(Z),13(E)-dienoic acid) was purchased from Upjohn Diagnostics (Kalamazoo, MI).

Platelet Antiaggregatory Activities. Blood was collected from normal human volunteers who reported to be free of medication for at least 10 days prior to blood collection. Platelet-rich plasma was prepared as described previously[4] and used for all studies with the exception of thrombin-induced AA release. For the latter experiments, washed platelets were prepared and suspended in a modified Tyrode's solution, pH 7.4 as previously described.[17] The platelet count was adjusted to 3×10^8/mL for aggregation, secretion, and biochemical studies.

Platelet aggregation studies were performed according to the turbidometric method of Born[18] in a Payton Model 600 dual-channel aggregometer interfaced to an Apple microcomputer for acquisition, quantitation, presentation, and management of platelet aggregation data.[19] All inducers were used at the minimum concentrations required to stimulate maximal aggregation. Inhibitors were added 1 min prior to induction of platelet activation, and inhibitory concentration-50 (IC_{50}) values for each inhibitor were determined from changes in the amplitude of light transmittance after 6 min for ADP and 4 min for AA and U46619. Secretion of the contents of platelet-dense granules was measured by monitoring the release of radioactivity from platelets prelabeled with [^{14}C]serotonin.[17] Malondialdehyde formation induced by AA was determined as previously described.[17]

To study the release of AA from platelet phospholipids, washed platelets were incubated with [^{3}H]AA (3.6 μCi/10^9 platelets) for 2 h at room temperature prior to the final centrifugation and resuspension of the washing procedure. Released [^{3}H]AA was quantified[17] from supernatants of stimulated platelet samples in a manner identical with the determination of serotonin secretion.

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Registry No. 3, 103620-90-2; 12, 4068-78-4; 13, 103620-87-7; 14, 103620-88-8; 14 (diol), 103620-89-9; ethyl (phenylmethoxy)-acetate, 32122-09-1.

Nonquaternary Cholinesterase Reactivators. 3. 3(5)-Substituted 1,2,4-Oxadiazol-5(3)-aldoximes and 1,2,4-Oxadiazole-5(3)-thiocarbohydroximates as Reactivators of Organophosphonate-Inhibited Eel and Human Acetylcholinesterase in Vitro

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As an extension of an earlier investigation (*J. Med. Chem.* **1984**, *27*, 1431), we prepared a series of 3-substituted 5-[(hydroxyimino)methyl]-1,2,4-oxadiazoles and the corresponding 5-thiocarbohydroximic acid 2-(*N*,*N*-dialkylamino)ethyl *S*-esters. The compounds were evaluated in vitro as reactivators of phosphonylated electric eel and human erythrocyte (RBC) acetylcholinesterases (AChE). The compounds were characterized with respect to (hydroxyimino)methyl acid dissociation constant, nucleophilicity, octanol/buffer partition coefficient, reversible AChE inhibition, and kinetics of reactivating ethyl methylphosphonylated AChE. One compound was also tested for effectiveness in preventing AChE phosphonylation. All of the tested compounds significantly reactivate ethyl methylphosphonylated AChE: the 3-*n*-octyl- and 3-(1-naphthyl)-substituted aldoximes are as reactive (within a factor of 5-10) toward the inhibited enzymes as the benchmark pyridinium reactivators, 2-PAM and HI-6. All of the substituted thiocarbohydroximic acid *S*-esters are powerful reversible inhibitors of AChE's: the 3-*n*-octyl- and 3-(1-naphthyl)-substituted thiocarbohydroximates inhibit eel AChE to 50% initial activity at concentrations <5 μM. When added to an eel AChE solution at concentrations between 5 and 50 μM, the 3-phenyl-substituted thiocarbohydroximate effectively antagonizes AChE inhibition by ethyl *p*-nitrophenyl methylphosphonate (EPMP), suggesting the potential utility of this compound for preventing anti-AChE-agent poisoning.

Various organophosphorus esters irreversibly inhibit acetylcholinesterase (AChE) by phosphonylating a serine hydroxyl at the enzyme active site.[1-7] Appropriately designed nucleophiles can reversibly bind to phosphonylated AChE and displace the phosphorus moiety, thereby restoring enzymatic activity. The therapeutic use of such cholinesterase "reactivators" to treat poisoning by organophosphorus anticholinesterase agents is well-known.[8-11] Our own research[12-15] has focused on compounds that combine the structural elements required for activity vs. phosphonylated AChE but that are devoid of any quaternary functionality. These nonquaternary reactivators should afford significant advantages over conventional

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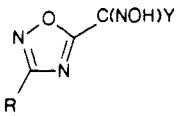
Table I. Selected Data for 3-Substituted 1,2,4-Oxadiazol-5-aldoximes and 1,2,4-Oxadiazole-5-thiocarbohydroximates

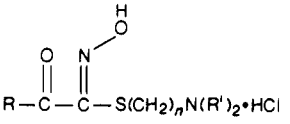


no.	R	Y	yield,[a] %	mp, °C	pK_a	log P[b]	formula[c]	analysis[c]	scheme
2a[d]	C_6H_5	H	7.9	166–168	7.94	2.61	$C_9H_7H_3O_2$	*d*	I/II/III
2b	t-C_4H_9	H	10	130.5–132	7.91	2.03	$C_7H_{11}N_3O_2$	C, H, N	I
2c	n-$(CH_2)_7CH_3$	H	43	84.5–85	8.22	>3.0	$C_{11}H_{19}N_3O_2$	C, H, N	I
2d(E)[d]	CH_3	H	7.0	163.5–164	8.11	0.47	$C_4H_5N_3O_2$	C, H, N	I
2d(Z)	CH_3	H	2.0	128–128.5	8.60	*e*	$C_4H_5N_3O_2$	C, H, N	I
2e	1-naphthyl	H	14	153–155	8.05	>3.0	$C_{13}H_9N_3O_2$	C, H, N	I
2f	2-thienyl	H	1.5	129–131.5	8.03	2.38	$C_7H_5N_3O_2S$	C, H, N, S	I
2g	$CH_2C_6H_5$	H	36	153–156	7.94	2.18	$C_{10}H_9N_3O_2$	C, H, N	II
2h	4-pyridyl	H	83	249–250	8.00	*e*	$C_8H_6N_4O_2$	C, H, N	III
2i	$C_6H_5(5)$[g]	H	84	162–163	8.12	2.33	$C_9H_7N_3O_2$	C, H, N	III
3a[d]	C_6H_5	$SCH_2CH_2N(C_2H_5)_2$	27	199–201	6.32	1.78	$C_{15}H_{20}N_4O_2S$	*d*	IV
3b	t-C_4H_9	$SCH_2CH_2N(C_2H_5)_2$	81	87–87.5	6.61	1.16	$C_{13}H_{24}N_4O_2S$	C, H, N, S	IV
3c	n-$(CH_2)_7CH_3$	$SCH_2CH_2N(C_2H_5)_2$	39	120–121	6.34	>3.0	$C_{17}H_{33}N_4O_2SCl$[f]	C, H, N, S, Cl	IV
3d[d]	CH_3	$SCH_2CH_2N(C_2H_5)_2$	63	92–93.5	6.32	−0.41	$C_{10}H_{18}N_4O_2S$	*d*	IV
3e	1-naphthyl	$SCH_2CH_2N(C_2H_5)_2$	84	193.5–196	6.50	2.92	$C_{19}H_{23}N_4O_2SCl$[f]	C, H, N, S	IV
3f	$CH_2C_6H_5$	$SCH_2CH_2N(C_2H_5)_2$	78	160–161	6.19	1.28	$C_{16}H_{23}N_4O_2SCl$[f]	C, H, N, S, Cl	IV
3g	$C_6H_5(5)$[g]	$SCH_2CH_2N(C_2H_5)_2$	98	175–176	7.36	1.83	$C_{15}H_{24}N_4O_2WCl$[f]	C, H, N, S, Cl	IV
15	C_6H_5	$NHCH_2CH_2N(C_2H_5)_2$	30	212–215	9.85	*e*	$C_{15}H_{22}N_5O_2Cl$[f]	C, H, N, Cl	IV

[a] Yield for production of target compound from immediate precursor. [b] log P is the octanol/buffer partition coefficient for 0.1 M, pH 7.6 phosphate buffer. [c] Elemental analytical results for indicated elements were within ±0.4% of theoretical. [d] Reported in ref 15. [e] Not determined. [f] Isolated as HCl salt. [g] Isomeric 5-aryl-3-[(hydroxyimino)methyl]-1,2,4-oxadiazole.

reactivators, such as 2-[(hydroxyimino)methyl]-1-methylpyridinium iodide (2-PAM), with respect to penetration through hydrophobic cell membranes into critical tissue regions (such as the central nervous system).

We previously examined a series of α-keto thiocarbohydroximic acid (*N*,*N*-dialkylamino)alkyl *S*-esters, **1**,[12] heteroaromatic aldoximes, **2**, and heteroaromatic thiocarbohydroximic acid (*N*,*N*-dialkylamino)alkyl *S*-esters, **3**,[15] for activity in vitro vs. electric eel AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate (EPMP). These



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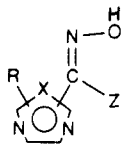
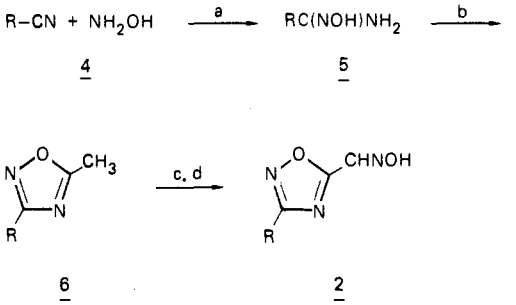


2, Z = H
3, Z = $SCH_2CH_2N(C_2H_5)_2 \cdot HCl$

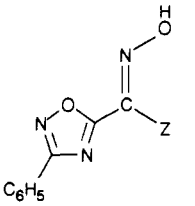
studies revealed particularly high reactivity for the 3-phenyl-1,2,4-oxadiazol-5-aldoxime (**2a**). Furthermore, the corresponding 3-phenyl-1,2,4-oxadiazole-5-thiocarbohydroximic acid 2-(*N*,*N*-diethylamino)ethyl *S*-ester (**3a**)

Scheme I. Alkyllithium-Induced Nitrosation[a]



[a] a, aqueous C_2H_5OH, Δ; b, $(CH_3CO)_2O$, Δ; c, n-BuLi, THF, −78 °C; d, i-C_3H_7ONO, H^+.

proved to be a powerful competitive, reversible inhibitor of AChE. These findings suggested the 1,2,4-oxadiazole



2a, Z = H
3a, Z = $SCH_2CH_2NEt_2 \cdot HCl$

ring system as a likely target for structural "fine-tuning" to improve maximal activity. Accordingly, 14 new type **2** and type **3** compounds representing the 1,2,4-oxadiazole ring system were synthesized. As before, these compounds were evaluated with respect to (hydroxyimino)methyl acid dissociation constant, nucleophilicity, lipophilicity, reversible inhibition of AChE, and kinetics of reactivating EPMP-inhibited electric eel AChE. We also extended our studies to test the in vitro activity of type **2** and type **3** compounds vs. EPMP-inhibited human erythrocyte AChE and their effectiveness in preventing eel AChE inhibition by EPMP. For comparison we also report results for the previously prepared 3-methyl and 3-phenyl type **2** and type **3** compounds[15] and for the well-known pyridinium reactivators, 2-PAM and 4-carbamoyl-2′-[(hydroxyimino)-

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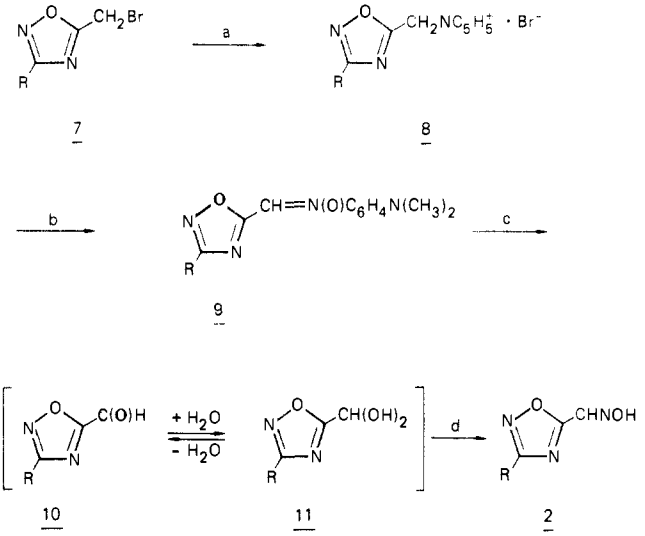
Scheme II. Hydrolysis of an Aldehyde Equivalent[a]



[a] a, 2 equiv of NC_5H_5, Δ; b, *N*,*N*-dimethylnitrosoaniline, NaOH; c, H^+; d, C_2H_5OH, NH_2OH.

methyl]-1,1′-oxydimethylenebis(pyridinium chloride), HI-6.

Results and Discussion

Synthesis and Structure. Fourteen new type **2** and type **3** compounds were prepared. Table I provides structures and selected physical data for these new compounds, as well as those previously reported materials **2a**, **3a**, **2d(E)**, and **3d**.

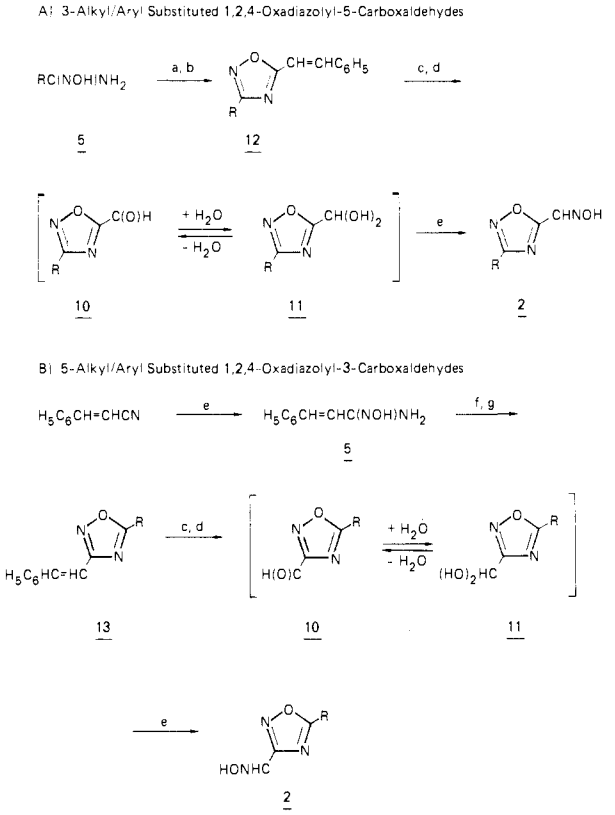
Three routes were used to prepare type **2** compounds. As previously described,[15] appropriately substituted nitriles, **4**, were converted to their corresponding amidoximes, **5**, and subsequently to the 3-substituted 5-methyl-1,2,4-oxadiazole, **6**, yielding the oxime precursors (Scheme I). Alkyllithium-induced nitrosation then provided the desired 1,2,4-oxadiazol-5-aldoximes, **2**.

As an alternative, compounds **2a** and **2g** were prepared via hydrolysis of an aldehyde equivalent, **9**, followed by oximation of the aldehyde, **10** (Scheme II). Compounds **2a** and **2h** were obtained via ozonolysis of the corresponding styryloxadiazole, **12**, followed by oximation of the resulting aldehyde (Scheme III). Scheme III provided **2a** under milder conditions and in higher yield than either Scheme I or Scheme II. Additionally the ozonolysis method gave a convenient route to the isomeric 5-substituted 1,2,4-oxadiazol-3-aldoxime **2i**.

Type **3** compounds were prepared (Scheme IV) from the corresponding type **2** compounds by chlorination and thioesterification as reported previously.[15] Treating the hydroxyimoyl chloride **14** with a primary amine gave the analogous 1,2,4-oxadiazolyl-substituted amidoxime **15**.

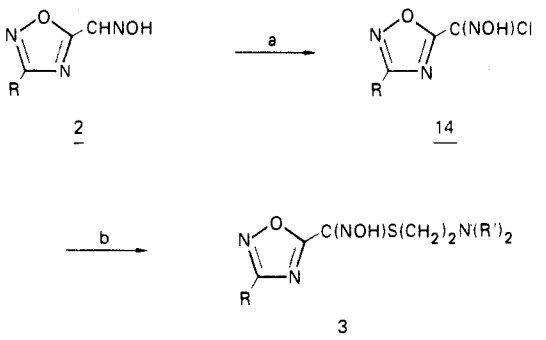
We separated and characterized the (*E*)- and (*Z*)-(hydroxyimino)methyl isomers derived from nitrosation of 3,5-dimethyl-1,2,4-oxadiazole **2d**. As expected, the physical properties differed substantially, as shown in Table I. The major component (80%) of the mixture exhibited an NMR proton resonance at δ 12.06 (NOH) and 8.26 (CH), whereas the minor isomer showed peaks at δ 12.34 (NOH) and 7.77 (CH). By analogy to literature examples,[16,17] we assigned the *E* and *Z* configurations, respectively, to the major and minor components of the **2d** isomer mixture. We postulate that the higher pK_a of the *Z* isomer results from hydrogen

Scheme III. Ozonolysis[a]



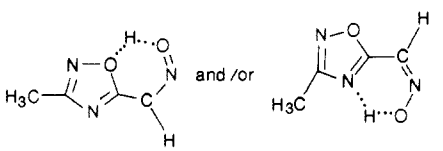
[a] a, Cinnamoyl chloride, K_2CO_3, DMK; b, Δ; c, O_3, CH_2Cl_2, −78 °C; d, Me_2S; e, NH_2OH, C_2H_5OH; f, acid chloride or anhydride, K_2CO_3, DMF; g, Δ.

Scheme IV. Thiocarbohydroximate Synthesis[a]



[a] a, NCS, DMF, Δ; b, $HSCH_2CH_2N(R')_2/NH_2CH_2CH_2N(R')_2$, CH_2Cl_2.

bonding between the hydroxyimino proton and ring heteroatoms. Such six-membered ring conformations were postulated to explain the remarkable stability of the 3- and 5-substituted geminal diols **11** over the generally preferred carboxaldehyde structures, **10**, in the 1,2,4-oxadiazole ring systems.[18]



Chemical and Physical Properties. All of the type **2** compounds listed in Table I exhibited (hydroxyimino)-methyl acid dissociation constant (pK_a) values near the

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Table II. Characteristic Constants for Reversible Inhibition of AChE (IC_{50}) and for the Rate of EPMP-Inhibited AChE Reactivation (k_{OX} and k_{HOX}) by Various Test Compounds

		eel AChE			human AChE		
compd[a]	substituent	IC_{50}, mM	k_{OX}, M^{-1} min^{-1}	k_{HOX}, M^{-1} min^{-1}	IC_{50}, mM	k_{OX}, M^{-1} min^{-1}	k_{HOX}, M^{-1} min^{-1}
2a	C_6H_5	>1.0	848	270	>1.0	141	48.5
2b	t-C_4H_9	>1.0	67.1	22.1	>1.0	23.4	31.6
2c	n-C_8H_{17}	>1.0	2170	419	>1.0	761	147
2d(E)	CH_3	>1.0	542	130	>1.0	47.4	11.2
2d(Z)	CH_3	>1.0	154	14.0	>1.0	24.0	2.4
2e	1-naphthyl	>1.0	2240	587	>1.0	390	102
2f	2-thienyl	>0.5	1550	327	>0.36	104	28.2
2g	$C_6H_5CH_2$	>1.0	368	116	>1.0	158	49.6
2h	4-pyridyl	[c]	[c]	[c]	>1.0	358	102
2i	$C_6H_5(5)$[b]	>1.0	3.1	0.72	>1.0	1.4	0.3
3a	C_6H_5	0.0075	21.9	20.8	0.006	33.2	31.6
3b	t-C_4H_9	0.099	0.94	0.85	0.39	[d]	[d]
3c	n-C_8H_{17}	0.0014	[c]	64.5	0.001	[c]	[c]
3d	CH_3	0.104	3.1	2.9	0.09	1.5	1.4
3e	1-naphthyl	0.0013	41.2	38.2	[c]	[c]	[c]
3f	$C_6H_5CH_2$	0.055	1.5	1.4	0.030	1.5	1.4
3g	$C_6H_5(5)$[b]	0.22	36.7	23.3	[c]	13.9	8.8
15	C_6H_5	0.89	327	19.4	0.26	[c]	[c]
2-PAM[e]		0.4	15260	4418	>1	1877	543
HI-6[e]		0.4	412	281	>1	5041	3435

[a] See Table I for structure and other details. [b] Isomeric 5-aryl-3-[(hydroxyimino)methyl]-1,2,4-oxadiazole. [c] Not determined. [d] No significant reactivation at highest test-compound concentration (0.5 mM). [e] 2-PAM, 2-[(hydroxyimino)methyl]-1-methylpyridinium halide; HI-6, 1-[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride.

optimal[8] value of pK_a = 8. Converting the type **2** compounds to the type **3** analogues lowered the dissociation constants by ca. 1.5–2 pK_a units. The amidoxime derivative **15** had a significantly higher pK_a value of 9.85, far outside the useful therapeutic range.

To assess the inherent nucleophilicities of the target compounds, we determined the bimolecular rate constant, k_n, for reaction of the anionic (oximate) form of selected test compounds toward acetylthiocholine (AcSCh) using methods described previously.[15] Combining k_n and pK_a values for the compounds listed in Table I with parallel data taken from Table II of ref 15 provided the linear least-squares regression line shown in eq 1 with n = 27 and

$$\log k_n = -3.48\ (\pm 0.33) + 0.63\ (\pm 0.043)pK_a \qquad (1)$$

squared correlation coefficient R^2 = 0.89. Thus the Brønsted behavior established previously for type **2** and **3** compounds extends to the new compounds reported herein.

The octanol/buffer coefficients were determined for the compounds listed in Table I. All the tested compounds partitioned primarily into the organic phase, whereas 2-PAM and HI-6 partition almost entirely into the aqueous phase (log P = −3.24).[19] Converting the type **2** aldoximes to the corresponding thiocarbohydroximates lowered the partition coefficient by ca. 0.8 log unit. Some of the type **2** compounds were extremely hydrophobic; for example, compounds **2c** and **2e** exhibited log P values greater than 3. Both **2c** and **2e** were poorly soluble in water, even at the low test-compound concentrations (10^{-3} to 10^{-5} M) employed in reactivation experiments (below). Consequently, all kinetic experiments were performed in pH 7.6 aqueous buffer with 4% ethanol added to prevent test-compound precipitation.

Reversible Inhibition and Reactivation of AChE. Our previous investigation[15] with EPMP-inhibited eel AChE established that the interactions of type **2** and type **3** compounds with ethyl methylphosphonylated AChE and with active enzyme are adequately described by eq 2–6

$$EI + R \overset{K_r}{\rightleftharpoons} [EI{\cdot}R] \qquad (2)$$

$$[EI{\cdot}R] \xrightarrow{k_r} E \qquad (3)$$

$$E + R \overset{K_i}{\rightleftharpoons} [E{\cdot}R] \qquad (4)$$

$$EI \xrightarrow{k_{sp}} E \qquad (5)$$

$$HOX \overset{K_a}{\rightleftharpoons} OX^- + H^+ \qquad (6)$$

where R is a test compound, EI is ethyl methylphosphonylated AChE, [EI·R] is a reversibly formed complex between reactivator and phosphonylated AChE, E is active enzyme, [E·R] is a reversibly formed (inactive) complex between AChE and reactivator, HOX is the protonated (oxime) form of added test compound, and OX^- is the anionic (oximate) form of added test compound. Suitable control experiments were performed with the EPMP-inhibited human erythrocyte AChE and showed that eq 2–6 also extend to the human enzyme.

In the absence of EPMP, test compounds inhibited AChE in a reversible, time-independent fashion. Therefore we express the reactivator–AChE inhibition as the IC_{50}, that is, the concentration of test compound that inhibits 50% of AChE activity.

With test compound added in exess over EPMP-inhibited enzyme, pseudo-first-order reactivation kinetics were

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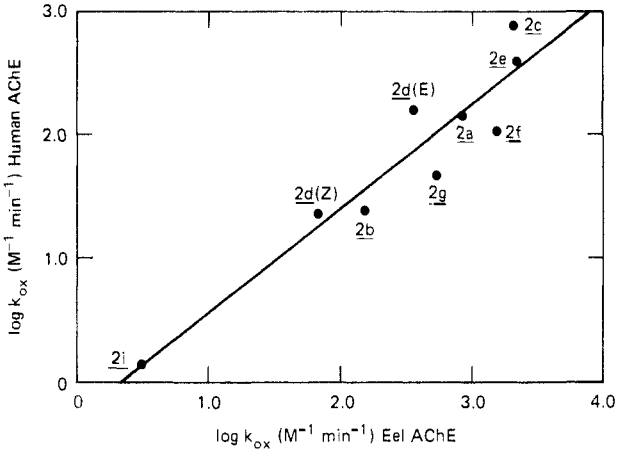

Figure 1. Correlation between the bimolecular rate constant k_{ox} for reactivation of EPMP-inhibited eel AChE vs. EPMP-inhibited human AChE for oxadiazole aldoximes **2**.

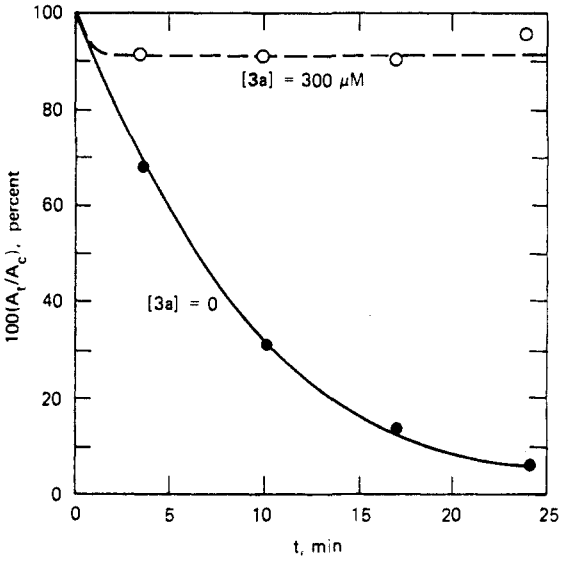

Figure 2. Percentage of control eel acetylcholinesterase activity, $100(A_t/A_c)$ vs. incubation interval, t, for reaction of enzyme (A_c = 5.11 μM min^{-1}) with 20 nM ethyl *p*-nitrophenyl methylphosphonate in the absence (●) or presence (○) of initially added **3a**.

observed. Following the previously derived kinetic treatment,[15] it is convenient to express reactivation potency as k_{OX} (= k_r/K_r), the bimolecular rate constant for reactivation in the limit of low reactivator concentration ([OX] ≪ K_r). Because various type **2** and type **3** compounds ionize to different extents at pH 7.6 (the pH of the buffer used in the in vitro evaluations), the effective rate constant for reactivating inhibited AChE, k_{HOX}, was defined as the product of k_{OX} and the fraction of added test compound present as oximate at pH 7.6: $k_{OX}[1 + \text{antilog}(pK_a - 7.6)]$. Table II summarizes data for reversible inhibition of AChE activity by the compounds and for reactivation of ethyl methylphosphonyl–AChE by the compounds.

The table shows that the reactivation data divide into two groups according to compound structure. Thus the type **2** compounds reversibly inhibit AChE poorly (or not at all) and reactivate EPMP-inhibited enzyme well, whereas the opposite is true for the type **3** compounds. The poor reactivation potency of the type **3** compounds derives from their low (hydroxyimino)methyl pK_a values and the concomitantly low nucleophilicities (see eq 1). Strong reversible AChE inhibition by the type **3** compounds suggests "two-site" binding to AChE via hydrophobic and Coulombic interactions as previously proposed.[15]

Oximes **2c** and **2e** exhibited the highest k_{OX} values of the nonquaternary compounds tested. Indeed these two compounds are the most potent nonquaternary reactivators of EPMP-inhibited AChE that we have prepared to date and are the first nonquaternary reactivators that approach (within 1 order of magnitude) the activity of 2-PAM.

It is interesting to note that (except for HI-6) the compounds tested showed parallel k_{OX} values toward both sources of AChE. Figure 1 is a plot of log k_{OX} values for reactivation of EPMP-inhibited eel vs. human enzymes. log–linear least-squares regression analysis of the data gave eq 7 with n = 10, R^2 = 0.961, and uncertainties about the

$$\log k_{OX}(\text{human}) = [\log k_{OX}(\text{eel})](0.884 \pm 0.068) - (0.761 \pm 0.087) \quad (7)$$

slope and intercept values expressed as ±95% confidence limits. For this series of compounds, the eel enzyme serves as a reasonable in vitro model for human AChE. It is unknown what factors contribute to the anomolously high reactivity of HI-6 vs. EPMP-inhibited human AChE.

Highly quantitative structure–activity relationships for the type **2** and type **3** reactivators were not apparent. The heterogeneity of structures presented in Table I probably accounts at least in part for the failure to observe quantitative relationships. Stepwise multiple linear regression of IC_{50} or k_{OX} values vs. pK_a values, plus steric, hydrophobic, and electronegativity substituent constants gave only modest correlations. The most significant correlation was eq 8 with n = 11, F = 9.2, and R^2 = 0.696. Probably,

$$k_{OX}(\text{eel}) = -4680 + 467 \log P + 588pK_a \quad (8)$$

hydrophobicity does play an important role in controlling activity of the nonquaternary type **2** and **3** compounds, but additional compounds must be prepared and tested to provide a quantitative basis for describing the influence of hydrophobicity on reactivation potency.

Finally, comparing k_{HOX} values from Table II for compounds **2a**, **2d**, **3d**, and 2-PAM with k_{HOX} values reported for the same compounds in Table V of ref 15 reveals 2- to 3-fold differences in reactivity toward ethyl methylphosphonylated eel AChE. Presumably the reactivity differences reside in the slightly different reaction conditions employed: the data reported herein pertain to buffer solutions containing 4% EtOH, whereas as the experiments reported in ref 15 did not use EtOH in the buffer solutions. It is recognized that organic solvents can influence AChE activity and thereby reactivation kinetics.[33-35] Because the data reported herein all used 4% ethanolic buffer, the results are internally consistent. However, direct comparisons of the data in Table II with results obtained in other reaction media will be of questionable validity.

Antagonism of AChE Inhibition by EPMP. The strong reversible inhibition of AChE by the type **3** compounds and the previous demonstration[15] that inhibition by **3a** is competitive suggested that it might be possible to protect the enzyme active site from irreversible phosphonylation by preincubating AChE with a thiocarbohydroximate prior to adding organophosphonate inhibitor. To test this, we incubated AChE with excess **3a** prior to

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Table III. Eel AChE Activities after Reaction of Enzyme with Ethyl p-Nitrophenyl Methylphosphonate in the Presence of Initially Added 3a

[3a], μM	incubation interval, min	$100(A_t/A_c)$[a]
100	25	102
30.0	25	91
10.0	33	79
3.00	33	46

[a] A_t corrected for AChE inhibition by 3a in the assay solution according to the expression A_t = (observed activity)[100/(100 − I)].

adding EPMP (which was also in excess over AChE) and assayed AChE activities as a function of 3a concentration and incubation interval after adding EPMP.

Figure 2 shows the dependence of AChE activity on incubation interval for the reaction of the enzyme with 20 nM EPMP in the absence of 3a and in the presence of 300 μM 3a. In the figure, the percentage observed activity at time t (A_t) relative to control activity (A_c) includes a correction for enzyme inhibition (I) by 3a in the assay using the expression A_t = (observed activity)100/(100 − I). The figure reveals pseudo-first-order loss of AChE activity in the absence of 3a. We repeated the inhibition experiment in the absence of 3a three times and calculated a bimolecular rate constant, k_i, for AChE inhibition by EPMP according to eq 9 and 10. For the three runs, we found k_i = 1.1 (±0.25) × 10^7 M^{-1} min^{-1}.

$$\ln (A_t/A_c) = k_9 t \quad (9)$$

$$k_9 = k_i[\text{EPMP}] \quad (10)$$

Figure 2 also reveals that incubating AChE with 300 μM 3a before adding EPMP markedly reduced the degree of inhibition at any incubation interval after addition of EPMP. The data for AChE activity in the presence of 3 showed a rapid initial loss of enzyme activity, followed by a plateau in activity corresponding to establishment of a steady state.

We also determined the AChE activity after adding EPMP as a function of 3a concentration. In these experiments, we preincubated AChE with 100 to 3.00 μM 3a, added 20 nM EPMP, and determined percentage AChE at an incubation interval (t = 17 to 33 min) corresponding to the plateau region in Figure 2. Table III summarizes these data. Here, the relationship between AChE activity and [3a] was clear: residual AChE activity increased monotonically with [3a]. Even at the lowest concentration of 3a (3 μM), AChE activity did not decrease below 40% of control during the experiment.

Conclusions

The present study reveals that, as a class, 3-substituted 1,2,4-oxadiazol-5-aldoximes are relatively potent reactivators of both eel and human AChE's inhibited in vitro by ethyl p-nitrophenyl methylphosphonate (EPMP). Within the class, the 3-n-octyl- and 3-(1-naphthyl)-substituted oximes (compounds 2c and 2e, respectively) are the most potent reactivators. Compounds 2c and 2e rival the benchmark reactivator, 2-PAM, for activity vs. EPMP-inhibited AChE and probably represent the most inherently active nonquaternary reactivators known.

Another important observation is that incubating eel AChE with compound 3a (the 3-phenyl-1,2,4-oxadiazole-5-thiocarbohydroximate) effectively protects the enzyme against irreversible inhibition by EPMP in vitro. This protection is significant even at 3a concentrations as low as 3 μM. The demonstrated antagonism of irreversible phosphonylation plus its modest reactivation potency suggest that compound 3a has potential as a pretreatment drug to *prevent* poisoning following exposure to organophosphonates.

This report concludes our synthesis and in vitro testing program of α-heteroaromatic aldoximes and thiocarbohydroximate reactivators. Structural "fine-tuning" of the 3-substituted 1,2,4-oxadiazole system has now provided several compounds with interesting properties. Although additional structural modifications, for example, to increase hydrophobicity, could lead to candidate compounds with even higher inherent activity, the current evidence suggests that activity gains would be modest. Also, increasingly hydrophobic reactivators face some practical dosage form design limitations because of very poor aqueous solubility. We now have several examples of nonquaternary reactivators with reasonably high inherent activity in vitro. Whether improved tissue distribution of the nonquaternary reactivators in vivo will afford significant antidotal efficacy gains relative to well-known pyridinium oximes is the important issue that remains to be established in future investigations.

Experimental Section

Materials. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associates EM-360 or EM-390 spectrometer. Chemical shifts are reported in parts per million (δ) from an internal tetramethylsilane standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Infrared (IR) spectra were obtained on a Perkin-Elmer Model 1420 spectrophotometer. Melting points were determined on a calibrated Fisher-Johns melting point apparatus. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and the results are reported in Table I.

Tetrahydrofuran (THF) was distilled from calcium hydride or benzophenone ketyl and stored under nitrogen over a 4A molecular sieve. Metalation reactions were performed under a positive nitrogen pressure. Analytical thin-layer chromatography (TLC) was performed on Analtech Uniplate silica gel GF (scored 10 × 20 cm plates, 250 μm); preparative TLC was performed on Uniplate silica gel GF (20 × 20 cm plates, 2000 μm). Column chromatography was done on reagent silica gel (90–200 mesh) obtained from Accurate Chemical and Scientific Corp. Eel acetylcholinesterase lyophilized powder was from Worthington. Human erythrocyte enzyme was obtained from Sigma. Ethyl p-nitrophenyl methylphosphonate (EPMP) was prepared by conversion of diethyl methylphosphonate to ethyl methylphosphonochloridate followed by reaction with p-nitrophenol.[20] **CAUTION!** EPMP is an extremely toxic anticholinesterase agent. It must be handled with gloves and in a fume hood or at high dilutions at all times.

General procedures for synthetic steps are given below along with details for individual representative compounds.

General Procedure for Preparation of Amidoximes 5. All amidoximes were prepared from the corresponding nitriles by use of previously reported procedures.[21-27] Generally, 1 equiv of sodium hydroxide dissolved in water was added to a solution of hydroxylamine hydrochloride (1 equiv) dissolved in 95% ethanol. The appropriate aryl or alkyl nitrile (0.25–1.0 mol) in 95% ethanol was added to the above solution. The mixture was heated under reflux for 8–24 h. After cooling, the solvent was removed in vacuo at 60 °C, the residue was treated with a slight excess of 3 M hydrochloric acid, and the mixture was heated to boiling. Insoluble material was filtered and the cool filtrate extracted with dichloromethane. The aqueous layer was adjusted to pH 8 with concentrated ammonium hydroxide. The precipitate was collected and recrystallized from an appropriate solvent, furnishing the desired amidoximes in 45–70% yield. The amidoximes prepared exhibited the following properties.

Benzamidoxime (5a). Benzamidoxime was prepared on a 1.0-molar scale. The crude product was recrystallized from benzene/hexane, giving 96.9 g (57%) of 5a, mp 70–71 °C (lit.[23] mp 70–70.5 °C).

2,2-Dimethylpropionamidoxime (5b) was prepared on a 0.5-molar scale. The crude product was recrystallized from

toluene, giving 26.1 g (45%) of **5b**, mp 112–114 °C (lit.[24] mp 115–116 °C).

***n*-Nonanamidoxime (5c)** was prepared on a 0.25-molar scale. The crude product was recrystallized from toluene, giving 20.2 g (59%) of **5c**, mp 160–163 °C.

Acetamidoxime (5d) was prepared on a 1.0-molar scale and was isolated as the hydrochloride salt in 46% yield, mp 140–142 °C (lit.[25] mp 140 °C).

1-Naphthalenamidoxime (5e) was prepared on a 0.25-molar scale. The crude product was recrystallized from 60% aqueous ethanol to give 25.6 g (55%) of **5e**, mp 149.5–151.5 °C (lit.[23] mp 148–149 °C).

2-Thiophenamidoxime (5f) was prepared on a 0.25-molar scale. The crude product was obtained as a black crystalline solid and was used without further purification after drying in vacuo, mp 88–90 °C (lit.[21] mp 91–92 °C).

Phenylacetamidoxime (5g) was prepared on a 0.5-molar scale. The crude product was recrystallized from toluene, giving 43.5 g (57%) of **5g**.

4-Pyridinamidoxime (5h) was prepared on a 0.20-molar scale. The crude product was recrystallized from ethanol, giving 18.0 g (53%) of **5h**, mp 207–208 °C.

4-Phenyl-2-oxobutanamidoxime (5i) was prepared on a 0.20-molar scale.[28] The crude product was recrystallized from diethyl ether to give 27.7 g (88%) of **5i**.

General Procedure for Preparing 3-Substituted 5-Methyl-1,2,4-oxadiazoles (6). The 1,2,4-oxadiazole ring system was prepared by treating the appropriate amidoxime (**5**) at reflux for 15–30 min with an excess of an anhydride or acid chloride. For example, the 3-substituted 5-methyl-1,2,4-oxadiazoles were prepared by heating the amidoximes **5** at reflux in an excess of acetic anhydride. The resulting solution was cooled and diluted with water, and the pH was adjusted to 8 with concentrated ammonium hydroxide. The mixture was extracted with ether, and the combined ether extracts were washed with brine and dried over anhydrous $MgSO_4$. Removal of solvent afforded the 3-substituted 5-methyl-1,2,4-oxadiazoles **6**, which were further purified by distillation or column chromatography on silca gel. The synthesized 3-substituted 5-methyl-1,2,4-oxadiazoles exhibited the following properties.

3-Phenyl-5-methyl-1,2,4-oxadiazole (6a). Benzamidoxime (106 g, 0.78 mol) was converted to 106 g (85%) of **6a**, which was isolated as white crystals, mp 36–37.5 °C (lit.[26] mp 40–41 °C).

3-*tert*-Butyl-5-methyl-1,2,4-oxadiazole (6b). 2,2-Dimethylpropionamidoxime (21.3 g, 0.184 mol) was converted to 23.2 g (90%) of **6b** isolated as a colorless liquid. A 5-g portion was distilled to furnish an analytical sample, bp 147–148 °C (760 torr).

3-*n*-Octyl-5-methyl-1,2,4-oxadiazole (6c). A mixture of 19.3 g (0.12 mol) of **5c** and 50 mL of acetic anhydride was refluxed for 20 min. Workup in the standard fashion afforded 19.8 g of material, which was purified by column chromatography on 450 g of silica gel. Elution with dichloromethane provided 13.5 g (68%) of **6c** as a light yellow oil, bp 97–100 °C (2.5 torr).

3,5-Dimethyl-1,2,4-oxadiazole (6d).[27] Acetamidoxime hydrochloride (20.0 g, 0.181 mol) was converted to 10.6 g (60%) of **6d**, which was isolated as a colorless liquid.

3-(1-Naphthyl)-5-methyl-1,2,4-oxadiazole (6e). A mixture of 20.0 g (0.107 mol) of **5e** and 50 mL of acetic anhydride was heated at reflux for 15 min. After general workup, removal of solvent afforded 25.0 g of material, which was placed on a column containing 450 g of silica gel. Elution with dichloromethane provided 17.0 g (75%) of **6e**, mp 28–29.5 °C. Recrystallization from petroleum ether gave needles melting at 29–30 °C (lit.[21] mp 36 °C).

3-(2-Thienyl)-5-methyl-1,2,4-oxadiazole (6f). 2-Thiophenamidoxime (**5f**; 24.5 g, 0.172 mol) was converted to 20.5 g (72%) of **6f**, which was recrystallized from petroleum ether, mp 50.5–52 °C.

3-Benzyl-5-(bromomethyl)-1,2,4-oxadiazole (7). Treatment of phenylacetamidoxime (36.0 g, 0.24 mol) with excess bromoacetyl chloride yielded 40.1 g (70%) of **7**, which was isolated as a yellow oil. The crude product was chromatographed on silica gel with dichloromethane as eluent, yielding an analytical sample of **7**: ^1H NMR ($CDCl_3$) δ 7.25 (s, 5 H, phenyl), 4.30 (s, 2 H, CH_2) 4.00 (s, 2 H, CH_2). Anal. ($C_{10}H_9N_2OBr$) C, H, N, Br.

3-(4-Pyridyl)-5-styryl-1,2,4-oxadiazole (12a). The styryl-substituted oxadiazoles were prepared via a two-step reaction sequence. To a cold (0 °C), stirred suspension of 10.0 g of **5h** and 4.0 g of K_2CO_3 in 100 mL of acetone was added dropwise 9.7 g of cinnamoyl chloride in 60 mL of acetone. The solution was allowed to warm to room temperature and stirred for an additional 2 h. The acetone was evaporated, and the residue was washed with water to give 14.5 g (94%) of the crude cinnamoyl acylated amidoxime. A portion of this material was recrystallized from ethanol (95%) to give white plates of analytically pure O-acylated amidoxime: mp 168–169 °C; ^1H NMR (Me_2SO-d_6) δ 8.75 (d, 2 H, J = 7.0 Hz, aryl), 7.92 (d, 1 H, J = 14.0 Hz, CH), 7.75 (d, 2 H, J = 7.0 Hz, aryl), 7.70 (m, 2 H, aryl), 7.40 (m, 3 H, phenyl), 7.23 (br s, 2 H, NH_2), 6.80 (d, 1 H, J = 14.0 Hz, CH). Anal. ($C_{15}H_{13}N_3O_2$) C, H, N.

The O-acylated amidoxime was converted to the desired 1,2,4-oxadiazole by heating under vacuum for 1–5 h. Thus, 8.2 g of the above O-acylated amidoxime was heated (neat) at 150 °C under vacuum for 2 h. The cyclized product crystallized on cooling to give 7.17 g (94% yield) of the desired crude styryl product (**12a**). Recrystallization from acetone/hexane gave an analytical sample of the styryl derivative: mp 109–110 °C; ^1H NMR (acetone-d_6) δ 8.90 (d, 2 H, J = 8.0 Hz, aryl), 8.13 (d, 1 H, J = 14.0 Hz, CH), 8.00 (d, 2 H, J = 8.0 Hz, aryl), 7.93 (m, 2 H, phenyl), 7.56 (m, 3 H, phenyl), 7.27 (d, 1 H, J = 14.0 Hz, CH). Anal. ($C_{15}H_{11}N_3O$) C, H, N.

5-Styryl-3-phenyl-1,2,4-oxadiazole (12b). A procedure similar to that described for **12a** above was used to prepare **12b** in 80% yield. Recrystallization from acetone/hexane gave an analytical sample of **12b** as light-yellow needles: mp 92–93 °C (lit.[28] 95–96 °C); ^1H NMR ($CDCl_3$) δ 8.16 (m, 2 H, phenyl), 7.72 (d, 1 H, CH), 7.42 (m, 8 H, phenyl), 6.95 (d, 1 H, CH).

3-Styryl-5-phenyl-1,2,4-oxadiazole (13). The 3-styryl-substituted oxadiazole was prepared as described above for **12a**. To a stirred solution of 16.0 g (99 mmol) of **5i** and 6.8 g of K_2CO_3 in 100 mL of acetone at 0 °C was added 11.4 mL of benzoyl chloride. The solution was warmed to room temperature and stirred overnight (15 h). The crude reaction mixture was decanted from the K_2CO_3 and the solvent evaporated in vacuo. A portion of this material was purified on a silica gel column using a 2% methanol/methylene chloride eluent. The O-acylated amidoxime was recrystallized from hexane/dichloromethane, yielding fine white needles: ^1H NMR ($CDCl_3$) δ 8.13 (d, 2 H, phenyl), 7.65 (d, 1 H, CH), 7.50 (m, 8 H, phenyl), 7.01 (d, 1 H, CH), 5.12 (br s, 2 H, NH_2). Anal. ($C_{16}H_{14}N_2O_2$) C, H, N.

The O-acylated amidoxime was converted to the desired 1,2,4-oxadiazole by heating at 170 °C under vaccum for 2 h. Crude **13** (20.4 g, 83%) was obtained by cooling the above crude reaction mixture. The cyclized product was purified on a silica gel column using a 1% methanol/dichloromethane eluent to give 10.0 g of **13**. Recrystallization from acetone/hexane gave an analytical sample of the styryl derivative as white needles: mp 100–101 °C; ^1H NMR ($CDCl_3$) δ 8.10 (d of d, 2 H, phenyl), 7.75 (d, 1 H, CH), 7.33 (m, 8 H, phenyl), 6.95 (d, 1 H, CH). Anal. ($C_{16}H_{12}N_2O$) C, H, N.

General Procedure for Preparation of 3-Substituted 1,2,4-Oxadiazol-5-aldoximes (2). Scheme I preparation involved alkyllithium deprotonation of the type **6** oxadiazole ring compounds followed by nitrosation with an alkyl nitrite. The general procedure was as follows.

To a solution of the appropriate 5-methyl-1,2,4-oxadiazole dissolved in THF at −70 °C was added 1 equiv of *n*-butyllithium (temperature generally rose to ca. −45 °C). The resulting dark-red solution was warmed to −20 °C over a 10-min period and then recooled to −70 °C with a dry ice–acetone bath. After 5 min, a slight excess of isopropyl nitrite was added, and the solution was warmed to room temperature. The crude reaction mixture was treated with 25 mL of 3 M HCl and extracted with ether (3 × 50 mL). The combined ether extracts were washed with 50 mL of brine and dried (Na_2SO_4). Removal of solvent yielded crude aldoxime **2**, which was partitioned between ether (50 mL) and 0.5 N sodium hydroxide (2 × 50 mL). The aqueous solution was adjusted to pH 6 with 3 M HCl and extracted with dichloromethane (2 × 50 mL). The organic solution was then washed with 40 mL of brine, dried (Na_2SO_4), and concentrated on the rotary evaporator. The crude acidic product was purified further by

column chromatography on silica gel followed by repeated recrystallizations from appropriate solvents. Details for specific type 2 compounds are given below.

3-Phenyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2a). See ref 15.

3-*tert*-Butyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2b). Compound **2b** was prepared from 6.10 g (43.5 mmol) of oxadiazole **6b** and purified by column chromatography. The crude acidic product (2.18 g) was placed on a column containing 100 g of silica gel. Elution with 10% ether/90% hexane gave 0.73 g (9.9%) of **2b**, mp 128–130 °C. Two recrystallizations from acetone/hexane afforded the analytical sample: mp 130.5–132 °C; IR (KBr) 3220, 3150, 3005, 2970, 2875, 1595, 1570, 1355, 1270, 1195, 1060, 1000 cm^{-1}; NMR (acetone-d_6) δ 1.41 (s, 9 H, *t*-Bu), 8.30 (s, 1 H, CH), 12.07 (s, 1 H, NOH); NMR (Me_2SO-d_6) δ 1.36 (s, 9 H, *t*-Bu), 8.38 (s, 1 H, CH), 13.01 (s, 1 H, NOH). Anal. ($C_7H_{11}N_3O_2$) C, H, N.

3-*n*-Octyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2c). Compound **2c** was prepared from 8.0 g (41 mmol) of oxadiazole **6c** and purified by column chromatography. Removal of solvent gave 3.64 g of crude material, which was placed on a chromatographic column (2.5 × 30 cm) containing 100 g of silica gel. Elution with 60% ether/40% hexane afforded 3.3 g (43%) of the crude aldoxime. Two recrystallizations from hexane plus a trace of acetone furnished the analytical sample of **2c**: mp 84.5–85.0 °C; IR (KBr) 3100, 3050, 2900, 2850, 1600, 1050, 850 cm^{-1}; NMR ($CDCl_3$) δ 0.9 (t, 3 H, CH_3), 1.35 (m, 10 H, $(CH_2)_5$), 1.8 (m, 2 H, CH_2), 2.85 (t, 2 H, H_2C), 8.3 (s, 1 H, CH), 11.1 (s, 1 H, NOH). Anal. ($C_{11}H_{19}N_3O_2$) C, H, N.

(*E*)-3-Methyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2d(*E*)). The crude acidic product (2.05 g) was placed on a column containing 100 g of silica gel. Elution with 20% ether/80% hexane provided 0.67 g (6.9%) of **2d**. Two recrystallizations from acetone/hexane afforded the analytical sample: mp 163.5–164 °C; ^1H NMR (acetone-d_6) δ 2.42 (s, 3 H, CH_3), 8.26 (s, 1 H, CH), 12.06 (s, 1 H, NOH); NMR (Me_2SO-d_6) δ 2.43 (s, 3 H, CH_3), 8.34 (s, 1 H, CH), 13.02 (br s, 1 H, NOH). Anal. ($C_4H_5N_3O_2$) C, H, N.

(*Z*)-3-Methyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2d(*Z*)). The minor component isolated from the above chromatography proved to be the *Z* isomer of **2d**. Two recrystallizations from petroleum ether/ether yielded an analytical sample of **2d(*Z*)**: mp 128–128.5 °C; ^1H NMR (acetone-d_6) δ 12.34 (s, 1 H, NOH), 7.77 (s, 1 H, CH), 2.45 (s, 3 H, CH_3). Anal. ($C_4H_5N_3O_2$) C, H, N.

3-(1-Naphthyl)-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2e). Compound **2e** was prepared from 1.6 g (7.6 mmol) of oxadiazole **6e**. Removal of solvent gave 0.68 g of material, which was placed on a column containing 60 g of silica gel. Elution with 20% ether/80% hexane afforded 0.25 g (14%) of crude oxime, mp 147–149 °C. Two recrystallizations from hexane plus a trace of acetone furnished the analytical sample: mp 153–154 °C; IR (KBr) 3220, 3165, 3060, 2885, 1570, 1005, 775 cm^{-1}; NMR (acetone-d_6) δ 7.4–8.4 (m, 5 H, arom), 8.48 (s, 1 H, CH), 8.98 (m, 2 H, arom), 12.26 (s, 1 H, NOH); NMR (Me_2SO-d_6) δ 7.37–8.50 (m, 5 H, arom), 8.59 (s, 1 H, CH), 8.70–9.13 (m, 2 H, arom), 13.25 (s, 1 H, NOH). Anal. ($C_{13}H_9N_3O_2$) C, H, N.

3-(2-Thienyl)-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2f). Compound **2f** was prepared from 16.6 g (0.1 mol) of oxadiazole **6f**. The crude acidic product (5.44 g) was placed on a column containing 100 g of silica gel. Elution with 10% ether/90% hexane afforded 1.28 g of material, which on recrystallization from acetone/hexane yielded 200 mg (1.5%) of the impure aldoxime. The oxime was purified on two preparative TLC silica gel plates with use of 2% methanol/dichloromethane as solvent and was recrystallized from hexane/acetone, yielding 140 mg of the oxime: mp 129–131.5 °C; NMR (Me_2SO-d_6) δ 7.3 (m, 1 H, arom), 8.0 (m, 2 H, arom), 8.45 (s, 1 H, CH). Anal. ($C_7H_5N_3O_2S$) C, H, N, S.

General Procedure for Preparation of 3-Phenyl- and 3-Benzyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazoles. Scheme II used hydrolysis of the *p*-nitroso-*N*,*N*-dialkylanilino derivative **9**, an aldehyde equivalent, followed by treating the resulting aldehyde **10** (or its hydrate, **11**) with hydroxylamine hydrochloride to yield the desired aldoximes.

3-Phenyl-5-(methylpyridino)-1,2,4-oxadiazole Bromide (8a). To 5 g (21 mmol) of 3-phenyl-5-(bromomethyl)-1,2,4-oxadiazole[18] in 25 mL of ethanol was added 2 equiv of pyridine (3.13 mL). The solution was refluxed for 20 h and cooled, and ether was added to precipitate the pyridinium bromide salt, yielding 5.85 g (87%) of a crude brown oil. The crude material was used in subsequent reactions without further purification.

***N*-[*p*-(*N*,*N*-Dimethylamino)phenyl]-α-(3-phenyl-1,2,4-oxadiazol-5-yl)nitrone (9a).**[18] To a stirred mixture of **8a** (5.85 g, 18.4 mmol) in 40 mL of water and *N*,*N*-dimethylnitrosoaniline (2.76 g, 17 mmol) in 50 mL of ethanol was added dropwise 40 mL of 1 N NaOH over a 30-min period. A slight rise in temperature was noted during base addition. The mixture was stirred for an additional 15 min, and the resulting precipitate was filtered, washed with 2 × 10 mL portions of water, and air-dried to yield 3.4 g (60%) of a dark-orange solid. This material was hydrolyzed without further purification.

3-Phenyl-5-formyl-1,2,4-oxadiazole Hydrate (11a). To 3.3 g (11 mmol) of the above compound in 150 mL of ether was added 150 mL of 3 N HCl. The mixture was vigorously shaken for 15 min, the ether layer was separated, and the aqueous layer was extracted with 2 × 50 mL portions of ether. The combined ethereal extracts were concentrated in vacuo, yielding 2.0 g (97%) of a brown solid. Recrystallization from hot water yielded 1.9 g of white needles: mp 111–112 °C (lit.[18] mp 105–108 °C); ^1H NMR (acetone-d_6) δ 8.15 (m, 2 H, phenyl), 7.60 (m, 3 H, phenyl), 6.70 (s, 2 H, OH), 6.30 (s, 1 H, CH). Anal. ($C_9H_8N_2O_3$) C, H, N.

3-Phenyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2a). To a stirred solution of the above geminal diol (2.0 g, 10 mmol) and 0.72 g (10 mmol) of hydroxylamine hydrochloride in 25 mL of absolute ethanol at 5 °C was added dropwise 1 equiv (1.43 mL) of triethylamine. The mixture was refluxed overnight and then cooled, and 10 mL of 0.5 N NaOH was added. The oxime precipitated from solution. Recrystallization from aqueous ethanol yielded 1.5 g (70%) of **2a**, mp 169.5 °C. All other physical data were consistent with those reported in ref 15.

3-Benzyl-5-(methylpyridino)-1,2,4-oxadiazole Bromide (8b). The pyridinium salt was prepared from **7** as described above for the phenyl derivative. Thus, treatment of **7** (10.9 g, 42.9 mmol) with 2 equiv of pyridine followed by refluxing for 20 h yielded 12.5 g (88%) of the crude pyridinium salt. The crude material was used in the subsequent reaction without further purification.

***N*-[*p*-(*N*,*N*-Dimethylamino)phenyl]-α-(3-benzyl-1,2,4-oxadiazol-5-yl)nitrone (9b).** Compound **9b** was prepared as described above for the phenyl compound. A 12.5-g (37.7 mmol) sample of the pyridinium salt was converted to 8.6 g (70%) of product, isolated as a reddish solid, mp 136.5–138 °C. This material was hydrolyzed without further purification.

3-Benzyl-5-formyl-1,2,4-oxadiazole (10b). To 8.5 g (26.0 mmol) of the compound described above in 150 mL of ether was added 150 mL of 3 N HCl. Shaking of the resulting mixture for 15 min followed by separation of the ether layer and extraction of the aqueous layer with 2 × 50 mL portions of ether gave, on concentration, 5.2 g (95%) of a red oil, which solidified on standing. The crude aldehyde was converted to the oxime without further purification.

3-Benzyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2g). To a stirred solution of the crude **10b** (3.4 g, 16.4 mmol) and 1.14 g of hydroxylamine hydrochloride in 25 mL of absolute ethanol was added dropwise 1 equiv of triethylamine. A workup similar to that described above yielded 1.2 g (36%) of **2g**. Recrystallization from benzene gave an analytical sample: mp 153–155 °C; ^1H NMR (Me_2SO-d_6) δ 12.95 (s, 1 H, NOH), 8.30 (s, 1 H, CH), 7.30 (s, 5 H, phenyl), 4.10 (s, 2 H, CH_2); IR (KBr) 3350–2800 (br, NOH), 1600, 1570, 1500, 1440, 1350, 1300, 1260, 1050, 920, 850, 780 cm^{-1}. Anal. ($C_{10}H_9N_3O_2$) C, H, N.

General Procedure for Ozonolysis of 5-Styryl-1,2,4-oxadiazoles. The 3-phenyl- and 3-(4-pyridyl)-5-styryl-1,2,4-oxadiazoles (**12a** and **12b**) were prepared by treating the corresponding amidoximes **5a** and **5h**, respectively, with cinnamoyl chloride to give *O*-acyl amidoxime followed by thermal rearrangement to the desired 1,2,4-oxadiazole as described above.

A solution of the 5-styryl-1,2,4-oxadiazole dissolved in dichloromethane and cooled to −70 °C was reacted with ozone generated from a Welsbach ozonator. On completion of the ozonolysis (as determined by TLC disappearance of starting styryl compound), excess dimethyl sulfide was added, and the reaction mixture was warmed to room temperature with stirring over a 2-h period. The methylene chloride solution was then washed

with 2 × 25 mL portions of water, dried over anhydrous Na_2SO_4, and evaporated, yielding the desired product contaminated with benzaldehyde. The mixture solidified on standing, and the resulting solid was washed with carbon tetrachloride to remove residual benzaldehyde. Recrystallization from appropriate solvents yielded the desired aldehyde **10** or hydrate **11**.

3-Phenyl-5-formyl-1,2,4-oxadiazole Hydrate (11a). This material was obtained in 32% yield and recrystallized from water to give analytically pure white needles of the hydrate, mp 97–98 °C (lit.[17] mp 105–108 °C). This material was identical to the sample of **11a** previously prepared above.

3-(4-Pyridyl)-5-formyl-1,2,4-oxadiazole Hydrate (11c). Due to precipitation of the styryloxadiazole **12a** from a −70 °C methylene chloride solution, the ozonolysis was conducted at room temperature. Workup gave the aldehyde hydrate in 72% yield. Recrystallization from ethanol–water gave an analytical sample: mp 119–120 °C; ^{1}H NMR (Me_2SO-d_6) δ 8.88 (d, 2 H, J = 8.0 Hz, aryl), 7.90 (d, 2 H, J = 8.0 Hz, aryl), 7.43 (br s, 1 H, OH), 7.34 (br s, 1 H, OH), 6.00 (t, 1 H, CH).

3-(4-Pyridyl)-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2h). A stirred solution of 1.0 g (5.2 mmol) of **11c**, 360 mg of hydroxylamine hydrochloride, and 7.0 mL of triethylamine in 50 mL of ethanol was refluxed for 5 h. The ethanol was evaporated, and the solid residue was washed with water to remove any salts, yielding 820 mg (83%) of the crude oxime. Recrystallization from ethanol gave analytically pure light-yellow needles of **2h**, mp 249–250 °C; ^{1}H NMR (Me_2SO-d_6) δ 13.25 (br s, 1 H, NOH), 8.92 (d, 2 H, J = 6.0 Hz, aryl), 8.43 (s, 1 H, CH), 7.93 (d, 2 H, J = 6.0 Hz, aryl). Anal. ($C_8H_6N_4O_2$) C, H, N.

5-Phenyl-1,2,4-oxadiazole-3-carboxaldehyde Hydrate (11b). The isomeric geminal diol was obtained from oxadiazole **13** in 79% yield and recrystallized from water to give analytically pure white needles of the hydrate: mp 80–82 °C (lit.[17] mp 90–91 °C); ^{1}H NMR (Me_2SO-d_6) δ 8.13 (m, 2 H, phenyl), 7.70 (m, 3 H, phenyl), 6.82 (br s, 2 H, OH), 6.00 (s, 1 H, CH). Anal. ($C_9H_8N_2O_3$) C, H, N.

3-[(Hydroxyimino)methyl]-5-phenyl-1,2,4-oxadiazole (2i). To a stirred solution of the above diol (770 mg) and 278 mg of hydroxylamine hydrochloride in 30 mL of ethanol was added 0.56 mL of triethylamine. The mixture was refluxed for 5 h, the solvent was evaporated, and the resulting solid was washed with water to remove any salts, yielding 640 mg (85%) of **2i**. Recrystallization from ethanol gave analytical flat plates of **2i**: mp 162–163 °C; ^{1}H NMR (acetone-d_6) δ 11.65 (br s, 1 H, NOH), 8.42 (s, 1 H, CH), 8.20 (m, 2 H, phenyl), 7.84 (m, 3 H, phenyl). Anal. ($C_9H_7N_3O_2$) C, H, N.

General Procedure for Preparation of Thiocarbohydroximic Acid *S*-Esters (3).[29] The intermediate hydroximoyl chlorides (**14**) were obtained by adding 1 equiv of *N*-chlorosuccinimide to a solution of the appropriate oxime **2** (0.9–1.5 mmol) in 6–12 mL of DMF at 23–24 °C. After 5 min, 10 mL of gas from the headspace of a concentrated hydrochloric acid bottle was added via syringe. After 10 min, the solution was heated to 45 °C and stirred for 40 min. The cooled mixture was poured into 50 mL of water and extracted with ether (3 × 50 mL). The combined ether layers were washed with water (2 × 50 mL) and brine (50 mL) and dried (Na_2SO_4). Removal of solvent furnished the crude hydroximoyl chloride **14** (90% yield), which was used in the subsequent conversion to thiocarbohydroximate **3** without further purification.

Triethylamine (excess) was added to a stirred solution of 1 equiv of **14** and 1 equiv of 2-(*N*,*N*-diethylamino)ethanethiol hydrochloride in dichloromethane. After 2 h, the solution was diluted with additional dichloromethane, washed with 1 M sodium bicarbonate and brine, and dried over anhydrous Na_2SO_4. The resulting crude products were purified by column chromatography, furnishing the desired thiocarbohydroximic acid *S*-esters in 60–90% yield. The individual *S*-esters are described below.

3-Phenyl-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester (3a). See ref 15.

3-*tert*-Butyl-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester (3b). Compound **3b** was prepared by using 283 mg (1.29 mmol) of **14b**. The crude product was purified by preparative TLC (silica gel, ether) to give 340 mg (81%) of **3b**, mp 86–87.5 °C. Recrystallizations from acetone/hexane and acetone afforded the analytical sample: mp 87–87.5 °C; ^{1}H NMR (acetone-d_6) δ 1.00 (t, 6 H, J = 7.5 Hz, CH_3), 1.43 (s, 9 H, CH_3), 2.56 (q, 4 H, J = 7.5 Hz, CH_2), 2.56–3.53 (m, 4 H, CH_2CH_2), 9.78 (br s, 1 H, NOH). Anal. ($C_{13}H_{24}N_4O_2S$) C, H, N, S.

3-*n*-Octyl-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester Hydrochloride (3c). Compound **3c** was prepared as described above by using 0.51 g (2.0 mmol) of **14c**. Removal of solvent yielded 0.5 g of crude product, which was placed on a chromatographic column containing 50 g of silica gel. Elution with dichloromethane yielded 0.3 g (39%) of the free base as a colorless oil. A solution of 200 mg of **3c** free base in 25 mL of ether was treated with ether saturated with HCl gas to provide 130 g (60%) of **3c**, mp 120–121 °C. Anal. ($C_{17}H_{33}N_4O_2SCl$) C, H, N, S, Cl.

3-Methyl-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester (3d). See ref 15.

3-(1-Naphthyl)-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester Hydrochloride (3e). Compound **3e** was prepared in the same fashion as **3a** by using 323 mg (1.18 mmol) of **14e**. Removal of solvent yielded 546 mg of crude product, which was placed on three silica gel preparative TLC plates using ether as developing solvent. Extraction of the lower R_f UV-active band with acetone yielded 366 mg (84%) of **3e** free base as a colorless oil: ^{1}H NMR (acetone-d_6) δ 0.95 (t, 6 H, J = 8 Hz, CH_3), 2.55 (d, 4 H, J = 8 Hz, CH_2), 2.7–3.7 (m, 4 H, CH_2CH_2), 7.48–9.25 (m, 7 H, aryl), 9.55 (s, 1 H, NOH). A solution of 338 mg of **3e** free base in 25 mL of ether was treated with ether saturated with HCl gas to provide 333 mg (90%) of the hydrochloride salt, mp 193.5–196 °C dec. Anal. ($C_{19}H_{23}N_4O_2SCl$) C, H, N, S.

3-Benzyl-1,2,4-oxadiazole-5-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester Hydrochloride (3f). Compound **3f** was prepared in the same fashion as **3a** by using 500 mg (2.1 mmol) of **14f** and 360 mg (2.1 mmol) of 2-(*N*,*N*-diethylamino)ethanethiol hydrochloride. Removal of solvent gave 550 mg (78% yield) of a yellow oil, which was chromatographed on a silica gel column using a diethyl ether eluent. Compound **3f** free base was obtained as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 10.02 (s, 1 H, NOH), 7.35 (s, 5 H, phenyl), 4.21 (s, 2 H, CH_2), 3.65 (m, 2 H, SCH_2), 2.74 (m, 6 H, CH_2), 1.20 (t, 6 H, CH_3). A solution of 200 mg of **3f** free base in 25 mL of ether was treated with ether saturated with HCl gas to provide 200 mg (90%) of the hydrochloride salt, mp 160–161 °C. Anal. ($C_{16}H_{23}N_4O_2SCl$) C, H, N, S, Cl.

5-Phenyl-1,2,4-oxadiazole-3-thiocarbohydroximic Acid 2-(*N*,*N*-Diethylamino)ethyl *S*-Ester (3g). Compound **3g** was prepared in the usual manner. Thus, 330 mg (1.5 mmol) of **14g** in 50 mL of methylene chloride containing triethylamine and 2-(*N*,*N*-diethylamino)ethanethiol yielded 470 mg (98%) of crude **3g**. The material was chromatographed on 10 g of silica gel with use of a diethyl ether eluent, from which 240 mg of the free base was recovered as an oil: ^{1}H NMR ($CDCl_3$) δ 10.80 (br s, 1 H, NOH), 8.23 (m, 2 H, phenyl), 7.55 (m, 3 H, phenyl), 3.27 (t, 2 H, CH_2S), 2.51 (m, 6 H, CH_2), 1.00 (t, 6 H, CH_3). The recovered material was converted to the HCl salt in THF; mp 175–176 °C. Anal. ($C_{15}H_{21}N_4O_2SCl$) C, H, N, S, Cl.

***N*-[2-(*N*,*N*-Diethylamino)ethyl]-3-phenyl-1,2,4-oxadiazole-5-amidoxime (15).** The hydroximoyl chloride **14a** (150 mg, 0.67 mmol), dissolved in 10 mL of THF, was treated with 78 mg (0.67 mmol) of 2-(*N*,*N*-diethylamino)ethylamine. The reaction mixture was stirred overnight at room temperature and then filtered, and the precipitate was dried in vacuo, yielding 60 mg (30%) of a white solid. Recrystallization from ethanol yielded 36 mg of analytically pure **15** as the hydrochloride salt: mp 212–215 °C; NMR ($CDCl_3$) δ 1.10 (t, 6 H, CH_3), 2.70 (m, 6 H, CH_2), 3.70 (m, 2 H, CH_2), 6.20 (m, 1 H, NH), 7.50 (m, 3 H, phenyl), 8.10 (m, 2 H, phenyl), 9.00 (br s, 1 H, NOH). Anal. ($C_{15}H_{22}N_5O_2Cl$) C, H, N, Cl.

Methods. Physical Measurements. Reactivator pK_a values were determined spectrophotometrically in 0.1 M phosphate buffer by the method of Albert and Sergeant.[30] Octanol/water partition coefficients were determined spectrophotometrically by the method of Fujita et al.[31] The aqueous phase for all log P determinations was pH 7.4, 0.1 M phosphate buffer.

Competitive Inhibition of Eel and Human Acetylcholinesterase. For analysis of AChE competitive inhibitory potency, AChE plus four to six different concentrations of the compounds

were dissolved in 0.1 M phosphate buffer containing 4% C_2H_5OH, and duplicates were assayed three times over a period of ca. 3 h. The results, invariant with time, were averaged for each concentration, and the IC_{50} determined from a plot of log (AChE activity) vs. log (inhibitor concentration). Only values between 10% and 90% inhibition were used for the calculation. The exact procedure was as follows.

Drug solutions were made up in aqueous ethanol at appropriate concentrations between 10^{-3} and 10^{-7} M. Aliquots of 50 μL of an AChE solution in pH 7.6, 0.1 M morpholinopropanesulfonic acid (MOPS) buffer at 1.5 U/mL were added, and the solution was mixed well and then incubated at 25 °C. To 960-μL aliquots were added 30 μL of 0.10 dithiobis(nitrobenzoic acid) (DTNB) and 10 μL of 0.075 M acetylthiocholine (AcSCh), and the increasing absorbance was monitored at 412 nm (AChE = 0.016 U/mL, DTNB = 3×10^{-3} M, AcSCh = 7.5×10^{-4} M).

Eel Acetylcholinesterase Reaction after EPMP Inhibition. Unless otherwise noted, all experiments were conducted at 25.0 ± 0.1 °C in pH 7.6, 0.1 M MOPS buffer plus NaN_3 (0.002%), $MgCl_2$ (0.01 M), and bovine serum albumin (0.01%). Enzyme activities were assayed by the Ellman[32] method on a Gilford-modified DU spectrophotometer coupled to an HP-85 laboratory computer for automatic rate determination. All rate constants were determined by least-squares linear regression analysis.

In general, eel AChE was reacted with the quantity of EPMP giving approximately 90% inhibition of activity in 20 min. Aliquots of inhibited enzyme were then withdrawn and diluted in MOPS buffer containing known concentrations of reactivators. The inhibited enzyme was incubated with reactivators for time intervals and assayed (in duplicate) for activity. In parallel experiments, uninhibited AChE and inhibited AChE in the absence of added reactivator were assayed for activity to determine, respectively, rates of enzyme denaturation and spontaneous reactivation. As a control in our AChE assay and to check for hydrolytic stability of the type **2** compounds, acetylthiocholine hydrolysis by the compounds was determined as a function of time. For each compound, observed thiocholine production rates (d[thiocholine]/dt) were invariant to within ±10% over the 3–4-h incubation period normally used in the in vitro assay of EPMP reactivation. Observed enzyme activities were corrected for inhibition by the test compounds and for spontaneous and reactivator-induced substrate hydrolysis. Reference 15 details specifics of the procedures employed.

Human Erythrocyte Acetylcholinesterase Reactivation after EPMP Inhibition. These experiments were performed generally as described for eel AChE reactivation, except that the initial reaction of enzyme with EPMP was conducted at pH 10.2 in borate buffer, to improve enzyme solubility. After the enzyme inhibition step, aliquots were diluted into 0.1 M MOPS buffer containing reactivator at the desired concentration. An actual procedure is as follows.

To inhibit the AChE, 100 μL of 5×10^{-7} M EPMP in borate buffer (freshly diluted from an alcohol stock) was added to 250 μL of 3 U/mL AChE in 0.01 M, pH 10.2 borate buffer containing 0.01% BSA. The uninhibited AChE control comprised 500 μL of 3 U/mL AChE in borate plus 20 μL of borate buffer. After 2 h of incubation at 25 °C, 120 μL of the AChE solution was added to pH 7.44 MOPS buffer containing the test compounds to a final volume of 3.5 mL. The final pH of the mixture should be 7.6 (the relative volumes and pH values to reach the appropriate final pH were determined each time a buffer was prepared). The solutions were incubated at 25 °C in stoppered 1.5-mL plastic tubes, and duplicate 300-μL aliquots were removed at 0.5, 1, 2, and 3 h. For assay, these aliquots are added to 660 μL of pH 8.5, 0.1 M phosphate buffer, 30 μL of 0.1 M DTNB, and 10 μL of 0.075 M acetylthiocholine (final pH in assay = 8.0), and absorbance was monitored for 3.4 min at 412 nm.

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Registry No. **2a**, 90507-20-3; **2b**, 99764-47-3; **2c**, 103499-02-1; (*E*)-**2d**, 99779-79-0; (*Z*)-**2d**, 103499-03-2; **2e**, 103499-04-3; **2f**, 103499-05-4; **2g**, 103499-06-5; **2h**, 103499-07-6; **2i**, 103499-08-7; **3a**, 90507-29-2; **3b**, 103499-09-8; **3c**, 103499-10-1; **3d**, 90507-31-6; **3e**, 103499-11-2; **3f**, 103499-12-3; **3g**, 103499-13-4; **4a**, 100-47-0; **4b**, 630-18-2; **4c**, 2243-27-8; **4d**, 75-05-8; **4e**, 86-53-3; **4f**, 1003-31-2; **4g**, 140-29-4; **4h**, 100-48-1; **4i**, 66582-22-7; **5a**, 613-92-3; **5b**, 42956-75-2; **5c**, 103499-15-6; **5d**, 22059-22-9; **5e**, 40019-43-0; **5f**, 53370-51-7; **5g**, 19227-11-3; **5h**, 1594-57-6; **5i**, 103499-16-7; **6a**, 1198-98-7; **6b**, 103499-17-8; **6c**, 103499-18-9; **6d**, 10403-80-2; **6e**, 103499-19-0; **6f**, 103499-20-3; **7** (R = $CH_2C_6H_5$), 103499-21-4; **7** (R = C_6H_5), 103499-27-0; **8a**, 103499-26-9; **8b**, 103499-28-1; **9a**, 73217-49-9; **9b**, 103499-29-2; **10b**, 103499-30-5; **11a**, 73217-59-1; **11b**, 73217-63-7; **11c**, 103499-31-6; **12a**, 103499-22-5; **12a** (O-acylated amidoxime), 103499-23-6; **12b**, 70395-63-0; **12b** (O-acylated amidoxime), 103499-25-8; **13** (R = C_6H_5), 103499-24-7; **14a**, 90507-40-7; **14b**, 103499-32-7; **14c**, 103499-33-8; **14e**, 103499-34-9; **14f**, 103499-35-0; **14g**, 103499-36-1; **15**, 103499-14-5; AChE, 9000-81-1; $BrCH_2COCl$, 22118-09-8; $C_6H_5CH{=}CHCOCl$, 102-92-1; C_6H_5COCl, 98-88-4; *i*-C_3H_7ONO, 541-42-4; 4-$ONC_6H_4N(CH_3)_2$, 138-89-6; $(CH_3CH_2)_2N(CH_2)_2SH{\cdot}HCl$, 1942-52-5; $(CH_3CH_2)_2N(CH_2)_2NH_2$, 100-36-7; pyridine, 110-86-1.