected results.[31] While compounds **1** and **2** were almost

**Table 2.** Reactivation ability of tested oximes (reactivation (%) ±SD).[a]

| Inhibitor | GA | | POX | | MePOX | | DFP | |
|---|---|---|---|---|---|---|---|---|
| Reactivator | 100 μM | 1 μM | 100 μM | 1 μM | 100 μM | 1 μM | 100 μM | 1 μM |
| pralidoxime (**1**) | −4.0±1.0 | −1.7±0.5 | 8.6±2.4 | 6.5±2.1 | 8.3±2.3 | 5.5±0 | 1.3±0.5 | 10.5±0.8 |
| HI-6 (**2**) | 0.9±2.2 | 2.6±1.4 | 9.6±1.6 | −3.0±0.6 | 7.3±1.6 | 6.6±1.5 | −7.0±3.7 | −2.0±1.8 |
| obidoxime (**3**) | 7.1±0.6 | 0.3±0.6 | 42.4±4.0 | 4.0±0.5 | 18.7±2 | 2.6±2.8 | 70.6±4.6 | 71.1±4.7 |
| TMB-4 (**4**) | −3.8±3.5 | 5.1±1.2 | 35.7±0.4 | 7.8±0.8 | 17.4±1.8 | 4.8±0.6 | 0.2±1.9 | 17.7±1.4 |
| MMB-4 (**5**) | 12.3±1.0 | 2.7±1.5 | −10.4±2.7 | −2.5±1.2 | −8.1±1.0 | 8.6±0.5 | −8.0±1.2 | 0.4±1.0 |
| K107 (**6**) | −0.6±1.2 | −0.9±0.3 | 5.2±1.6 | 0.9±1.6 | −4.0±2.9 | 1.8±1.8 | 0.2±2.0 | 5.8±1.3 |
| K108 (**7**) | −0.8±1.9 | 0.9±0.6 | 52.4±4.6 | 21.5±3.6 | 4.1±2.3 | 11.4±2.5 | −8.0±3.7 | 11.3±1.7 |
| K203 (**8**) | 15.9±2.2 | 0.1±1.0 | 62.5±3.1 | 30.2±1.9 | 20.1±1.1 | 5.6±1.3 | 8.8±2.7 | 0±2.1 |
| **9** | 9.6±3.9 | −0.3±0.6 | 0.3±0.8 | 1.1±0.7 | 0.9±0.7 | 7.1±0.6 | −0.6±0.4 | −0.1±0.3 |
| **10** | −3.5±2.1 | −3.0±0.9 | 10.8±3.1 | −2.0±4.1 | −3.0±0.6 | 8.5±2.6 | −6.0±3.2 | 1.8±2.1 |
| **11** | 4.9±0.6 | −1.5±0.9 | 7.8±4.6 | 2.6±3.3 | 1.4±2.2 | 12.5±4.7 | 5.0±3.0 | −6.0±1.9 |
| **12** | 3.9±0.3 | 3.1±4.0 | 39.3±1.0 | 43.2±1.5 | 14.3±3.6 | 20.0±2.4 | 3.6±2.8 | 2.3±0.3 |
| **13** | −1.5±0.4 | −3.2±0.6 | 0.8±1.7 | 1.7±1.5 | 8.3±0.3 | 1.6±0.4 | −3.0±0.4 | 5.0±1.8 |
| **14** | 4.3±1.2 | −0.1±1.3 | −5.0±3.6 | 4.0±3.0 | 19.8±0.8 | 9.5±2.3 | 1.0±1.8 | 0.3±2.7 |
| **15** | −0.9±1.3 | −2.3±2.0 | 2.2±2.3 | 0.8±3.1 | 2.8±1.1 | 8.4±1.3 | −2.0±2.7 | 3.8±3.1 |
| **16** | −1.5±2.1 | 0.5±0.7 | 3.3±1.3 | −4.0±4.5 | 2.5±1.4 | 9.9±2.9 | −4.0±1.9 | 0±2.0 |
| **17** | −2.4±1.6 | 0.3±0.6 | 14.4±4.3 | −8.0±1.5 | 1.7±1.9 | 2.9±1.2 | −2.0±3.0 | −6.0±0.5 |
| **18** | −0.7±0.3 | 0.6±0.2 | 3.9±0.3 | −2.0±1.2 | −2.0±2.9 | 8.3±2.4 | 0.8±1.4 | −1.0±0.7 |
| **19** | 2.2±0.3 | 0.3±1.2 | 5.5±3.3 | −1.0±1.3 | −3.0±4.6 | 4.7±1.7 | −5.0±2.8 | −3.0±0.5 |
| **20** | 0.4±0.4 | −2.3±0.9 | −4.0±3.4 | 0.2±2.2 | 5.1±2.6 | 10.3±2.0 | −5.0±0.6 | −1.0±1.3 |
| **21** | −3.2±1.5 | −3.4±1.0 | 7.3±1.3 | 6.2±1.6 | 6.5±0.5 | 9.9±1.1 | 0.8±0.6 | 1.7±0.5 |
| **22** | 0.6±2.8 | 0.6±2.2 | −2.0±1.6 | 5.4±3.9 | −2.0±3.1 | 6.0±4.3 | 1.7±3.1 | 3.3±0.7 |
| **23** | −4.0±1.4 | −0.8±0.6 | −6.0±4.7 | −2.0±1.4 | −1.0±1.3 | 5.2±0.5 | −6.0±1.6 | −2.0±0.2 |
| **24** | −0.1±2.2 | 0.3±0.4 | 7.8±0.8 | 4.4±0.6 | 7.8±0.7 | 8.9±3.5 | −6.0±1.2 | −1.0±1.1 |
| **25** | 1.2±0.3 | 1.0±0.8 | 10.8±2.9 | 16.2±3.8 | 0±2.9 | 6.9±0.7 | 0.6±3.8 | −4.0±2.0 |
| **26** | −1.9±0.2 | −4.2±0.5 | 8.0±1.5 | 20.9±2.5 | −1.0±2.2 | 5.3±1.7 | 0.7±0.6 | 0.7±0.9 |
| **27** | 0.0±1.5 | 0.3±1.7 | 11.9±2.7 | 16.2±0.1 | 2.6±0.1 | 5.3±0.7 | 3.7±1.7 | −2.0±0.4 |
| **28** | 2.0±2.7 | 0.4±0.4 | 22.9±3.5 | 22.5±1.8 | 9.2±5.0 | 8.9±0.5 | 1.2±1.3 | 0.7±1.3 |
| **29** | −9.2±2.1 | −3.1±2.4 | 10.2±0.9 | 18.1±1.6 | 0±1.9 | 4.5±0.9 | −7.0±1.7 | −3.0±0.8 |
| **30** | −3.1±0.6 | 1.2±0.3 | 10.7±1.4 | 15.8±0.7 | 3.3±1.2 | 0.2±1.7 | −7.0±1.5 | −6.0±1.4 |
| **31** | −3.2±1.8 | −0.3±0.8 | 7.4±1.2 | 10.9±3.0 | 2.9±1.9 | 1.8±0.8 | −1.0±1.5 | 1.6±1.1 |
| **32** | −2.2±0.9 | 0.6±1.4 | 4.5±1.8 | −2.0±2.3 | 8.7±4.0 | 10.9±2.9 | 1.5±2.3 | −2.0±2.6 |
| **33** | 3.7±0.7 | −2.0±1.6 | −1.0±0.9 | −3.0±3.8 | −4.0±4.0 | 2.5±0.9 | −1.1±3.8 | −0.5±5.9 |
| **34** | −4.5±2.4 | −10.4±0.6 | −7.0±2.5 | −3.0±1.1 | −0.7±2.8 | 6.6±4.2 | −6.0±3.5 | −3.0±2.1 |

[a] %, mean value of three independent determinations ±SD; time of inhibition 5 min; time of reactivation by AChE reactivators 15 min; pH 7.4; temperature 25°C

ineffective against tabun, derivatives **3**–**5** showed some reactivation ability. Interestingly, compound **5** showed the best in vitro results of all commercial compounds against tabun-inhibited AchE at a concentration of 100 μM. Conversely, compounds **1** and **4** presented increased oximolysis—the hydrolysis of used substrate (thiocholine) by oxime itself that interferes with reactivation for highly concentrated oximes.[32] Known compounds **6**–**8** presented divergent results; although xylene-linked compounds **6**–**7** showed mainly oximolysis, compound **8** (K203) surpassed all commercial compounds and was also found to be the best reactivator of tabun-inhibited AChE among all tested derivatives.[22–23]

Regarding the pesticide-inhibited AChE, known compounds presented different reactivation ability for various pesticides. For POX-inhibited AChE, compound **8** was the most promising agent at both screening concentrations.[28] Against MePOX-inhibited AChE, reactivator **8** showed the best ability at 100 μM, however, xylene-linked bis-oxime **7** showed the best ability at 1 μM. Concerning DFP-inhibited AChE, reactivator **3** exceeded all other commercial compounds at both concentrations tested.

The newly prepared mono-oximes showed interesting differences in reactivation of tabun. Although most of them presented only oximolysis, several compounds (**9, 11**–**12, 14, 33**) had some reactivation ability at 100 μM. Compound **9** was found to be the best, but did not exceed the reactivator ability of known oxime **8**.

On the other hand, different mono-oximes showed improved reactivation of pesticide-inhibited AChE. Namely, compounds **12** and **28**) exceeded all other newly prepared compounds against POX intoxication at both screening concentrations. Unfortunately, their ability to reactivate POX-inhibited AChE was lower compared with the known compound **8**. In the case of MePOX-inhibited AChE, compound **12** was again found to be the most potent among the newly prepared agents. Moreover, the ability of compound **12** to reactivate MePOX-inhibited AChE at 1 μM surpassed all other tested compounds. Regarding DFP-inhibited AChE, all newly prepared compounds showed minor reactivation of the enzyme, when compared with the best commercial compound against DFP intoxication (**3**).

### Acute toxicity evaluation

The acute toxicity of the reactivator ($LD_{50}$) is considered to be an important factor for further evaluation and development. Literature data suggests that oxime HI-6 is the least toxic reactivator among the commercial compounds (Table 3), however, it is ineffective against tabun or organophosphate pesticides.[33] Similarly, methoxime **5** showed low toxicity, but also low ability to reactivate organophosphate pesticide- or DFP-inhibited enzymes.[34] Conversely, the more effective commercial AChE reactivators against tabun and organophosphate pesticides **3**–**4** showed increased toxicity (lower $LD_{50}$ values).[33,35] Interestingly, K203 (**8**) was found to be more toxic than compounds **3**–**4** in mice, whereas its toxicity in rat was lower compared with **3**–**4**.[25] Among the xylene compounds, the very high toxicity of symmetrical bisoximes was previously seen in rat.[36] In our experiments, these data were confirmed for mice also, where compounds **6**–**7** were the most toxic compounds (Table 3). Regarding the K203 (**8**) results in rat, the replacement of one oxime by a carbamoyl moiety in xylene-linked compounds was hypothesised to decrease their toxicity. This hypothesis was supported when compound **12**—a promising reactivator of POX- and MePOX-inhibited AchE—displayed toxicity similar to compound **8**. However, the toxicity of the newly prepared compounds in mice was found to be higher than the toxicity of the commercial reactivators.

**Table 3.** Toxicity of selected compounds in mice.[a]

| Compound | $LD_{50}$ [mg kg$^{-1}$] |
|---|---|
| pralidoxime (**1**) | 263.6 (253.7–273.8)[33] |
| HI-6 (**2**) | 671.3 (627.4–718.3)[33] |
| obidoxime (**3**) | 188.4 (156.3–208.0)[33] |
| TMB-4 (**4**) | 150.5 (142.1–159.4)[35] |
| MMB-4 (**5**) | 641.8 (590.5-716.0)[34] |
| K107 (**6**) | 6.6 (4.4–8.0) |
| K108 (**7**) | 2.2 (0.8–3.2) |
| K203 (**8**) | 95.0 (88.4–102.2)[23] |
| K646 (**12**) | 100.3 (78.9–134.7) |

[a] 95% confidence limits given in parentheses were calculated by probit-logarithmic method.

### Docking studies and SAR discussion

The docking studies were performed on three compounds (**3, 8, 12**) to rationalise possible interactions within tabun-inhibited AChE, the main organophosphate of interest.[23] Obidoxime (**3**) was chosen as the commercial reactivator with proposed ability to reactivate AchE inhibited by tabun.[33] Its top-scored docking pose (−10.01 kcal mol$^{-1}$; Figure 2) showed mainly interactions with aromatic residues of the internal cationic site (ICS) and peripheral anionic site (PAS): namely, T-stacking with Tyr 337 (3.4 Å) from the ICS and sandwiching between Trp 286 (3.9 Å) and Tyr 124 (4.3 Å) from the PAS. Moreover, the non-reactivating oxime moiety hydrogen bonds to Ser 298 (2.1 Å). Even though compound **3** was considered to be one of the best commercial reactivators of tabun-inhibited AChE, modelling studies showed that the distance between the oxime moiety and the tabun phosphorus atom is long (5.7 Å). This distance limits its reactivation ability towards GA-inhibited AChE. This finding was also supported by in vitro reactivation data (Table 2).

Oxime K203 (**8**) is a known, very promising reactivator of tabun-inhibited AChE.[22–23] Its top-scored docking pose (−9.87 kcal mol$^{-1}$; Figure 3) showed interactions with aromatic residues of the ICS and PAS similar to compound **3**: again, T-stacking with Tyr 337 (3.6 Å) from the ICS and sandwiching between Trp 286 (3.6 Å) and Tyr 124 (3.8 Å) from the PAS. However, unlike compound **3**, the carbamoyl moiety forms a hydrogen bonded with Trp 286 (3.4 Å) and Glu 285 (2.9 Å). Moreover, the π-electrons of the double bond in the connecting linker displayed possible T-stacking to Phe 297 (3.7 Å) and Phe 338

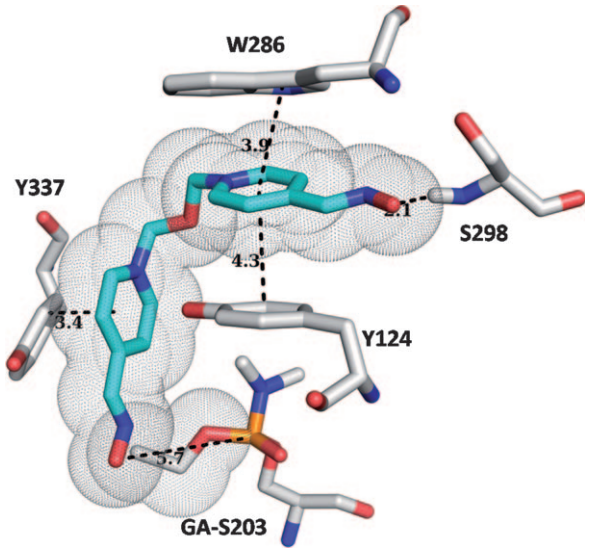



**Figure 2.** Molecular docking study on obidoxime (**3**; in blue).

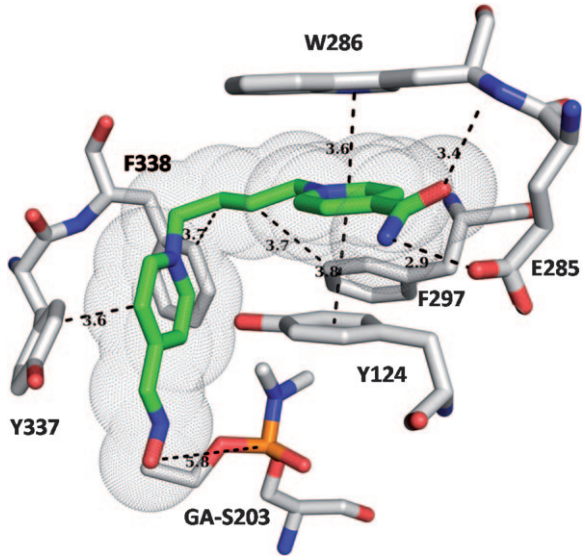



**Figure 3.** Molecular docking study on K203 (**8**; in green).

(3.7 Å). Although compound **8** was found to be the best reactivator of tabun-inhibited AChE from the tested series, the distance of the oxime moiety from the phosphorus atom of tabun was found to be similar to that of compound **3** (5.8 Å). Consequently, the improved reactivation ability of compound **8** compared with **3** can be attributed to the slightly stronger interactions with aromatic residues and stronger hydrogen bonding of the carbamoyl moiety (Table 2).

Compound **12** was chosen for docking studies from the new series of reactivators in order to rationalise the low reactivation of tabun-inhibited AChE. While compound **12** showed minor reactivation of tabun-inhibited AChE in vitro, like compounds **3** and **8**, it was a promising reactivator of POX- and MePOX-inhibited enzymes. Hence, this lack of enzyme reactivation by compound **12** became the object of interest. The top-scored docking pose (−12.51 kcal mol$^{-1}$; Figure 4) showed interactions with aromatic residues of the ICS and PAS similar to compounds **3** and **8**—namely, T-stacking with Tyr337 (3.0 Å) from

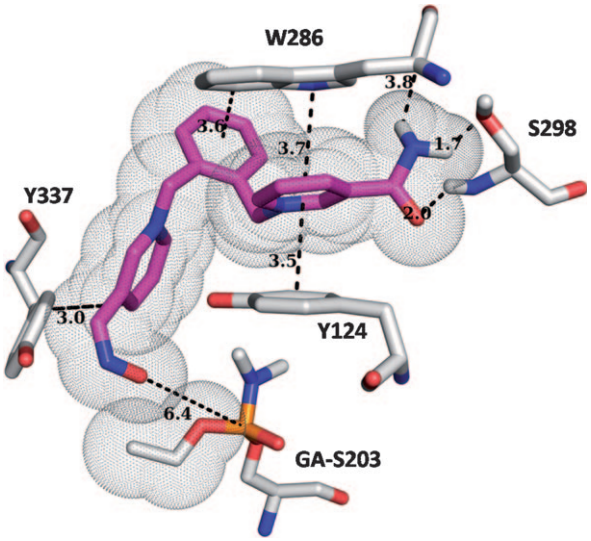



**Figure 4.** Molecular docking study on compound **12** (in magenta).

the ICS, sandwiching between Trp286 (3.7 Å) and Tyr124 (3.5 Å) from the PAS and one more T-stacking interaction between the xylene linker and Trp286 (3.6 Å) from the PAS. Similar to compound **8**, the carbamoyl moiety is involved in a hydrogen bond with Trp286 (3.8 Å) and Ser298 (1.7 Å and 2.0 Å). Nevertheless, the distance of the oxime moiety from the tabun phosphorus atom (6.4 Å) in presented model is too great, and this correlates with the minor reactivation activity against tabun-inhibited AChE in vitro.

The lack of correlation between the in vitro reactivation data (Table 2) and docking calculations (Figure 2–4) can be explained by the chosen AChE conformation (PDB: 2JEZ), where crystal structure data do not cover the real conformation of the flexible protein, but only one particular conformation found by crystallographic experiments at a given resolution.

The structure–activity relationship (SAR) properties valuable for increased reactivation ability should be mentioned:[37] the position and quantity of the oxime groups, structure of the connecting linker, presence of the heteroarenium rings and various functional groups. Concerning the oxime group, the mono-oxime compounds with a hydroxyiminomethyl functionality in position 4 were formerly highlighted as required groups for AChE reactivation after tabun and organophosphate pesticide intoxication reword.[22,2] The known compounds fulfilled this hypothesis, especially the best compound (**8**) against tabun.[22] However, the newly prepared reactivators with oxime in position 2 or 3 (**9, 11**–**12**) showed the same or increased reactivation ability when compared to similar compounds with oxime in position 4 (**14, 33**). This phenomenon was also observed for a previous series of reactivators with xylene linkage and is apparently caused by the additional aro-

matic ring available for π–π interactions.[19–20] From this point of view, the connecting linker plays an important role via interactions with the active sites. Although *m*- or *p*-xylene linkers (**6**–**7**) were formerly considered as more promising, *o*-xylene linker (**9, 12**) seemed to have an impact on reactivation ability also.[20] Such interactions were formerly hypothesised also for a (*E*)-but-2-ene linker (**8**).[26–28] Apparently, heteroarenium (pyridinium) moieties took part in the same cation–π interactions within the enzyme active sites.[33] Conversely, the second oxime or non-oxime moiety were responsible for hydrogen bonding. A carbamoyl group in position 4 was formerly highlighted as the most promising substituent (compound **8**).[22] Regarding the newly prepared compounds (particularly **9, 11**–**12, 14**, and **33**), there is no evidence that the carbamoyl position was favourable for reactivation of tabun- or organophosphate-inhibited AChE. On the other hand, the introduction of a carbamoyl moiety decreased the toxicity of the xylene-linked compounds approximately twofold (Table 3).

## Conclusions

A new series of monooxime-monocarbamoyl xylene-linked bispyridinium compounds has been developed. Their potency was tested on a model of tabun-, POX-, MePOX- and DFP-inhibited AChE in vitro. Their reactivation ability against tabun did not exceed that of known inhibitor K203 (**8**), but one compound was able to exceed the standard commercial reactivators (including obidoxime and trimedoxime) in the reactivation of MePOX-inhibited AChE. In the case of POX- and DFP-inhibited AChE, the novel compounds were not able to surpass the reactivation ability of K203 or obidoxime. The acute toxicity of the newly prepared compounds in mice was greater than that of the commercial compounds **3**–**4** and comparable with K203 (**8**), but improved over the formerly prepared xylene-linked compounds (**6**–**7**). The introduction of a carbamoyl moiety decreased the toxicity in mice approximately twofold. The molecular docking studies confirmed π–π and cation–π interactions within the enzyme active sites. Hydrogen bonding was found to be an important reactivator–AChE interaction also. Regarding the SAR, compounds with an oxime moiety in position 2 or 3 showed increased reactivation activity against tabun- or organophosphate pesticide-inhibited AChE in contrast with previous findings. Apparently, the xylene linkage and carbamoyl moiety strongly influenced the reactivation ability of the newly prepared compounds.

## Experimental Section

### Chemistry

Solvents (acetone, DMF, MeCN) and reagents were purchased from Fluka and Sigma–Aldrich, and were used without further purification. Reactions were monitored by TLC using DC-Alufolien Cellulose F plates (Merck, Germany) with BuOH/$CH_3COOH$/$H_2O$ (5:1:2) as the mobile phase. Plates were visualised using a solution of Dragendorff reagent (10 mL $CH_3COOH$, 50 mL $H_2O$, 5 mL stock solution: prepared from two fractions, A: 850 mg $Bi(NO_3)_3$, 40 mL $H_2O$, 10 mL $CH_3COOH$; B: 8 g KI, 20 mL $H_2O$). Melting points were measured on a microheating stage PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany) and were uncorrected.

NMR spectra were recorded on a Varian Gemini 300 (Palo Alto, USA): $^1H$ 300 MHz, $^{13}C$ 75 MHz. In all cases, the chemical shift values (δ) in ppm relative to the residual solvent peaks: $^1H$, $CHD_2SO_2CD_3$ ($\delta = 2.50$), $D_2O$ ($\delta = 4.79$); $^{13}C$, [$D_6$]DMSO ($\delta = 39.43$). Signals are quoted as s (singlet), d (doublet), t (triplet) and m (multiplet).

Mass spectra were measured on a LCQ FLEET ion trap and evaluated using Xcalibur v 2.5.0 software (both Thermo Fisher Scientific, San Jose, USA). The sample was dissolved in deionized water (Goro, s.r.o., Prague, Czech Republic), and injected continuously (8 μL $min^{-1}$) by Hamilton syringe into an electrospray ion source. The parameters of the electrospray were set up as follows: sheath gas flow rate 20 arbitrary units, aux gas flow rate 5 arbitrary units, sweep gas flow rate 0 arbitrary units, spray voltage 5 kV, capillary temperature 275 °C, capillary voltage 13 V, tube lens 100 V.

The monoquaternary monooxime salts were prepared according to the previously published procedure.[20] Full spectral data for compounds **9**–**34** can be found in the Supporting Information.

#### *Preparation of bispyridinium salts (9–34)*

**General procedure**: A solution of monoquaternary salt (0.50 g, 1.3 mmol) and the corresponding carbamoylpyridine (0.30 g, 2.4 mmol) in DMF (10 mL) was stirred at the indicated temperature for the appropriate length of time (Table 4). The reaction mixture was cooled to RT, diluted with acetone (50 mL) and left overnight at 5 °C. The liquid was decanted off and the solid crude product was dissolved in MeCN (50 mL) and left at RT overnight. The crystalline product was collected by filtration, washed with MeCN (3× 20 mL) and recrystallized from MeCN. The purity of all compounds was determined by NMR, ESI-MS and elemental analysis.

**Table 4.** Reaction conditions for the synthesis of compounds **9**–**34**.

| Compd | Temp °C | Time [h] | Yield [%] | Compd | Temp °C | Time [h] | Yield [%] |
|---|---|---|---|---|---|---|---|
| **9** | 50 | 79 | 6 | **22** | 70 | 21.5 | 64 |
| **10** | 70 | 4 | 78 | **23** | 50 | 28 | 13 |
| **11** | 70 | 3 | 59 | **24** | 70 | 7.5 | 61 |
| **12** | 70 | 4.5 | 73 | **25** | 70 | 7.5 | 63 |
| **13** | 70 | 3 | 97 | **26** | 70 | 100 | 21 |
| **14** | 70 | 30.5 | 7 | **27** | 70 | 3.5 | 98 |
| **15** | 70 | 5.5 | 79 | **28** | 70 | 3.5 | 98 |
| **16** | 70 | 3.5 | 88 | **29** | 50 | 57 | 13 |
| **17** | 50 | 50 | 16 | **30** | 100 | 23 | 80 |
| **18** | 70 | 12.5 | 60 | **31** | 100 | 22 | 80 |
| **19** | 70 | 11.5 | 95 | **32** | 50 | 46.5 | 17 |
| **20** | 70 | 70 | 3 | **33** | 100 | 3 | 92 |
| **21** | 70 | 22.5 | 49 | **34** | 100 | 3 | 96 |

**2-Carbamoyl-2′-hydroxyiminomethyl-1,1′-(1,2-phenylenedimethyl)bispyridinium dibromide (9)**: The reaction mixture was stirred at 50 °C and stopped after 79 h (yield = 6%): $R_f = 0.15$; mp: 179–181 °C; $^1H$ NMR (300 MHz, [$D_6$]DMSO): $\delta = 9.19$–8.95 (m, 2H, H-6,6′), 8.87–8.51 (m, 7H, -N$H_2$, -C$H$=NOH, H-3,3′,5,5′), 8.38–8.15 (m, 2H, H-4,4′), 7.47–7.23 (m, 2H, Ph), 6.69–6.25 ppm (m, 4H, Ph, -C$H_2$); $^{13}C$ NMR (75 MHz, [$D_6$]DMSO): $\delta = 148.1$, 146.3, 145.9, 141.2, 131.5,

129.1, 128.3, 125.8, 125.3, 57.7 ppm; Anal. calcd for $C_{20}H_{20}Br_2N_4O_2$: 47.27 C, 3.97 H, 11.02 N; found: 46.82 C, 4.09 H, 11.22 N; MS (ESI+): *m/z*: 174.1 $[M/2]^{2+}$.

**2-Carbamoyl-2′-hydroxyiminomethyl-1,1′-(1,3-phenylenedimethyl)bispyridinium dibromide (17)**: The reaction mixture was stirred at 50 °C and stopped after 50 h (yield = 16%): $R_f$ = 0.15; mp: 225–227 °C; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 9.30–9.19 (m, 2H, H-6,6′), 8.87 (s, 1H, -$NH_2$), 8.84–8.59 (m, 4H, H-3,3′,-$NH_2$,-$CH$=NOH), 8.47–8.40 (m, 1H, H-4), 8.34–8.16 (m, 3H, H-4′,5,5′), 7.52–7.25 (m, 4H, Ph), 6.12 (s, 2H, -$CH_2$), 5.98 ppm (s, 2H, -$CH_2$); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 148.2, 147.3, 147.1, 146.7, 146.3, 146.0, 141.3, 134.8, 134.5, 129.8, 129.0, 128.8, 127.9, 127.9, 127.4, 126.1, 60.4, 59.8 ppm; Anal. calcd for $C_{20}H_{20}Br_2N_4O_2$: 47.27 C, 3.97 H, 11.02 N; found 47.27 C, 4.17 H, 10.71 N; MS (ESI+): *m/z*: 174.1 $[M/2]^{2+}$.

**2-Carbamoyl-2′-hydroxyiminomethyl-1,1′-(1,4-phenylenedimethyl)bispyridinium dibromide (26)**: The reaction mixture was stirred at 70 °C and stopped after 100 h (yield = 21%): $R_f$ = 0.15; mp: 218–220 °C; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 9.29 (d, 2H, $J$ = 6.2 Hz, H-6,6′), 8.88–8.73 (m, 4H, H-3,3′,-$NH_2$,-$CH$=NOH), 8.69–8.63 (m, 2H, H-4,-$NH_2$), 8.34–8.24 (m, 3H, H-3′,5,5′), 7.43 (s, 4H, Ph), 6.01 ppm (s, 4H, -$CH_2$); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 148.4, 147.5, 147.1, 141.6, 134.8, 129.3, 128.2, 128.1, 60.6 ppm; Anal. calcd for $C_{20}H_{20}Br_2N_4O_2$: 47.27 C, 3.97 H, 11.02 N; found 47.01 C, 4.25 H, 10.71 N; MS (ESI+): *m/z*: 174.1 $[M/2]^{2+}$.

### In vitro assay

The reactivation ability of the test compounds was measured on a multichannel Sunrise spectrophotometer (Tecan, Salzburg, Austria). The previously used Ellman's procedure was slightly modified.[29] The standard polystyrene microplates with 96 wells (Nunc, Rockilde, Denmark) were chosen as reaction vessels. Human erythrocyte AChE (Sigma–Aldrich) was used in all experiments. Pesticides paraoxon (POX), methylparaoxon (MePOX) and diisopropylfluorophosphate (DFP) were purchased from Sigma–Aldrich. 50 mM phosphate buffer (pH 7.4) was used in all experiments.

The activity of the enzyme was adjusted to 0.002 U μL$^{-1}$. Enzyme solution (15 μL), phosphate buffer (60 μL), 5,5′-dithiobis(2-nitrobenzoic)acid (DTBN; 0.4 mg mL$^{-1}$, 20 μL) were combined in a well. The enzyme was inhibited via addition of 5 μL of a solution of pesticide in propan-2-ol: tabun (0.1 mM), POX (0.1 mM), MePOX (0.1 mM), DFP (1 mM). Propan-2-ol was used as a control. The mixture was left for 5 min. After inhibition, cholinesterase was reactivated by the addition of a solution of test compound in the phosphate buffer (100 or 1 μM). Enzyme activity was measured after 15 min incubation via addition of 1 mM acetylthiocholine chloride (20 μL, ATChCl). Oximolysis was determined similarly by displacing enzyme with a solution of albumin in phosphate buffer (1 mg mL$^{-1}$, 15 μL). The microplate was gently shaken prior to measurement. Absorbance was measured against phosphate buffer at 412 nm. The reactivation ability was calculated according to Equation (1):

$$(\%) = \frac{A_r - A_{ox}}{A_0 - A_i} \times 100 \quad (1)$$

where $A_r$ is the absorbance at 412 nm provided by reactivated cholinesterase; $A_{ox}$ is the absorbance provided by oximolysis; $A_0$ is the absorbance provided by intact cholinesterase; $A_i$ is the absorbance provided by inhibited cholinesterase.

All measurements were carried out in triplicate and the reactivation data were expressed as average value ± standard deviation (SD).

### Acute toxicity evaluation

Experiments involving animals were performed in accordance with the guidelines set forth by the Ethics Committee of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic).

Female BALB/C mice (25–30 g) were purchased from Konarovice (Czech Republic). They were kept in an air-conditioned room with a standard 12 h light on/off protocol (light on: 07:00; light off: 19:00 h), with free access to standard food and water. The acute toxicity of selected compounds (intramuscular administration (i.m.), standard saline solution, dosage in mg kg$^{-1}$) was estimated as $LD_{50}$ values for each reactivator (see Table 3). The 95% confidence limits were determined using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of each oxime at 4–6 different doses with six animals per dose.[38] Abnormal behaviour was defined as convulsions, apathy, or increased locomotion, and appeared within a few minutes of administration (2–15 min, dependent on dose). Death occurred within 2 h and survival remained then same after 24 h.

### Molecular docking

Docking calculations were carried out using Autodock 4.0.1.[39] The structure of mus musculus AChE was taken from the crystal structure (pdb code 2JEZ) and prepared using Autodock Tools 1.5.2.[39–40] The three-dimentional affinity grid box was designed to include the full active and peripheral sites of AChE. The number of grid points in the x-, y- and z-axes was 110, 110 and 110, with grid points separated by 0.253 Å. Ligands **3, 8, 12** were drawn in ChemDraw 11.0 and minimised with UCSF Chimera 1.3 (amber force field) in charged form.[41] Docking calculations were set to 50 runs. At the end of a calculation, Autodock performed cluster analysis. The visualisations of enzyme–ligand interactions (Figure 2–4) were prepared using Pymol 1.1.[42]

## Acknowledgements

*The authors express their appreciation to Mrs. M. Hrabinova for her technical assistance. The work was supported by the Ministry of Defence (FVZ0000501) and by Ministry of Education, Youth and Sports of Czech Republic (MSM 0021620822).*

**Keywords:** acetylcholinesterases · molecular modeling · organophosphates · reactivators · tabun

[1] *Cholinesterases and Cholinesterase Inhibitors*, (Ed.: E. Giacobini), Martin Dunitz, London, **2000**.
[2] E. Giacobini, *Pharmacol. Res.* **2004**, *50*, 433 – 440.
[3] J. Bajgar, *Adv. Clin. Chem.* **2004**, *38*, 151 – 216.
[4] T. C. Marrs, *Pharmacol. Ther.* **1993**, *58*, 51 – 66.
[5] *Toxicology of organophosphate & carbamate compounds*, (Ed.: R. C. Gupta), Elsevier Academic Press, London, **2006**; pp. 5 – 69, 703 – 714.
[6] A. Saxena, W. Sun, C. Luo, T. M. Myers, I. Koplovitz, D. E. Lenz, B. P. Doctor, *J. Mol. Neurosci.* **2006**, *30*, 145 – 148.
[7] A. Lucic, B. Radic, M. Peraica, M. Mesic, I. Primozic, Z. Binenfeld, *Arch. Toxicol.* **1997**, *71*, 467 – 470.

[8] A. Lucić Vrdoljak, M. Čalić, B. Radić, S. Berend, D. Jun, K. Kuča, Z. Kovarik, *Toxicology* **2006**, *228*, 41 – 50.
[9] E. J. Poziomek, B. E. Hackley, G. M. Steinberg, *J. Org. Chem.* **1958**, *23*, 714 – 717.
[10] A. Lüttringhaus, I. Hagedorn, *Arzneim. Forsch.* **1964**, *14*, 1 – 5.
[11] I. Hagedorn, W. H. Gündel, K. Schoene, *Arzneim. Forsch.* **1969**, *19*, 603 – 606.
[12] F. Worek, P. Eyer, N. Aurbek, L. Szinicz, H. Thiermann, *Toxicol. Appl. Pharmacol.* **2007**, *219*, 226 – 234.
[13] A. Hörnberg, A.-K. Tunemalm, F. Ekstrom, *Biochemistry* **2007**, *46*, 4815 – 4825.
[14] N.-J. Park, Y.-S. Jung, K. Musilek, D. Jun, K. Kuca, *Bull. Korean Chem. Soc.* **2006**, *27*, 1401 – 1404.
[15] H. Kalász, M. Y. Hasan, R. Sheen, K. Kuca, G. Petroianu, K. Ludanyi, A. Gergely, K. Tekes, *Anal. Bioanal. Chem.* **2006**, *385*, 1062 – 1067.
[16] R. Odzak, M. Calic, T. Hrenar, I. Primozic, Z. Kovarik, *Toxicology* **2007**, *233*, 85 – 96.
[17] G. A. Petroianu, K. Arafat, K. Kuca, J. Kassa, *J. Appl. Toxicol.* **2006**, *26*, 64 – 71.
[18] D. E. Lorke, M. Y. Hasan, K. Arafat, K. Kuca, K. Musilek, A. Schmitt, G. A. Petroianu, *J. Appl. Toxicol.* **2008**, *28*, 422 – 429.
[19] K. Musilek, K. Kuca, D. Jun, V. Dohnal, M. Dolezal, *J. Enzyme Inhib. Med. Chem.* **2005**, *20*, 409 – 415.
[20] K. Musilek, K. Kuca, D. Jun, V. Dohnal, M. Dolezal, *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 425 – 432.
[21] D. E. Lorke, S. M. Nurulain, M. Y. Hasan, K. Kuca, K. Musilek, G. A. Petroianu, *J. Appl. Toxicol.* **2008**, *28*, 920 – 928.
[22] K. Musilek, D. Jun, J. Cabal, J. Kassa, F. Gunn-Moore, K. Kuca, *J. Med. Chem.* **2007**, *50*, 5514 – 5518.
[23] K. Kuca, J. Bielavsky, J. Cabal, M. Bielavska, *Tetrahedron Lett.* **2003**, *44*, 3123 – 3125.
[24] K. Kuca, J. Bielavsky, J. Cabal, J. Kassa, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3545 – 3547.
[25] J. Kassa, J. Karasova, K. Musilek, K. Kuca, *Toxicology* **2008**, *243*, 311 – 316.
[26] K. Musilek, O. Holas, K. Kuca, D. Jun, V. Dohnal, V. Opletalova, M. Dolezal, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3172 – 3176.
[27] K. Musilek, K. Kuca, D. Jun, V. Dohnal, M. Dolezal, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 622 – 627.
[28] K. Musilek, O. Holas, D. Jun, V. Dohnal, F. Gunn-Moore, V. Opletalova, M. Dolezal, K. Kuca, *Bioorg. Med. Chem.* **2007**, *15*, 6733 – 6741.
[29] M. Pohanka, D. Jun, K. Kuca, *Talanta* **2008**, *77*, 451 – 454.
[30] J. E. Tattersall, *Br. J. Pharmacol.* **1993**, *108*, 1006 – 1015.
[31] K. Musilek, O. Holas, J. Hambalek, K. Kuca, D. Jun, V. Dohnal, M. Dolezal, *Lett. Org. Chem.* **2006**, *3*, 831 – 835.
[32] G. Sinko, M. Calic, A. Bosak, Z. Kovarik, *Anal. Biochem.* **2007**, *370*, 223 – 227.
[33] J. Kassa, D. Jun, K. Kuca, *Int. J. Toxicol.* **2006**, *25*, 397 – 401.
[34] J. Kassa, *Neurotoxic. Res.* **2006**, *9*, 59 – 62.
[35] J. Kassa, *Acta Med. (Hradec Kralove, Czech Repub.)* **1998**, *41*, 19 – 21.
[36] S. M. Nurulain, D. E. Lorke, M. Y. Hasan, M. Shafiullah, K. Kuca, K. Musilek, G. A. Petroianu, *Neurotoxic. Res.* **2009**, *16*, 60 – 67.
[37] K. Kuca, D. Jun, K. Musilek, *Mini-Rev. Med. Chem.* **2006**, *6*, 269 – 277.
[38] R. J. Tallarida, R. B. Murray, Manual of Pharmacologic Calculation with Computer Programs, Springer-Verlag, New York, **1987**, p. 145.
[39] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639 – 1662.
[40] available online via the Protein Data Bank: http://www.pdb.org.
[41] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605 – 1612.
[42] W. L.DeLano, *The PyMOL Molecular Graphics System*, **2002**: http://www.pymol.org.

Received: November 3, 2009
Published online on January 7, 2010