Design of a Potent Reactivator of Tabun-Inhibited Acetylcholinesterase—Synthesis and Evaluation of (*E*)-1-(4-Carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene Dibromide (K203)

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Acetylcholinesterase reactivators are crucial antidotes for the treatment of organophosphate intoxication. Among the organophosphates, with the exception of soman, tabun (GA) intoxications are the least responsive to treatment with commercially available therapeutics. A rational design was used to increase reactivation ability and decrease the toxicity of the novel reactivator. (*E*)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (K203) has better properties than previously tested compounds in vitro and, therefore, is a potential candidate for the treatment of GA intoxication in vivo.

Although there are many natural and synthetic compounds that inhibit the enzyme acetylcholinesterase (AChE,[a] EC 3.1.1.7), the organophosphorus inhibitors (OPI; e.g., nerve agents, pesticides, flame retardants) remain one of the most dangerous and deleterious series of compounds developed by man.[1] Namely, the OPI moiety is covalently bound to the hydroxyl moiety of the serine residue within the enzyme's active site, so that the AChE is not able to cleave the neuromediator acetylcholine, which causes the permanent activation of muscarinic and nicotinic receptors. This leads to a central cholinergic crisis with symptoms of lacrimation, salivation, miosis, but additionally, neuromuscular and breathing difficulties occur; death arises from suffocation.[2]

Various treatments are used to counteract the toxic effects of OPI either pre- or post-intoxication.[2,3] For example, the pretreatment of potentially threatened soldiers as first responders include weak AChE inhibitors (e.g., pyridostigmine) to sequester the enzyme, other esterases (e.g., human butyrylcholinesterase) to scavenge OPI, or use of oxime to reactivate (e.g., HI-6) AChE. Post-treatment regimens include oxime reactivators of AChE in combination with atropine and diazepam.[2−4] Reactivators of AChE (e.g., pralidoxime, HI-6, obidoxime, trimedoxime; **1**−**4**; Figure 1)[5−7] are commonly used in the treatment of OPI intoxications.[2,3] They contain a nucleophilic oxime group (oximate anion), which is able to cleave the OPI moiety from AChE and thereby restore its function. However, the group of OPI called nerve agents (e.g., tabun (GA), sarin, soman, VX; Figure 2)[1] undergo a process called "aging", where some parts of the OPI moiety are cleaved and replaced by a hydroxyl group with a negative charge.[8] After this aging process, oxime are unable to counteract the inhibited AChE.[9]

While OPI nerve agents were prepared in the first half of the 20th century, there is still no reactivator of AChE able to counteract the full spectrum of different OPI.[2] Presently, **2** is the most broad reactivator for nerve agent intoxication with the exception of GA.[10,11] Moreover, the changes induced by GA on AChE's amino acids leads to a partial closing of the enzyme's active site such that only a few reactivators (**3**−**4**) are able to counteract its effect.[8,9] However, oximes **3** and **4** exhibit increased toxicity compared to **2**.[12]

Here we report the synthesis and evaluation of a potential reactivator of GA-inhibited AChE. Our previous SAR studies confirmed the hypothesis of steric hindrance by some bispyridinium molecules.[13] The K-oximes (e.g., K027, K048, K074, and K075; **5**−**8**; Figure 3)[14−16] were developed by us to increase their effectiveness at reactivating GA-inhibited AChE. The partial success of these compounds led us to the production of the compound (*E*)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**9**). In contrast to **2** and **3**, this compound has one oxime moiety present (as in **5** and **6**), with the second oxime moiety replaced by a carbamoyl group (as in **5** and **6**). A four atom (*E*)-but-2-ene connecting linker was chosen because it provided better reactivation (as in **8**)[17,18] and slightly lower toxicity (compare **7** to **8**). Using these molecular moieties (oxime group, carbamoyl group, and a connecting linker), we generated a new reactivator of AChE (**9**).

A two-step synthesis was performed beginning with commercially available compounds via two routes (Scheme 1). In the first step, an excess of alkylating agent was used to obtain monoquaternary products (**9a**,**b**) in adequate yields (93−95%), which were purified by recrystallization from acetonitrile (MeCN), in which the bisquaternary compounds as side products are insoluble.[19] These monoquaternary compounds were then treated by the addition of a corresponding pyridine derivative in DMF and again recrystallized from MeCN. The purity of compound **9** was established by using NMR, MS, and elemental analysis. Next we determined the ability of **9** and previously investigated oximes (**1**−**8**) to reactivate AChE.[20] The in vitro model used GA-inhibited AChE and three concentrations of the oxime being tested (Table 1). In addition, the toxicity (LD_{50}) of these reactivators was determined at 24 h after intramuscular

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[a] Abbreviations: AChE, acetylcholinesterase; OPI, organophosphorus inhibitors; GA, tabun.

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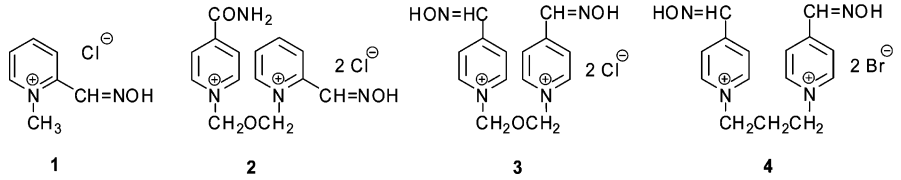


Figure 1. Commercially available acetylcholinesterase reactivators.

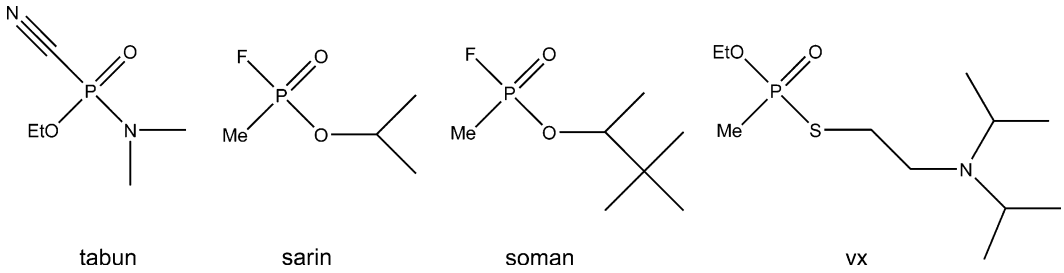


Figure 2. OPI nerve agents—organophosphorus inhibitors with lethal effects.

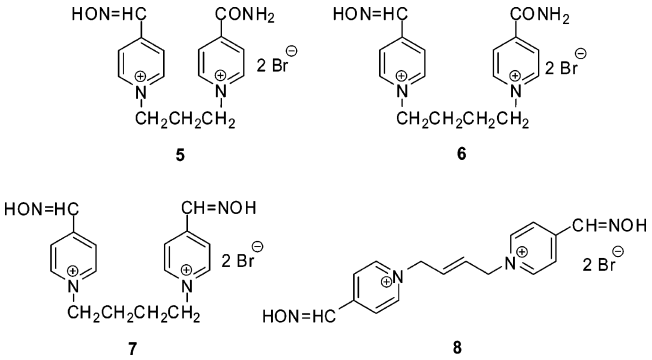


Figure 3. Reactivators developed for tabun-mediated inhibition of acetylcholinesterase.

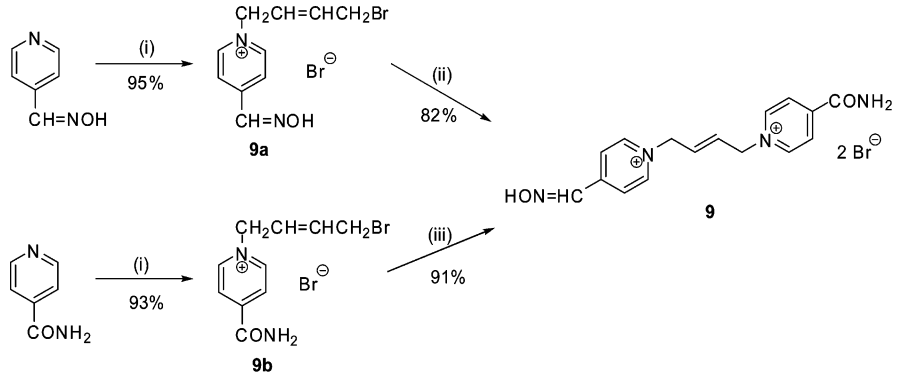
administration of five different concentrations using six rats per dose (Table 1).[21]

The reactivation potency in vitro should exceed 10% to warrant further investigation in vivo.[22] Although monoquaternary compounds such as **1** have a very low reactivation of GA intoxication, they are still used worldwide for other OPI threats as antidotal or pretreatment therapy.[2,22,23] It was also already known that the oxime **2** has a very low reactivation of GA-inhibited AChE, but despite this, it is currently the most broad spectrum reactivator.[23] Two other commercially available compounds (**3**, **4**) improved over the potency of **1** and **2** at all concentrations. The K-oximes (**5**−**9**) differed in reactivation ability. Compound **9** showed the best reactivation ability among all tested reactivators at 10^{-3} M, closely followed by compounds **7** and **8**. However, the highest concentration attainable for human use is 10^{-4} M, and as such, compound **3** is the best from commercial available reactivators and compound **9** among all tested reactivators.[24] Subsequently, compound **3** has the best reactivation ability at 10^{-5} M, followed by K-oximes (**8** > **7** > **9**), with only minor differences between them. Notably, **5** and **6** have lower reactivation ability when compared to **3** and **7**−**9** at all concentrations.

The kinetic constants describing the reactivation process obtained in our experiments were calculated (Table 2).[20,25] First, an affinity of the reactivators toward GA-inhibited AChE is characterized by a K_R value. The best affinity (the lowest value) to the inhibited enzyme was the commercial compound **3** with a slightly lower affinity compared to **2** and **9**; other reactivators had a moderately (**5**−**8**) or substantially decreased affinity (**1** and **4**) toward GA-inhibited AChE. Second, a rate constant k_R is the first-order rate constant that characterizes the ability of oxime to interrupt the covalent bond between enzyme and OPI. The K-oxime **9** showed the highest ability (the highest value) among the tested compounds to break the bond between OPI and AChE, followed by the commercial compound **4** and K-oximes (**7** and **8**), whereas the other reactivators (**3**, **5**, and **6**), especially **1** and **2**, had a very low capability. Additionally, a rate constant k_r (the second-order rate constant) characterizes the velocity of the whole reactivation process. Its value is calculated from the ratio between k_R and K_R. The highest velocity (the highest value) for GA-inhibited AChE has K-oxime **9**. The other compounds showed a higher (**2**−**8**) or substantial decrease in the reactivation velocity (**1**).

The reactivation potency depends on the structure of the reactivator.[13,26] A hetero-aromatic ring with a quaternary nitrogen has been reported to increase reactivation ability,[26] where a pyridinium ring gave better results compared to an imidazolium ring.[13,27−29] At least one hydroxyiminomethyl group in position 4 on the heteroaromatic ring (**3**−**9**) is necessary for proper reactivation of GA-inhibited AChE (in contrast to **1** and **2** with hydroxyiminomethyl group in position 2). Two hydroxyiminomethyl groups at position 4 (bis-oximes **3** and **4**, **7** and **8**) exhibited better reactivation properties for all concentrations than mono-oxime (**5** and **6**), with the exception of **9**. This exception correlated with other structural factors—a connecting linker and a second functional group. The better reactivation ability of a 3- and 4-atom connecting linker (equivalent of three or four carbon−carbon bonds) compared to shorter or longer linkers has been previously reported (**3**−**8**) for GA-inhibited AChE.[14−16,26] Compound **3** showed the best potency at 10^{-4} M and 10^{-5} M from the reactivators bearing a 3-atom connecting linker (**3**−**5**). Compound bearing a 4-atom connecting linker (**7**) had increased potency at 10^{-3} M and 10^{-4} M compared to **3**. Notably, **8** and **9** with the (*E*)-but-2-ene linker increased capability to reactivate GA-inhibited AChE at 10^{-3} M and 10^{-4} M compared to **3**. The (*E*)-but-2-ene connecting linker length (**8** and **9**) is slightly shorter than a four-atom linker (**6** and **7**) but longer than a three-atom linker (**3**−**6**) due to the carbon−carbon double bond.[30] Presumably, compounds **8** and **9** had the length of the connecting linker in the range of three

Scheme 1. Two-step Synthesis of (*E*)-1-(4-Carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene Dibromide[a]



[a] Reagents and conditions: (i) (*E*)-1,4-dibromobut-2-ene (5 equiv), acetone, reflux, 2 h; (ii) 4-carbamoylpyridine (2 equiv), DMF, 100 °C, 2 h; (iii) 4-hydroxyiminomethylpyridine (2 equiv), DMF, 100 °C, 2 h.

Table 1. Ability of Tested Compounds to Reactivate Tabun-Inhibited Acetylcholinesterase In Vitro[a]

	% reactivation ± SD (concentration)			
reactivator	10^{-3} M	10^{-4} M	10^{-5} M	LD_{50}[b] (rat; mg/kg)
1	4 ± 0	1 ± 0	0	377.5 (325.7−437.4)
2	2 ± 0	6 ± 0	4 ± 0	781.3 (738.4−826.6)
3	37 ± 1	34 ± 0	28 ± 2	211.07 (176.4−252.6)
4	41 ± 1	15 ± 0	6 ± 0	150.5 (142.1−159.4)
5	11 ± 0	11 ± 0	1 ± 0	>1200
6	25 ± 1	15 ± 0	6 ± 0	>1200
7	46 ± 1	38 ± 1	15 ± 0	49.0 (43.5−55.3)
8	42 ± 1	42 ± 1	19 ± 0	71.3 (60.9−83.6)
9	55 ± 1	51 ± 0	14 ± 0	326.4 (285.4−373.2)

[a] Mean value of three independent determinations; time of inhibition, 30 min; time of reactivation by AChE reactivators, 10 min; pH 7.6; temperature 25 °C. [b] 95% confidence limits were calculated by probit-logarithmic method[21]

Table 2. Kinetic Parameters of the Tested Compounds[a]

reactivator	K_R ± SD [μM]	k_R ± SD [min^{-1}]	k_r [min^{-1} M^{-1}]
1	575 ± 43	0.006 ± 0.001	10
2	6 ± 1	0.007 ± 0.002	1111
3	3 ± 1	0.020 ± 0.002	6250
4	460 ± 41	0.079 ± 0.007	172
5	54 ± 4	0.015 ± 0.002	273
6	93 ± 6	0.032 ± 0.003	348
7	29 ± 3	0.056 ± 0.006	1931
8	19 ± 2	0.056 ± 0.004	2947
9	6 ± 1	0.096 ± 0.003	16000

[a] K_R = dissociation constant of inhibited enzyme-reactivator complex; k_R = the first-order rate constant of reactivation; k_r = the second-order rate constant of reactivation (obtained as the ratio k_R/K_R).

to four carbon atoms and due to this better reactivation at 10^{-3} M and 10^{-4} M.

The second functional group attached to the other pyridinium ring, which is not included in the reactivation process (not an oxime), is probably interacts with the enzyme's active site.[26,31] Significantly, a second oxime group (**3** and **4**, **7** and **8**) enhanced the reactivation probably due to the greater possibility to interact with the inhibited AChE.[17] In contrast, the carbamoyl group (**5**, **6**) could not affect the reactivation in this manner. However, **9** showed better reactivation of GA-inhibited AChE at 10^{-3} M and 10^{-4} M compared to **3** and **4** and **7** and **8**, despite that the latter compounds are bis-oximes.

The toxicity of a reactivator is an important factor for its use in humans.[2,22] The commercially available compounds able to reactivate GA-inhibited AChE (**3** and **4**) had an increased toxicity (indicated by a decrease in the LD_{50}) in rats compared to compounds **2** (the reactivator with the lowest toxicity among the commercial compounds)[23] and **1**. Compound **3**, with the connecting chain bearing a heteroatom, had a slightly lower toxicity in comparison with **4**. When two oxime groups and a connecting linker one unit longer were utilized (**7** and **8**), the reactivation slightly decreased whereas the toxicity increased three or four times compared to compound **3**. In contrast, the reactivators with one carbamoyl group (**5** and **6**) had almost half the reactivation ability at 10^{-4} M when compared to **3**, while the toxicity decreased to below detectable levels. Compound **9**, which was developed by a combination of utilizing one oxime group, one carbamoyl group, and a (*E*)-but-2-ene connecting linker, was the best reactivator for GA-inhibited AChE at a concentration of 10^{-3} M and 10^{-4} M and the least toxic when compared to compounds **3** and **4** and **7** and **8**.

Although the AChE reactivators are very hydrophilic compounds (containing one or two quaternary nitrogen and hydroxyiminomethyl group), a penetration through the blood−brain barrier has been confirmed in the case **1**.[32] Apparently, the increased hydrophilic bisquaternary compounds such as **2**−**9** have lower levels of blood−brain barrier penetration, but have yet to be tested.[22] However, reactivation outside of the central nervous system is equally important, especially shortly after intoxication and, in the case of OPI, can deposit within lipophilic tissues.[33,34]

In summary, the (*E*)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide was prepared as a new potent reactivator for GA-inhibited AChE. Its reactivation potency was better than commercially available or previously evaluated compounds for GA-inhibited AChE at a concentration permissible for human use. In addition, the calculated kinetic data showed that it has the high affinity to the enzyme−inhibitor complex, the highest ability to break the enzyme−inhibitor bond, and the highest velocity of the whole reactivation process among all tested compounds. The reactivator's low toxicity is an improvement over commercially available compounds suitable for GA-inhibited AChE. The relationship between activity and toxicity of this new reactivator suggests its potential use for further in vivo application.

Experimental Section

(*E*)-1-(4-Bromobut-2-enyl)-4-hydroxyiminomethylpyridinium Bromide (9a). A solution of the 4-hydroxyiminomethylpyridine (2.0 g, 16.4 mmol) and (*E*)-1,4-dibromobut-2-ene (17.51 g, 81.9 mmol) in acetone (60 mL) was stirred at reflux for 2h. The reaction mixture was cooled to the room temperature; the crystalline crude product was collected by filtration, washed with acetone (3 × 20

mL), and recrystallized from MeCN. Yield 95%, mp 187−191 °C. ^{1}H NMR spectrum (300 MHz, DMSO-d_6): δ (ppm) 9.02 (d, 2H, J = 6.0 Hz, *H-2* + *H-6*), 8.45 (s, 1H, −*CH*═NOH), 8.27 (d, 2H, J = 6.0 Hz, *H-3* + *H-5*), 6.26−6.09 (m, 2H, −*CH*═*CH*−), 5.32 (d, 2H, J = 4.9 Hz, −CH_2—Br), 4.17 (d, 2H, J = 6.0 Hz, N—CH_2−). ESI-MS: *m*/*z* 254.9 $[M]^+$; calcd for $[C_{10}H_{12}BrN_2O]^+$ 255.01. Anal. ($C_{10}H_{12}Br_2N_2O$) C, H, N.

(*E*)-1-(4-Bromobut-2-enyl)-4-carbamoylpyridinium Bromide (9b). A solution of the 4-carbamoylpyridine (2.0 g, 16.4 mmol) and (*E*)-1,4-dibromobut-2-ene (17.51 g, 81.9 mmol) in acetone (60 mL) was stirred at reflux for 2 h. The reaction mixture was cooled to the room temperature; the crystalline crude product was collected by filtration, washed with acetone (3 × 20 mL), and recrystallized from MeCN. Yield 93%, mp 183−185 °C. ^{1}H NMR spectrum (300 MHz, DMSO-d_6): δ (ppm) 9.23 (d, 2H, J = 6.2 Hz, *H-2* + *H-6*), 8.71 (bs, 1H, −NH_2), 8.48 (d, 2H, J = 6.2 Hz, *H-3* + *H-5*), 8.29 (bs, 1H, −NH_2), 6.28−6.10 (m, 2H, −*CH*═*CH*−), 5.38 (d, 2H, J = 4.7 Hz, −CH_2—Br), 4.18 (d, 2H, J = 5.9 Hz, N—CH_2−). ESI-MS: *m*/*z* 254.9 $[M]^+$; calcd for $[C_{10}H_{12}BrN_2O]^+$, 255.01. Anal. ($C_{10}H_{12}Br_2N_2O$) C, H, N.

(*E*)-1-(4-Carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene Dibromide (9). A solution of the monoquaternary salt (0.50 g, 1.5 mmol) and corresponding pyridine (0.38 g, 3.0 mmol) in DMF (10 mL) was stirred at 100 °C. The reaction mixture was cooled to the room temperature and portioned with acetone (50 mL); the crystalline crude product was collected by filtration, washed with acetone (3 × 20 mL), and recrystallized from MeCN. Yield 82−91%, mp 240−241 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 9.06 (d, 2H, J = 6.0 Hz, *H-2* + *H-6*), 8.84 (d, 2H, J = 6.0 Hz, *H-2′* + H-6′), 8.44−8.36 (m, 3H, H-3 + H-5 + −*CH*═NOH), 8.23 (d, 2H, J = 6.0 Hz, *H-3′* + H-5′), 6.43−6.25 (m, 2H, −*CH*═*CH*−), 5.43 (d, 2H, J = 4.8 Hz, −CH_2—N), 5.34 (d, 2H, J = 4.8 Hz, −CH_2—N′). ESI-MS: *m*/*z* 149.1 $[M]^{2+}$; calcd for $[C_8H_9N_2O]^+$, 149.17. Anal. ($C_{16}H_{18}Br_2N_4O_2$) C, H, N.

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Supporting Information Available: Experimental details for the synthesis and characterization of compounds (EA, standard reactivation test). This material is available free of charge via the Internet at http://pubs.acs.org.

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