Sulfur Derivatives of 2-Oxopropanal Oxime as Reactivators of Organophosphate-Inhibited Acetylcholinesterase in Vitro: Synthesis and Structure-Reactivity Relationships

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We have prepared four new oximes, **1b–e**, which conform to the general structure $RCH_2COCH{=}NOH$ where R = CH_3S, CH_3SO, CH_3SO_2, and $(CH_3)_2S^+$, respectively, and have the same *E* configuration as the parent 2-oxopropanal oxime **1a** (R = H, MINA). The pK_a values range from 6.54 (**1e**) to 8.16 (**1b**), as compared with 8.30 for **1a**. Rates of reaction (k_1) with 4-nitrophenyl acetate indicate that the oximate anions have a much higher nucleophilicity than common oxyanions of similar basicities: the α effects measured for **1a–e** are of the order of 200–250. The abilities of **1b–e** to reactivate acetylcholinesterase (AChE) inhibited by organophosphates have been evaluated. In vitro experiments reveal a significant reactivation potency of **1b–e** against VX-, sarin-, and paraoxon-inhibited immobilized eel AChE. The highly lipophilic methylthio oxime **1b** ($\log P > 1$) is intrinsically (k_2) 3 times more reactive than the more basic MINA ($\log P < 1$). The sulfonium oxime **1e** is a potent reactivator against paraoxon. Interestingly, both **1b** and **1e** have a low toxicity and they exhibit a significant antidotal effect at a relative low dose against paraoxon in rats.

Organophosphates inhibit acetylcholinesterase (AChE) by phosphorylation of a serine residue in the active site.[1-4] Standard therapy against this organophosphate poisoning is based on coadministration of anticholinergics and of AChE reactivators.[4-7] These must be efficient nucleophiles capable to displace the phosphoryl residue from the active site, thereby restoring the enzymatic activity.

In a search for useful reactivators, special attention has been drawn to compounds having an oxime functionality.[4,7-15] This is because many oximes have pK_a values in the region of physiological pH, and the corresponding oximate anions behave as typical α-nucleophiles, i.e., they exhibit an exceptionally high nucleophilic reactivity compared to common oxygen nucleophiles of similar basicities.[1,6-19] However, the efficiency of a reactivator is also highly dependent on its affinity for the inhibited enzyme.[4,11] In this respect, it was found that the incorporation of cationic centers into the potential reactivators plays a very positive role so that most studied oxime antidotes have been pyridinium oximes.[4,6,7,10]

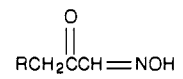
Although the therapeutic utility of pyridinium oximes is largely developed, their cationic nature limits their penetration into the central nervous system, which occurs at a very low rate compared to the relatively lipophilic organophosphorus esters.[4,11] On this basis, it is not surprising that the possibility of improving therapy of organophosphate poisoning by use of nonpositively charged reactivators has been considered by some authors.[4,8,9,12] Besides well-known compounds like butanedione oxime (DAM)[2] or 2-oxopropanal oxime (MINA),[9,15] a number of neutral heteroaromatic aldoximes, notably various oxadiazole and thiadiazole carboxaldoximes, have been studied.[9,12] Concomitantly, efforts have been made to design quaternary-type reactivators, which would show an intrinsic reactivity comparable to that of pyridinium oximes but would also exhibit a higher susceptibility to penetrate into hydrophobic cell membranes.[4,11-13] In this context, we report here the synthesis as well as the physicochemical and reactivating properties of three new neutral oximes, **1b–d**, which derive from the parent MINA (**1a**) by substitution of one of the methyl hydrogens by a methylthio, a methylsulfinyl, and a methylsulfonyl group, respectively.

Table I. Selected NMR Data and pK_a Values for Oximes 1a–e and 2a,b

oxime	δ_{OH}[a]	δ_{CH}[a]	$\delta_{OH} - \delta_{CH}$[a]	pK_{a_1}[b]	pK_{a_2}[b]
1a	12.57	7.45	5.12	8.30	⩾13
	12.44[c]	7.42[c]	5.02[c]		
1b	12.70	7.56	5.14	8.16	⩾13
1c	12.96	7.63	5.33	7.74	12.23
1d	13.13	7.67	5.46	7.46	11.10
1e	13.20	7.74	5.46	6.54	10.55
5a	12.50[c]	8.02[c]	4.48[c]	8.50[c,d]	
	12.63[e]	8.05[e]	4.58[e]		
5b	12.78[f]	8.01[f]	4.77[f]		

[a] NMR data in Me_2SO or Me_2SO-d_6, δ in ppm. [b] pK_a values at 25 °C. [c] Reference 21. [d] At 20 °C. [e] Reference 22. [f] Reference 24a.

Data for the 3-dimethylsulfonium derivative **1e**, a positively charged oxime, are also reported.



1a: R=H
b: R=CH_3S
c: R=CH_3SO
d: R=CH_3SO_2
e: R=$(CH_3)_2S^+$

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Table II. Kinetic Parameters for Reaction of Oximate Anions with PNPA (k_1) and VX-, Sarin-, and Paraoxon-Inhibited Immobilized Eel AChE

					VX		sarin		paraoxon	
oxime	pK_{a_1}[a]	log P[b]	k_1,[c] M^{-1} s^{-1}	α effect	k_2,[d] M^{-1} min^{-1}	k_{eff},[e] M^{-1} min^{-1}	k_2,[d] M^{-1} min^{-1}	k_{eff},[e] M^{-1} min^{-1}	k_2,[d] M^{-1} min^{-1}	k_{eff},[e] M^{-1} min^{-1}
1a	8.30	0.34	24.8	166	73.9	28	132.2	50	75.5	28.5
1b	8.16	1.21	24.5	204	192.7	79.2	160	66	162.1	66.6
1c	7.74	-1.02	17.1	263	56.4	29	83.1	42.6	49.9	25.6
1d	7.46	-0.84	11.6	263	68.1	39.7	50.4	29.3	65	37.9
1e	6.54	<-2	2.4	220	33.9	26.4	26.3	20.4	152.1	118.5
contrathion	7.99[f]	-3.24[g]	16[f]	190	9500	3700	3800	1470	19000	7400

[a] pK_{a_1} at 25 °C. [b] log P is the octanol/buffer partition coefficient for 0.1 M, pH 7.8, phosphate buffer. [c] Second-order rate constant for attack on PNPA at t = 25 °C. [d] k_2 is the intrinsic bimolecular reactivation rate constant, as defined by eq 7, at 25 °C. [e] k_{eff} is the effective bimolecular rate constant for reactivation at pH 7.8 and 25 °C as calculated from k_2 by eq 9. [f] Reference 11. [g] Reference 9.

Results and Discussion

Synthesis and Structure of 1b–e. The methylsulfinyl and methylsulfonyl oximes **1c** and **1d** were prepared as shown in eq 1. The dimethylsulfonium oxime **1e** was

$$EtOOCCH{=}NOSi(CH_3)_3 + RCH_2^- \rightarrow RCH_2COCH{=}NOH \quad (1)$$

1c: R = CH_3SO
1d: R = CH_3SO_2

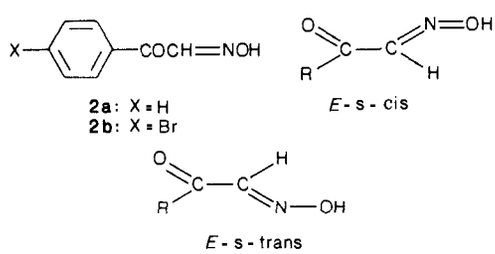
obtained as a bromide salt under the conditions indicated in eq 2. Thermic decomposition of this sulfonium salt

$$BrCH_2COCH{=}NOH + (CH_3)_2S \xrightarrow[20\ °C]{\text{acetone}} (CH_3)_2{}^+SCH_2COCH{=}NOH\ Br^- \xrightarrow[\text{excess } (CH_3)_2S,\ 65\ °C]{CH_3CN} CH_3SCH_2COCH{=}NOH \quad (2)$$

1e → **1b**

gives the methylthio oxime **1b**. Details of the synthesis are given in the Experimental Section.

Inasmuch as the configuration of the oxime functionality can have a profound influence on the chemical and biological reactivity of **1b–e**,[11,20] the possibility of E and Z isomerization in these derivatives was considered. First, the ^1H NMR spectra showed only one signal ascribable to the OH or CH protons of **1b–e** in DMSO, indicating that these compounds formed as pure E or Z isomers. As can be seen in Table I, the oxime proton chemical shifts range from δ 12.70 for **1b** to 13.20 for **1e**, comparing well with the δ values for **1a** or the monoximes **2a** and **2b**.[21,22] This together with the observation of similar $\Delta\delta = \delta_{OH} - \delta_{CH}$ values suggested that all these oximes adopt the same configuration as MINA, i.e., the E configuration.[21,23,24,27] Lehn has shown that the measurement of the ^{15}N-H_x coupling in the CH_x=NOH fragment allows an unambiguous assignment of the configuration of aldoximes; this coupling ranges from 10 to 16 Hz for the Z isomers and from 2 to 4 Hz for the E isomers.[25] The 500-MHz ^{15}N NMR spectrum recorded for the sulfoxide **1c** shows $J_{^{15}N\text{-}H_x}$ = 4 Hz, confirming the E geometry for this derivative and, by analogy, for all the aldoximes listed in Table I. IR spectra of dilute solutions of **1a** and **1b** in CCl_4 did not show the presence of OH bands in the region (~2500 cm^{-1}) typical for intramolecular hydrogen bonding but exhibited sharp OH bands near 3560 cm^{-1}, which are ascribable to free OH groups.[11,21,24] These observations are consistent with each of the two E-s-cis or E-s-trans configurations for **1a–e**.[26] However, it has been conclusively established by gas-phase electron diffraction that **1a** exists in the E-s-trans form.[27] The same configuration is therefore likely for **1b–e**. The assignment of the E configuration to **1a–e** is of special interest since it has been noted that oximes that exhibit notable antipoisoning activity usually have this geometry.[28]



Acidity of 1a–e. The acidity of the NOH group of **1a–e** (K_{a_1}) was measured by standard potentiometric methods using oximate buffer solutions with various [oximate]/[oxime] ratios.[29,30] From the data summarized in Table I, it can be seen that the pK_a values obey the sequence H > CH_3S > CH_3SO > CH_3SO_2 > $(CH_3)_2S^+$, which is the reverse of that observed for the δ_{NOH} values and is consistent with the known electron-withdrawing effects of the substituents.[31] Accordingly, the oximes **1c–e** will be more largely dissociated than MINA at pH suitable for reactivation.

Since **1a–e** are α-keto aldoximes, we have considered the possibility that the substitution of a hydrogen from the methyl group of **1a** by electron-withdrawing substituents

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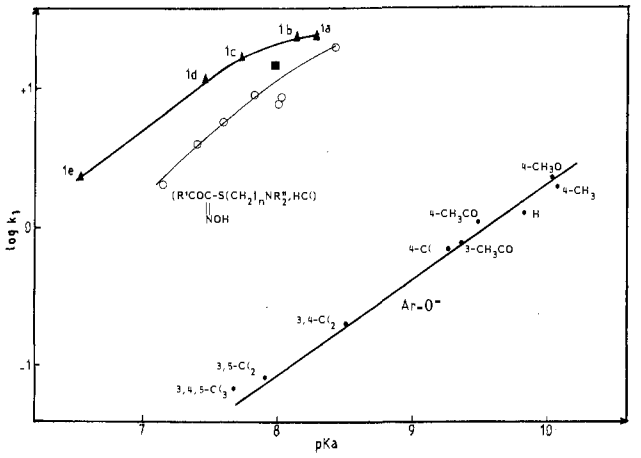


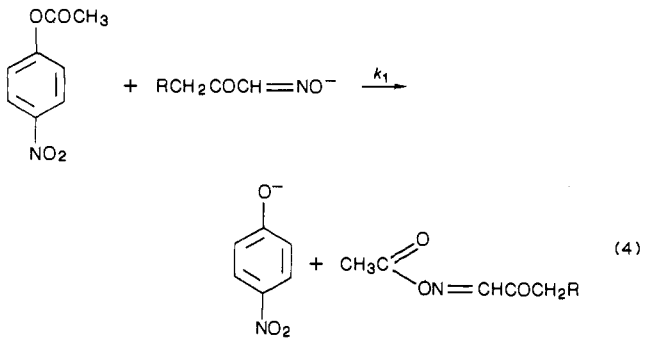
Figure 1. Brønsted plots for the reactions of oximate anions and phenoxide anions (ArO^-) with PNPA at t = 25 °C in aqueous solutions. (■) 2-PAM.

like CH_3SO_2 (σ_i = 0.55)[31] and $(CH_3)_2S^+$ (σ_i = 0.89)[31] increases the acidity of the methylene group to such an extent that the concentration of the dianion D (or the corresponding species for **1e**) would not be negligible at pH used for biological assays (eq 3). We succeeded in

$$\underset{\textbf{1a-e (OxH)}}{RCH_2C(=O)CH=NOH} \overset{K_{a_1}}{\rightleftharpoons} \underset{\textbf{Ox}}{RCH_2C(=O)CH=NO^-} \overset{K_{a_2}}{\rightleftharpoons} \underset{\textbf{D}}{R^-CHC(=O)CH=NO^-} \quad (3)$$

determining potentiometrically the K_{a_2} values for **1c-e**, while those for **1a** and **1b** were too low to be accurately assessed. Table I shows that pK_{a_2} values are all greater than 10.55 and much higher than the corresponding pK_{a_1} values ($\Delta pK \geq 3.64$). This indicates that ionization of the CH_2 group does not overlap with that of the NOH group and is a negligible process at pH ≤8. Changes in pK_{a_2} parallel those in pK_{a_1}, but the effect of the substitution is somewhat greater on pK_{a_2}, in agreement with the fact that the R substituent is directly attached to the site of ionization. For an assessment of the strength of the carbon acidity of **1a-e**, the pK_{a_2} values in Table I may be compared with the pK_a value for acetylacetone (pK_a = 9.03).[32]

Nucleophilicity of 1a-e. The ability of the oxime anions to act as nucleophiles has been assessed by studying their intrinsic Brønsted reactivity toward *p*-nitrophenyl acetate (PNPA) (eq 4). Values of the second-order rate

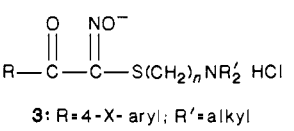


(4)

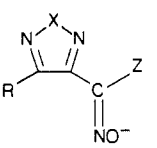
constant k_1 determined from experiments in oximate buffers are given in Table II for the various oximates. The k_1 value found for **1a** agrees fairly well with previous reports.[33]

The rate constants k_1 follow a nonlinear Brønsted-type correlation (Figure 1), which is similar in nature to that observed for the reactions of PNPA with various oxyanions.[34-36] According to recent suggestions by Jencks, the observation of such curvatures in correlations for nucleophilic attack of oxyanions on esters would largely result from a requirement for partial desolvation of the anions before nucleophilic attack, which is more difficult with more basic anions.[36,37] However, it is to be noted that the curvature appears at lower pK_a for oximate anions **1a-e** than for common oxyanions, and it may be that other factors than desolvation play a role in the leveling off of the reactivity of **1a-e**. Data reported for various oximate anions previously studied are included in Figure 1, which shows that the reactivity of these nucleophiles is strongly dependent on the moiety bearing the NO^- functionality.[11] On the other hand, oximate ion attack on PNPA is certainly rate determining in the reactions of **1a-d**, which are all more basic than the leaving *p*-nitrophenoxide anion (pK_a = 7.1), but one cannot exclude that leaving group departure is present to a significant extent in the rate-determining step of the reaction of **1e**, which has a pK_a of 6.54, similar to that of the leaving group.[36] Hence, the use of the k_1 value for this derivative in the Brønsted correlation is perhaps not satisfactory, making a comprehensive analysis of the observed curvature difficult at this stage.

Notwithstanding the difficulties just mentioned, it is interesting to note that the k_1 values determined by Kenley et al. for a homogeneous set of moderately basic thiohydroximate anions of general structure **3** define a linear Brønsted plot that is essentially parallel to the linear portion of the correlation drawn for **1a-e** at the lower pK_a range.[11] The β_{Nu} value determined from the slope is of ~0.70 in both cases, a value that is the same as that determined for phenoxide anions.[36] Similar β_{Nu} values have been observed in reactions of **3** as well as the heteroaromatic aldoximates of general structure **4** with acetylthiocholine.[12]



3: R = 4-X-aryl; R' = alkyl



4: R = H, CH_3; X = O, S; Z = $SCH_2CH_2NEt_2$ HCl, H

The extrareactivity exhibited by α-type nucleophiles is commonly assessed with reference to that of phenoxide ions as the normal nucleophiles. On the basis of the Brønsted correlations in Figure 1, the α effects given in Table II have been derived. These appear to be among the highest α effects so far measured.

Reactivation of Phosphylated AChE.[38] The in vitro reactivating potency of **1a-e** was evaluated with reference to their ability to reactivate immobilized eel AChE inhibited by various organophosphorus compounds. These were selected to give differently phosphylated AChE (EI).

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Thus, we have studied systems with I = $P(O)(OEt)_2$ (paraoxon), $P(O)CH_3(OEt)$ (VX), $P(O)CH_3(O\text{-}i\text{-}Pr)$ (sarin), $P(O)CH_3(OCH(CH_3)C(CH_3)_3)$ (soman), and $P(O)NMe_2(OEt)$ (tabun).

A recently described continuous-flow method adapted from Ellman's technique was employed to follow reactivation kinetics.[39] Acetylthiocholine (ASCh) was used as the substrate. A major advantage of the continuous-flow method is to allow independent inhibition and reactivation of the enzyme. Accordingly, side processes such as oximate-induced hydrolysis of the substrate or enzyme retroinhibition by the resulting phosphylated oxime, which are potentially susceptible to affect the degree of the restored enzymatic activity, are either strongly minimized or suppressed.[39]

The kinetics of AChE reactivation was investigated as a function of the concentration of test oximes **1a–e**. We first controlled that spontaneous reactivation of the inhibited enzyme proceeded at a negligible rate. Also, it was found that oximes do not act as an inhibitor of immobilized AChE, thus eliminating the occurrence of reversible inhibition in our AChE assays. Under our experimental conditions, restoration of the enzymatic activity was found to occur when poisoning eel AChE with VX, sarin, and paraoxon but not with soman and tabun.

In the three active systems, the reactivation of the inhibited enzyme proceeds according to the classical equation

$$EI + Ox \overset{K_r}{\rightleftharpoons} [EI\text{-}Ox] \xrightarrow{k_r} E + I\text{-}Ox \qquad (5)$$

where E is the active enzyme and K_r is an equilibrium constant describing the affinity of the oximate reactivator for the inhibited enzyme EI; k_r is the rate constant for nucleophilic displacement of the phosphyl residue from EI.

In all experiments, the concentration of the reactivator was much lower than the K_r value but in large excess over phosphylated enzyme. Hence, the restoration of the enzymatic activity was expected to follow pseudo-first-order kinetics according to the equation

$$\ln (A_0 - A_t) = -k_{obsd}t + \ln (A_0 - A_i) \qquad (6)$$

where A_0, A_i, and A_t are observed activities before inhibition, after inhibition, and after incubation with reactivator for time t. The observed rate constant, k_{obsd}, is given by the equation

$$k_{obsd} = (k_r/K_r)[Ox] = k_2[Ox] \qquad (7)$$

where the ratio of k_r to K_r is equivalent to k_2, an apparent bimolecular rate constant that measures the intrinsic capability of the oximate ion reactivator to restore the enzymatic activity under the aforementioned experimental conditions. However, at pH 7.8 used for reactivation, the oximate ion concentration is only a fraction of the total oxime concentration since we have

$$[Ox] = [OxH][1 + 10^{(pK_a-7.8)}]^{-1} \qquad (8)$$

Hence, eq 7 may be rewritten as

$$k_{obsd} = k_2[1 + 10^{(pK_a-7.8)}]^{-1}[OxH] = k_{eff}[OxH] \qquad (9)$$

where k_{eff} is now a bimolecular rate constant that measures the effective capability of the oxime to reactivate inhibited AChE at pH 7.8.

Equation 9 predicts a linear dependence of k_{obsd} on the oxime concentration. As shown in Figures 2 and 3, which refer to the in vitro reactivation experiments of immobilized eel AChE inhibited by sarin and paraoxon, respectively, the experimental results were consistent with this expectation. The corresponding k_2 and k_{eff} values for the systems studied are given in Table II, which includes for comparison the data that we have obtained for 2-[(hydroxyimino)methyl]-1-methylpyridinium methyl sulfate, most commonly known as contrathion,[40] under similar experimental conditions. Also indicated in Table II are the octanol/phosphate buffer partition coefficients (log P) assessing the lipophilicity of the oximes studied.

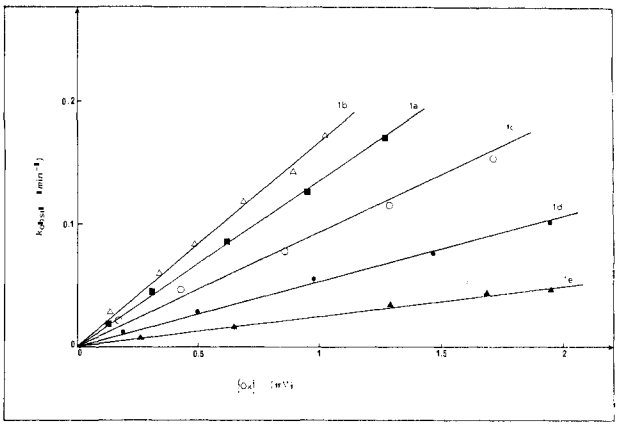

Figure 2. Plots of the observed rate constants k_{obsd} vs oximate concentration for reactivation of sarin-inhibited AChE by the oximes **1a–e** at 25 °C, pH 7.8.

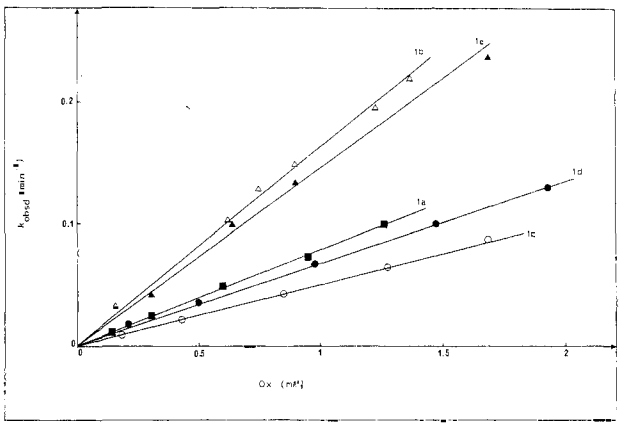

Figure 3. Plots of the observed rate constants k_{obsd} vs oximate concentration for reactivation of paraoxon-inhibited AChE by the oximes **1a–e** at 25 °C, pH 7.8.

Structure–Activity Relationships. The basicity and the lipophilic character of the oxime and the nature of the organophosphorus moiety of the inhibited enzyme are main factors susceptible to determine the reactivation potency of **1a–e**. Inspection of the k_2 values in Table II shows that the intrinsic reactivation potency of **1a–e** against VX- and sarin-inhibited AChE decreases in the approximate order **1b** > **1a** > **1d** ~ **1c** > **1e**. Against paraoxon-inhibited AChE, the reactivity sequence is markedly different: **1b** ~ **1e** > **1a** > **1d** > **1c**. These results, together with the finding of different k_2 values for reactivation of VX-, sarin- or paraoxon-inhibited AChE by a given oxime confirm that restoration of the enzymatic activity by **1a–e** is, other things being equal, strongly dependent on the organophosphorus poison.[4,8,9,11-13] Other evidence for this dependence is obviously the ineffective-

(39) Kiffer, D.; Minard, P. *Biochem. Pharmacol.* **1986**, *35*, 2527.

(40) Depending on its association with methyl sulfate or methanesulfonate anion, 2-[(hydroxyimino)methyl]-1-methylpyridinium cation is commercialized as contrathion (Specia) or P_2S, respectively.

Table III. Acute Toxicity and Protective Index of Contrathion and of Oximes 1a–e against Paraoxon

products	LD50[a] mg/kg	LD50[a] mol/kg	sign free dose mg/kg	sign free dose mol/kg	protective index[b]
1a	50[c]	5.74×10^{-4}	30[d]	3.45×10^{-4}	
1b	>1000	$>7.5 \times 10^{-3}$	50	3.76×10^{-4}	8.4
1c	>400	$>2.68 \times 10^{-3}$			
1d	198 (182–215)	1.2×10^{-3}			
1e	254 (240–269)	1.11×10^{-3}	68	2.98×10^{-4}	22.0
contrathion	317 (274–368)[e]	1.36×10^{-3}	150[e]	6.46×10^{-4}	3.3[e,f]
paraoxon	0.36 (0.35–0.38)	1.31×10^{-6}			

[a] Read after 24 h, 95% confidence limits. [b] The protective index of atropine sulfate alone (17.4 mg/kg) is 1.55. [c] See ref 57. [d] See ref 58. [e] See ref 59. [f] Contrathion was used at 40 mg/kg with atropine sulfate (17.4 mg/kg); use of a greater dose of contrathion causes a decrease in the LD50 of paraoxon.[59]

ness of **1a–e** against soman- and tabun-inhibited AChE. The lack of reversibility of the inhibition with soman is (probably) due to rapid conversion of the phosphorylated enzyme into a nonreactivable species.[39,41] This process known as "aging" is typical for AChE inhibited by this poison. In contrast, tabun-inhibited AChE is much less prone to "aging", and it is efficiently reactivated by some oximes, e.g., 1-benzyl-2-[(hydroxyimino)methyl]pyridinium bromide.[14,39,42,43] According to Schoëne and co-workers, the steric demand of the NMe_2 group of tabun would cause a decrease in the affinity of many oximes for the corresponding inhibited AChE as well as a decrease in the rate constant k_r for the nucleophilic displacement of the organophosphate moiety by these reactivators.[42] The ineffectiveness of **1a–e** in the tabun system may be understood in these terms.

The k_2 reactivity sequences mentioned above reveal two other noteworthy features. The first relates to the greater intrinsic reactivity of the methylthio oxime **1b** compared to that of the slightly more basic unsubstituted analogue **1a** (MINA) in the three systems: the ratio $k_2(\mathbf{1b})/k_2(\mathbf{1a})$ is equal to 2.6, 1.2, and 2.15 for VX, sarin, and paraoxon, respectively. This result may be the reflection of the higher lipophilicity of **1b** since the expected reactivity order, i.e., **1a** > **1b**, is found in comparing the intrinsic Brønsted nucleophilicity (k_1) of these two oximes toward PNPA. The second and probably most significant result of this work is the finding that the dimethylsulfonium oxime **1e**—the more acidic oxime in the series—exhibits an intrinsic reactivity that is essentially the same as that of **1b** against paraoxon-inhibited AChE. For this compound, which features a cationic moiety, increased Coulombic interactions with the phosphorylated enzyme appear to govern the in vitro reactivation kinetics.[4,8–14,43–45] Though to a much lower extent, the intrinsic reactivity of **1e** against VX- and sarin-inhibited AChE is also greater than expected on the basis of its acidity. The ratio $k_2(\mathbf{1e})/k_2(\mathbf{1b})$ is equal to about 0.17 in both systems as compared with a ratio $k_1(\mathbf{1e})/k_1(\mathbf{1b})$ of about 0.10 for the Brønsted nucleophilicities. However, it is clear from Table II that none of the oximes **1a–e** has an intrinsic reactivity that compared favorably with that of contrathion.

Because the various oximes ionize to different extents at pH 7.8, the rate constants k_{eff}, measuring the effective ability of **1a–e** to restore the enzymatic activity, vary much less than the k_2 values with changing the oximate structure. The methylthio derivative **1b** remains the most potent reactivator of the series against the three active systems, being for example 2 times more effective than **1d** and 3 times more effective than **1a**, **1c**, and **1e** against VX-inhibited AChE. Of course, the finding that the effective reactivation potency of the dimethylsulfonium oxime **1e** does not differ very much from that of the more basic analogues **1a–d** is not only due to its positively charged character but also to its low pK_a value (**1e** is about 95% ionized at pH 7.8). Comparison with contrathion shows that this latter compound is finally 1 order of magnitude more potent than **1a–e** against VX, sarin, and paraoxon in vitro.

From preliminary experiments with rats, LD50 determinations have been made that show that the acute toxicity of the new oximes **1b–e** is lower than that of MINA, both on a weight and a molar basis. Accurate determinations could not be obtained, however, for **1b** and **1c** because of the large quantities of oxime needed. When tested as therapeutic agents against paraoxon in rats, **1b** and **1e** exhibited a significant antidotal effect at a relatively low dose. It was controlled that this effect was not related to the presence of atropine sulfate (17.4 mg/kg), which we used as an anticholinergic agent in the experiments.

The various observations pertaining to **1b–e** which are summarized in Table III are consistent with results obtained in vitro. A noteworthy feature is that the LD50 of contrathion is in the same range as those of **1d** and **1e** but is lower than those of **1c** and **1b**. Similarly, the protective index of this compound appears to be weaker than those of **1b** and **1e**. This makes the antidotal effect of **1b** and **1e** of interest, even though their reactivation potency is lower than that of the pyridinium aldoxime derivative.

Experimental Section

Materials. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker WP-200 (^1H, ^{13}C) and -500 (^{15}N) spectrometers with Me_2SO or Me_2SO-d_6 and CCl_4 as solvents. Chemical shifts are reported in parts per million (δ) from an internal tetramethylsilane standard; signals are designated as s (singlet) and d (doublet). Infrared (IR) spectra were obtained on a Bruker Model IFS 85 spectrophotometer. UV–vis measurements were performed by means of a Hewlett-Packard HP-8451 spectrophotometer, the cell compartment of which was thermostated at 25 ± 0.1 °C. Melting points were determined by a capillary method with a Büchi 501 apparatus. Microanalyses were performed by the CNRS Laboratory.

Literature methods were used to prepare MINA (**1a**)[46] and 3-bromo-2-oxopropanal oxime.[47] (Hydroxyimino)acetic acid ethyl ester was prepared according to the method of Kissinger and Ungnade,[48] by use of ethyl diethoxyacetate instead of its hemi-

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(42) Schoëne, K.; Oldriges, H. *Arch. Int. Pharmacodyn. Ther.* **1973**, *204*, 110.
(43) De Jong, L. P. A.; Wolring, G. Z. *Biochem. Pharmacol.* **1978**, *27*, 2229.
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(48) Kissinger, L. W.; Ungnade, H. E. *J. Org. Chem.* **1959**, *24*, 1517.

acetal as the starting material, mp 36 °C (lit.[48,49] mp 35 °C). All commercially available compounds and solvents used in this work were either of analytical grade or purified according to standard recrystallization or distillation procedures.

Electrophorus electricus E.C. 3.1.1.7 type VI S AChE was used as supplied by Sigma. The organophosphorus reagents were prepared and purified according to literature methods. The atropine sulfate was purchased from Merck and Co.

Ethyl [[(Trimethylsilyl)oxy]imino]acetate.[50] To a stirred solution of 48.8 g (0.42 mol) of (hydroxyimino)acetic acid ethyl ester in 400 mL of dry benzene was added dropwise 0.45 mol (46 g) of triethylamine at 20 °C. Then, the reaction mixture was cooled to ca. −5 °C, and 0.42 mol (46 g) of freshly distilled trimethylsilyl chloride was added. The resulting solution was allowed to warm to room temperature. After 1 h, the mixture was filtered, and the solvent was removed to give **2** as a crude product. Distillation yielded analytically pure ethyl [[(trimethyl)silyl]oxy]acetate (59.2 g, 75%) as a colorless oil: $bp^{0.4}$ 37 °C. Anal. ($C_7H_{15}NO_3Si$) C, H, N.

2-Oxo-3-(methylsulfinyl)propanal Oxime (1c).[50] Via the classical procedure by Corey,[52] a solution of the methylsulfinyl carbanion was prepared by heating a mixture of 7.8 g (0.26 mol) of sodium hydride in 60 mL of dry dimethyl sulfoxide at 55–60 °C and under a nitrogen atmosphere for 1 h. To this solution at room temperature was added with stirring 60 mL of dry THF. The resulting mixture was cooled at 5 °C, and 0.13 mol (24.6 g) of the ester **2** was added dropwise. After 1 h of being stirred at room temperature, the green solution turned orange, and excess THF was added until precipitation of the sodium salt of **1c** resulted. The precipitate was washed with THF and diethyl ether and ther. dissolved in the minimum amount of water. By addition of concentrated aqueous HCl, the pH was adjusted to 3.5, resulting in precipitation of a gray solid (6.6 g, 35%), which was filtered and washed with cooled water. Recrystallization in ethanol or acetonitrile afforded the analytical example of **1c**: mp 133 °C; NMR (Me_2SO-d_6) δ 12.96 (s, 1 H, NOH), 7.63 (s, 1 H, CH=NOH), 4.28 (d, AB system 2 H, J = 17 Hz, CH_2), 2.67 (s, 3 H, CH_3); IR (KBr) 3033 (m), 1676 (s), 1618 (m), 1040 (s), 982 (s) cm^{-1}; UV (H_2O) λ_{max} 234 nm (ϵ = 10 250 M^{-1} cm^{-1}), λ_{max} (oximate) 288 nm (ϵ = 17 200 M^{-1} cm^{-1}). Anal. ($C_4H_7NO_3S$) C, H, N, S.

2-Oxo-3-(methylsulfonyl)propanal Oxime (1d). A stirred mixture of 9 g (0.22 mol) of sodium hydride and 28.2 g (0.3 mol) of pure dimethyl sulfone in 140 mL of dry dimethyl sulfoxide was heated at 55–60 °C under nitrogen for 1 h. Dry THF (100 mL) was added to the resulting solution of the methylsulfonyl carbanion, and the mixture cooled at 5 °C prior to the dropwise addition 22.7 g (0.12 mol) of the ester **2**. The reaction mixture turned yellow, and some precipitate was formed, which disappeared on heating. After 2 h at 50 °C, excess of THF was added at room temperature, resulting in the precipitation of a crude salt, which was filtered, washed with THF and diethyl ether, and then redissolved in water. Acidification at pH 10 by HCl caused the precipitation of gray crystals that proved to be the sodium salt of **1d** (12.7 g): mp 175 °C. Anal. ($C_4H_6NNaO_4S$) C: calcd 25.67, found 25.25; H, N, Na. Further acidification at pH 4 gave the oxime **1d** (9.5 g, 50%), which was recrystallized from benzene: mp 114 °C. NMR (Me_2SO-d_6) δ 13.13 (s, 1 H, NOH), 7.67 (s, 1 H, CH=NOH), 4.72 (s, 2 H, CH_2), 3.12 (s, 3 H, CH_3); IR (KBr) 3355 (s), 1679 (s), 1605 (m), 1283 (s), 1138 (s), 941 (s), 577 (m) cm^{-1}; UV (H_2O) λ_{max} 236 nm (ϵ = 9600 M^{-1} cm^{-1}), λ_{max} (oximate) 290 nm (ϵ = 15 500 M^{-1} cm^{-1}). Anal. ($C_4H_7NO_4S$) C, H, N, S.

[3-(Hydroxyimino)-2-oxopropyl]dimethylsulfonium Bromide (1e). To a solution of 2-oxo-3-bromopropanal oxime (33 g, 0.2 mol) in 100 mL of acetone was added with stirring 22 g (0.35 mol) of dimethyl sulfide. The mixture turned brown rapidly, and some precipitate was formed within a few hours. After 3 days at room temperature, the resulting solid was filtered and recrystallized in ethanol to yield 31.2 g (68.8%) of pure **1e**: mp 133–134 °C; NMR (Me_2SO-d_6) δ 13.20 (s, 1 H, NOH), 7.74 (s, 1 H, CH=NOH), 5.18 (s, 2 H, CH_2), 3.01 (s, 6 H, CH_3); IR (KBr) 3369 (m), 1682 (s), 1601 (m), 922 (m) cm^{-1}; UV (H_2O) λ_{max} 234 nm (ϵ = 10 600 M^{-1} cm^{-1}), λ_{max} (oximate) 292 nm (ϵ = 11 800 M^{-1} cm^{-1}). Anal. ($C_5H_{10}BrNO_2S$) C, H, N; S: calcd 14.05, found 13.57.

2-Oxo-3-(methylthio)propanal Oxime (1b). A mixture of 20 g (0.088 mol) of **1e** and 16.3 g (0.26 mol) of dimethyl sulfide in acetonitrile was heated at 65 °C for 8 h.[53] Removal of the solvent gave an oil, which crystallized on cooling with ice water. The crude material was collected and recrystallized in a 80:20 hexane/benzene mixture to yield 7.1 g (62%) of pure **1b**: mp 78 °C; NMR (Me_2SO-d_6) δ 12.70 (s, 1 H, NOH), 7.56 (s, 1 H, CH=NOH), 3.60 (s, 2 H, CH_2), 2.06 (s, 3 H, CH_3); IR (KBr) 3224 (s), 1657 (s), 1608 (s), 933 (s); UV (H_2O) λ_{max} 230 nm (ϵ = 9650 M^{-1} cm^{-1}), λ_{max} (oximate) 284 nm (ϵ = 15 000 M^{-1} cm^{-1}). Anal. ($C_4H_7NO_2S$) C, H, N, S.

Physical Measurements. The acidity constants of **1a–e** were measured by potentiometry at 25 °C by using an electronic pH meter (Tacussel Isis 20000). For the determination of the pK_{a_1} values, buffer solutions with [Ox]/[OxH] ratios equal to 1/3, 1/1, and 3/1 were prepared so that the molarity of the charged species was equal to 0.01 M, i.e., the ionic strength I = 0.01 M. Under these experimental conditions at t = 25 °C, the pK_{a_1} values were related to the measured pH by the relation[29,30]

$$pK_{a_1} = pH - \log [Ox]/[OxH] \pm 0.5\sqrt{I} \quad (10)$$

The plus sign applied to neutral oximes while the minus sign applied to the sulfonium oxime. A similar procedure was used to measure the pK_{a_2} values for the ionization of the methylene group of **1c–e**. In these instances, the buffer solutions were prepared so that the molarity of the basic species was always equal to 0.01 M, but the ionic strength was kept constant at I = 0.06 M by adding KCl as needed. In addition, the appropriate corrections for the hydrolysis of the dibasic species were made in using eq 10 for **1c** (pK_{a_2} = 12.13) and **1d** (pK_{a_2} = 11.10).

Rate measurements were made with use of oximate buffers, varying the concentration of the acid and basic buffer components at constant pH and following spectrophotometrically the appearance of the resulting *p*-nitrophenoxide anion at 400 nm in a conventional spectrophotometer. All experiments were carried out at 25 °C under pseudo-first-order conditions with a large excess of the base reagent ([PNPA] = 5×10^{-5} M; [Ox] = 10^{-3}–10^{-2} M). Both the reactions with water and hydroxyl anion were negligible under these experimental conditions so that the observed first-order rate constant k_{obsd} obeyed the equation

$$k_{obsd} = k_1[Ox] \quad (11)$$

The log P values were calculated according to the fragmentation method of Rekker[55] by using the log P value previously reported by Benschop for MINA (**1a**) as a reference.[9] The aqueous phase for these determinations was pH 7.8, 0.1 M phosphate buffer.

Acetylcholinesterase Determinations in Vitro. The kinetics of the in vitro reactivation of immobilized eel AChE after inhibition with the appropriate organophosphorus ester were performed in the presence of substrate (ASCh), as recently described in detail in a paper dealing with the flow method used in this work.[39] The inhibition was effected at 95 ± 3% by flowing solutions of VX (10^{-6} M), sarin (2×10^{-6} M), and paraoxon (5×10^{-5} M) for 5, 2, and 1 min, respectively. The oxime concentrations used in reactivation experiments were in the range 0.001–0.01 M in a Tris buffer 0.05 M at ionic strength 0.14 M maintained by NaCl (pH 7.80). A new AChE fiberglass disk was used for each reactivating assay.

In Vivo Experiments. Acute toxicity was determined in male albino rats (Wistar; 180–200 g) with intramuscular coadministration of the oximes **1b–e** and atropine sulfate (17.4 mg/kg)

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(50) The methods used to prepare **2** and **1c** were first elaborated by L. Laloi in the laboratory.[51]
(51) Laloi, L., personal communication.
(52) Corey, E. J.; Chaykowsky, M. *J. Am. Chem. Soc.* **1965**, *87*, 1345.
(53) The thermic decomposition of **1e** is consistent with recent findings by Labuschagne et al. that many symmetrical and unsymmetrical organic sulfides can be prepared by heating of sulfonium halides under essentially nonbasic conditions.[54]
(54) Labuschagne, A. J. H.; Malherbe, J. S.; Meyer, C. J.; Schneider, D. F. *Tetrahedron Lett.* **1976**, 3571.
(55) Rekker, R. F. In *The Hydrophobic Fragment Constant*; Elsevier: Amsterdam, 1977.

dissolved in physiological salt. LD50 values were determined on five groups of 10 animals each and calculated according to the method of Litchfield and Wilcoxon.[56]

The doses of the oximes **1b** and **1e** producing no observable symptoms, i.e., the so-called sign free dose, were determined under similar experimental conditions.

The protective effects of **1b** and **1c** were evaluated against paraoxon in male albino rats (Wistar), with use of sign free dose conditions. Paraoxon dissolved in physiological salt was administrated subcutaneously while the oxime and atropine sulfate (17.4 mg/kg) were administered intramuscularly 1 min after intoxication. The protective index was calculated as the ratio LD50 (paraoxon + oxime)/LD50 (paraoxon). The protective index of atropine sulfate (17.4 mg/kg) was similarly calculated.

Acknowledgment. We are indebted to Dr. M. P. Simonnin for assistance in the analysis of NMR spectra and to Isabelle Callebat for therapeutic determinations.

Registry No. **1a**, 306-44-5; **1b**, 112740-61-1; **1c**, 112740-56-4; **1c**·Na, 112740-55-3; **1d**, 112740-58-6; **1d**·Na, 112740-57-5; **1e**, 112740-60-0; **2**, 112740-54-2; AChE, 9000-81-1; Me_2S, 75-18-3; PNPA, 830-03-5; VX, 50782-69-9; $(CH_3)_3SiCl$, 75-77-4; (*E*)-HON=$CHCO_2Et$, 31767-15-4; $MeSOCH_2^-$, 13810-16-7; $MeSO_2CH_2^-$, 29119-74-2; (*E*)-$BrCH_2COCH$=NOH, 112740-59-7; *p*-$MeOC_6H_4OH$, 150-76-5; *p*-MeC_6H_4OH, 106-44-5; PhOH, 108-95-2; *p*-AcC_6H_4OH, 99-93-4; *m*-AcC_6H_4OH, 121-71-1; *p*-ClC_6H_4OH, 106-48-9; 3,4-Cl_2-1-OHC_6H_3, 95-77-2; 3,5-Cl_2-1-OHC_6H_3, 591-35-5; 3,4,5-Cl_3-1-OHC_6H_3, 609-19-8; sarin, 107-44-8; paraoxon, 311-45-5.

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Methotrexate Analogues. 31. Meta and Ortho Isomers of Aminopterin, Compounds with a Double Bond in the Side Chain, and a Novel Analogue Modified at the α-Carbon: Chemical and in Vitro Biological Studies[1]

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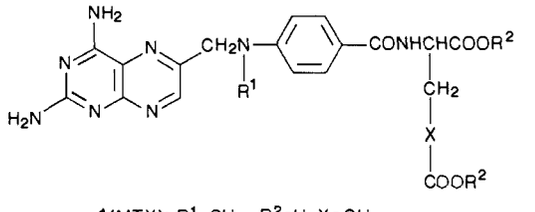
Five heretofore undescribed analogues of methotrexate (MTX) and aminopterin (AMT) were synthesized and tested as dihydrofolate reductase (DHFR) inhibitors and tumor cell growth inhibitors. The meta isomer of AMT was obtained from 2,4-diamino-6-(bromomethyl)pteridine and *m*-(aminobenzoyl)-L-glutamic acid, while the ortho isomer was obtained via the same route by using α-methyl γ-*tert*-butyl *o*-(aminobenzoyl)-L-glutamate instead of the free acid. Analogues of MTX and AMT containing a double bond in the side chain were prepared from dimethyl D,L-2-amino-4-hexenedioate and 4-amino-4-deoxy-N^{10}-methylpteroic acid and 4-amino-4-deoxy-N^{10}-formylpteroic acid, respectively. Finally, a positional isomer of MTX with the CH_2CH_2COOH moiety moved from the α-carbon to the adjacent carboxamide nitrogen was synthesized from 3-[*N*-(carboxymethyl)amino]propanoic acid diethyl ester and 4-amino-4-deoxy-N^{10}-methylpteroic acid. The positional isomers of AMT were weak DHFR inhibitors and showed very little growth-inhibitory activity against L1210 murine leukemia cells or the MTX-resistant L1210/R81 mutant line in culture. The MTX and AMT analogues with the CH_2CH_2COOH moiety replaced by a CH_2CH=$CHCOOH$ side chain showed anti-DHFR activity similar to that of the previously described saturated compound *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-2-aminoadipic acid, but were less potent than the parent drugs. The MTX analogue with the CH_2CH_2COOH side chain displaced from C to N was weakly bound to DHFR, confirming the importance of an intact CONH moiety, and showed greatly diminished cell growth inhibitory potency relative to MTX. None of the compounds was a substrate for folylpolyglutamate synthetase (FPGS) from mouse liver. Furthermore, inhibition of folic acid polyglutamylation in vitro at equimolar 500 μM concentrations of drug and substrate was negligible. The structural changes embodied in these five novel compounds are therefore too great for binding to the FPGS active site.

Structural analogues of methotrexate (MTX, **1**) and aminopterin (AMT, **2**) have been studied extensively as means of gaining a better understanding of the molecular features that are optimal for enzyme binding, cell membrane penetration, and in vitro/in vivo antitumor activity.[2]



1 (MTX): R^1 = CH_3; R^2 = H; X = CH_2
2 (AMT): R^1 = R^2 = H; X = CH_2
5: R^1 = CH_3; R^2 = H; X = CH=CH
6: R^1 = R^2 = H; X = CH=CH
16: R^1 = CH_3; R^2 = Me; X = CH=CH

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[§] Medical College of Ohio.

Work in this laboratory has focused mainly on the amino acid side chain,[1,3-13] though this is clearly not the only

(1) For paper 30 in this series, see: Rosowsky, A.; Solan, V. C.; Forsch, R. A.; Delcamp, T. J.; Baccanari, D. P.; Freisheim, J. H. *J. Med. Chem.* **1987**, *30*, 1463.
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