Associates, Milford, MA, by a procedure similar to that previously described.[24] Following incubation, the sample was quenched with 3 vol of methanol and centrifuged to remove precipitated proteins. An aliquot of the supernatant was applied to a C-18 Sep Pak (Waters Associates, Milford, MA), which had been washed with 2 mL of MeOH and 2 mL of SIK/PIC A buffer (0.25 M sucrose, 0.025 M imidazole, 0.5 M KCl, 0.005 M PIK A, and pH 7.2). After application, the Sep Pak was washed with SIK/PIC A buffer and the sample eluted with 2 mL of MeOH.

The two carboxylated products were separated by ion-exchanged HPLC on a Dupont Zorbax analytical column. The sample was applied to the column and eluted for 10 min with 25% buffer A (5 mM NH_4OAc, pH 6.0/20% MeOH) and 75% buffer B (500 mM NH_4OAc, pH 6.0/20% MeOH). At 25 min the elution was altered to 10% buffer A and 90% buffer B and at 27 min to 100% buffer B. The eluate was monitored at 230 nm for the first 10 min (to detect Boc tripeptide) and at 257 nm after 10 min (to detect quinone tripeptide). Fractions of about 600 μL were collected at 0.5-min intervals, and radioactivity was determined by liquid scintillation spectrometry.

Acknowledgment. This work was supported by the School of Pharmacy and the College of Agricultural and Life Sciences of the University of Wisconsin–Madison and in part by Grants AM-21472 and AM-14881 from NIH, USPHS.

Registry No. 1, 82376-80-5; 2, 90764-20-8; 3, 77302-67-1; 4, 77302-88-6; 5, 82376-81-6; 6, 82376-79-2; 7, 90764-21-9; 8, 90764-22-0; 9, 90764-23-1; 10, 90764-24-2; 11, 82376-82-7; 13, 82376-83-8; 14, 82376-85-0; 15, 90764-25-3; 16, 90764-26-4; 17, 90790-64-0; 18, 90790-65-1; 1,4-naphthoquinone, 130-15-4; digluteroyl peroxide, 10195-54-7; vitamin K dependent carboxylase, 81181-72-8.

Nonquaternary Cholinesterase Reactivators. 2. α-Heteroaromatic Aldoximes and Thiohydroximates as Reactivators of Ethyl Methylphosphonyl–Acetylcholinesterase in Vitro

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We prepared six pairs of α-heteroaromatic aldoximes, RC(=NOH)H, and thiohydroximates, RC(=NOH)S-$(CH_2)_2N(C_2H_5)_2$, where R represents various oxadiazole and thiadiazole rings. Each compound was characterized with respect to the following: structure, (hydroxyimino)methyl acid dissociation constant, nucleophilicity toward trigonal carbon and tetrahedral phosphorus, octanol–buffer partition coefficient, reversible inhibition of eel acetylcholinesterase (AChE), and in vitro reactivation of AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate. Eight of the twelve compounds significantly reactivate ethyl methylphosphonyl–AChE, but inherent reactivities are moderate to low: the most potent nonquaternary reactivator, 3-phenyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole, is 17 times less reactive than the well-known reactivator 2-[(hydroxyimino)methyl]-1-methylpyridinium iodide (2-PAM). One of the nonquaternary compounds, 3-phenyl-1,2,4-oxadiazole-5-thiohydroximic acid 2-(diethylamino)ethyl *S*-ester, is a powerful reversible inhibitor of AChE (I_{50} = 7.5 μM). The observed relationships between nonquaternary compound structure, reactivation potency, and anti-AChE activity reveal important molecular requirements for high reactivity toward phosphonylated AChE.

Many organophosphorus (OP) compounds irreversibly inhibit acetylcholinesterase (AChE).[1–7] Therapy of intoxication by these nerve poisons relies on coadministration of cholinolytics (e.g., atropine) and so-called cholinesterase reactivators.[8–11] The latter drugs combine strong reversible binding to inhibited AChE with high inherent nucleophilicity to effect rapid displacement of inhibitor from the enzyme. For all practical purposes, pyridinium aldoximes, such as 2-[(hydroxyimino)methyl]-1-methylpyridinium halide (2-PAM), are the only cholinesterase reactivators currently available for clinical or emergency first-aid application. As quaternary ammonium salts, the pyridinium aldoximes penetrate poorly from the serum into hydrophobic cell membranes. Limited tissue distribution (especially within the central nervous system[12–14]) for pyridinium oximes is a potentially serious disadvantage that could be improved upon, in principle, by developing nonquaternary cholinesterase reactivators.

In our search for improved anticholinesterase agent antidotes, we previously prepared and evaluated[15,16] a series of α-keto thiohydroximic acid (dialkylamino)alkyl *S*-esters, 1. Our objective was to incorporate into a single molecule

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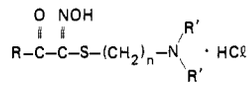
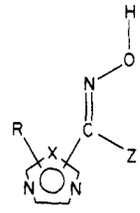


1

the following elements: a good nucleophile (the (hydroxyimino)methyl moiety); a protonated (dialkylamino)alkyl chain to mimic substrate (acetylcholine) binding characteristics; and an electron-withdrawing group (the α-carbonyl) to bring the (hydroxyimino)methyl acid dissociation constant near the optimal[6] value of $pK_a \sim 8$.

We evaluated the type 1 compounds with respect to kinetics of reactivation of diisopropylphosphoryl-AChE[15] and of ethyl methylphosphonyl-AChE.[16] All of the type 1 compounds investigated reactivate phosphylated[17] AChE, and we established useful structure–activity relationships for the series. However, none of the type 1 compounds compares favorably with 2-PAM in vitro; p-$BrC_6H_4C(=O)C(=NOH)SCH_2CH_2N(C_2H_5)\cdot HCl$ (**1a**) is one-fiftieth as potent as 2-PAM toward ethyl methylphosphonyl-AChE.

In the following sections, we describe the synthesis and characterization of two different classes of cholinesterase reactivators: α-heteroaromatic aldoximes, **2**, and the corresponding α-heteroaromatic thiohydroximates, **3**. The reactivators are given by the general formula:

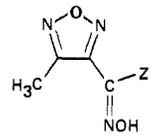


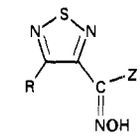
2, Z = H
3, Z = $SCH_2CH_2NEt_2$

where X = O or S and R = H, CH_3, or C_6H_5. Our selection of the five-membered heterocyclic functionality was based on the report by Benschop et al.[18] that certain thiadiazolyl aldoximes function as cholinesterase reactivators, although only to a moderate degree. We wished to extend the original work of Benschop et al. to a greater variety of ring systems to better define the molecular parameters that govern reactivity of the type **2** compounds toward phosphylated AChE.

For the type **3** compounds, we anticipated that the protonated (dialkylamino)alkyl functionality would contribute binding interactions with anionic regions[7] of the AChE active site and therefore enhance the inherent reactivity of the (hydroxyimino)methyl heteroaromatic systems. As described in the following sections, we find strict geometric and functional group requirements for heteroaromatic aldoxime reactivity toward phosphonylated AChE. We also observe that several type **3** compounds exhibit particularly high affinity for the AChE active site. However, this affinity does not necessarily mean that the compounds bind strongly to phosphonylated AChE.

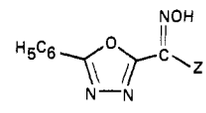
Scheme I

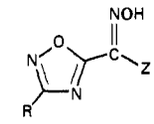




2a (Z = H)
3a (Z = $SCH_2CH_2NEt_2 \cdot HCl$)

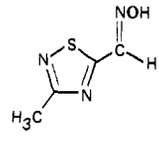
2b (R = H, Z = H)
3b (R = H, Z = $SCH_2CH_2NEt_2$)
2c (R = CH_3, Z = H)
3c (R = CH_3, Z = $SCH_2CH_2NEt_2 \cdot HCl$)



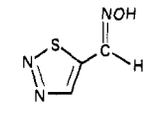


2d (Z = H)
3d (Z = $SCH_2CH_2NEt_2 \cdot HCl$)

2e (R = C_6H_5, Z = H)
3e (R = C_6H_5, Z = $SCH_2CH_2NEt_2$)
2f (R = CH_3, Z = H)
3f (R = CH_3, Z = $SCH_2CH_2NEt_2$)

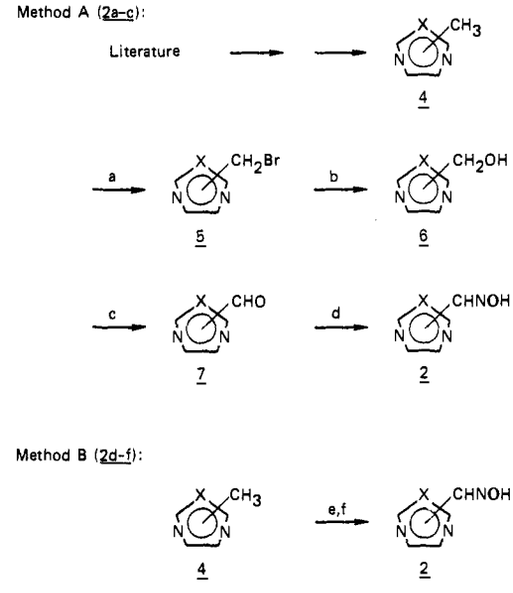


2g



2h

Scheme II



where X = O,S: a, 1 eq. NBS; b, K_2CO_3, H_2O, Δ; c, $(NH_4)_2Ce(NO_3)_6$, H_2O, HNO_3; d, NH_2OH; e, n-BuLi, i-C_3H_7ONO; f, H^+.

Results and Discussion

Synthesis and Structure. We prepared six pairs of type **2** and type **3** compounds given by the structures shown in Scheme I. Table I provides selected data on the type **2** and type **3** compounds prepared to date. For comparison, we have included the thiadiazolyl aldoximes **2g** and **2h** that Benschop et al.[18] investigated previously as potential cholinesterase reactivators.

We used two different routes to prepare the desired type **2** compounds. These are summarized in Scheme II.

The type **3** compounds were obtained from the corre-

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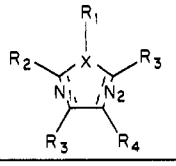
Table I. Selected Data for α-Heteroaromatic Aldoximes and Thiohydroximates



code	no.	X	N_1[a]	N_2[a]	R_2	R_3	R_4	R_5	yld,[b] %	mp, °C	NMR[c] (=NOH), δ	pK_a[d]	log P[e]	anal.[f]
4185-94	**2a**	O	2	5		C(NOH)H	CH_3		24	54–56	12.42	9.15		C, H, N
4185-96	**3a**	O	2	5		C(NOH)Z[g]	CH_3		41	121–122[h]	13.14	7.59	1.10	C, H, N, S, Cl
11879-10	**2b**	S	2	5		C(NOH)H	H		16	128–129	12.10 (*Z*)	9.45		C, H, N, S
										164–165	12.53 (*E*)			
11879-14	**3b**	S	2	5		C(NOH)Z[g]	H		18	122–124		7.69	0.74	C, H, N
11879-7	**2c**	S	2	5		C(NOH)H	CH_3		73	131–134	12.05	9.61		C, H, N, S
11879-12	**3c**	S	2	5		C(NOH)Z[g]	CH_3		39	179–182[h]	12.72	8.65[i]	1.02	C, H, N, S, Cl
4811-5	**2d**	O	3	4	C(NOH)H			C_6H_5	3	189–190	11.37[j]	8.41	1.96	C, H, N
4811-28	**3d**	O	3	4	C(NOH)Z[g]			C_6H_5	52	214–215[h]	10.95[h]	6.35	1.13	C, H, N, S
4185-17	**2e**	O	2	4		C_6H_5		C(NOH)H	8	166–168	12.87	7.94	2.61	C, H, N
4185-54	**3e**	O	2	4		C_6H_5		C(NOH)Z[g]	27	199–201	13.88[j]	6.32	1.78	C, H, N
4811-43	**2f**	O	2	4		CH_3		C(NOH)H	7	163.5–164	13.04	8.10	0.47	C, H, N, S
4811-49	**3f**	O	2	4		CH_3		C(NOH)Z[g]	63	92–93.5	6.73[j]	6.30		C, H, N, S
3-Me-TDA-5	**2g**	S	2	4		CH_3		C(NOH)H	*l*	*l*	13.77	6.97	0.66[l]	*l*
TDA-5	**2h**	S	2	3			H	C(NOH)H	*l*	*l*	13.63	7.64	0.72[l]	*l*

[a] N_1, N_2 indicates ring positions of the nitrogen heteroatoms. [b] Yield of target compound from immediate precursor. [c] In Me_2SO-d_6 unless otherwise noted. [d] Determined spectrophotometrically in 0.1 M buffer. [e] P is the octanol-buffer partition coefficient. [f] Analysis agrees within ±0.4% of theoretical except for those elements marked with an asterisk (see Experimental Section for analytical data). [g] Z = $SCH_2CH_2N(C_2H_5)_2$. [h] Isolated as hydrochloride salt. [i] Determined potentiometrically in 0.1 M $NaClO_4$. [j] In CD_3COCD_3. [k] In $CDCl_3$. [l] See ref 18.

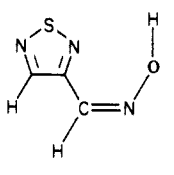
sponding aldoximes, **2**, via chlorination to the hydroximoyl chlorides and subsequent thioesterification:

$$RC(=NOH)H \xrightarrow[DMF]{NCS} RC(=NOH)Cl$$

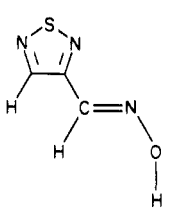
$$RC(=NOH)Cl + HSCH_2CH_2NEt_2 \xrightarrow[CH_2Cl_2]{Et_3N} RC(=NOH)SCH_2CH_2NEt_2$$

3

Of the heteroaromatic aldoximes shown in Table I, only one (**2b**) exhibited two sets of NMR proton signals, indicating a mixture of (*E*)- and (*Z*)-(hydroxyimino)methyl isomers. The major component (80%) of the mixture exhibited proton resonances at δ 12.10 (=NOH), 9.12 (=CH), and 8.43 (Ar H), whereas the minor isomer showed peaks at δ 12.53 (=NOH), 9.50 (=CH), and 7.95 (Ar H). By analogy to literature examples,[19-22] we assign the *E* and *Z* configurations, respectively, to the minor and major components of the **2b** isomer mixture.



(*Z*)-**2b**
(major component)



(*E*)-**2b**
(minor component)

We hoped to establish configurations for the other examples of type **2** compounds in Table I, but such assignments are treacherous in the absence of pairs of *E* and *Z* isomers or crystallographic data.[21-24] Therefore, at present we do not assign isomeric configurations for aldoximes other than **2b**.

Chemical and Physical Properties. High nucleophilicity is a primary requirement for cholinesterase reactivators. In accordance with the usual[25,26] Brønsted relationships, nucleophilicities of oximate anions increase with increasing pK_a of the conjugate acid (hydroxyimino)methyl groups. The relationship between oximate, nucleophilicity and basicity combines with the requirement for a high degree of dissociation to the oximate form at physiological pH to dictate an optimal pK_a of ~8 for cholinesterase reactivators.[6]

We spectrophotometrically[27] determined pK_a values for all of the type **2** and type **3** compounds listed in Table I. Table I shows that the type **2** and type **3** compounds spanned a wide range of acidities—from a low of pK_a = 6.3 (**3d–f**) to a high of pK_a > 9 (**2a–c**). Of the 14 com-

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Table II. Rate Constants for Reaction of AcSCh with Aqueous Buffer (k_0) and with Type 2 and Type 3 Compounds (k_n) at 25 °C, pH 8

compd[a]	pK_a	k_n,[b,c] M^{-1} min^{-1}	$10^{-3}k_0$,[b,d] min^{-1}
2a	9.15	128 ± 9.0	0.27 ± 0.014
3a	7.59	37.7 ± 0.62	0.28 ± 0.008
2b	9.45	207 ± 17	0.51 ± 0.25
3b	7.69	25.6 ± 0.44	0.28 ± 0.005
2c	9.61	237 ± 33	0.62 ± 0.38
3c	8.65	71.2 ± 0.36	0.28 ± 0.002
2d	8.41	79.1 ± 1.9	0.15 ± 0.011
3d	6.35	2.28 ± 0.56	0.12 ± 0.02
2e	7.94	51.8 ± 1.3	0.31 ± 0.01
3e	6.32	4.35 ± 0.35	0.26 ± 0.006
2f	8.10	91.6 ± 0.2	0.24 ± 0.02
3f	6.32	3.81 ± 0.46	0.16 ± 0.01
2g	6.97	5.68 ± 4.1	0.25 ± 0.01
2h	7.64	42.7 ± 9.7	0.25 ± 0.02
		mean	0.28 ± 0.13

[a] See Table I for structures. [b] Error limits are ±SD from linear least-squares regression analysis. [c] k_n is the biomolecular rate constant for reaction of oximate with 7.5×10^{-4} M AcSCh in 0.1 M phosphate buffer. Calculated according to eq 1 and 2. [d] k_0 is the rate constant for spontaneous hydrolysis of 7.5×10^{-4} M AcSCh in 0.1 M phosphate buffer.

pounds tested, six (**2d–f**, **2h**, **3a** and **3b**) exhibited pK_a values in the useful 7.5–8.5 range. Typically, converting type **2** compounds to the corresponding thiohydroximic acid esters lowered the pK_a by approximately 1.5 units.

To provide a direct measure of the inherent nucleophilicities of all type **2** and type **3** compounds (and also as a control in our AChE assays), we determined bimolecular rate constants for reaction of the compounds with acetylthiocholine (AcSCh).

We incubated three or more concentrations of each test compound with excess AcSCh and monitored thiocholine production as a function of time. We followed the reactions to very low conversions and observed pseudo-zero-order kinetics. Under these conditions, the thiocholine production rate obeyed eq 1, where +d[thiocholine]/dt is

$$+\mathrm{d[thiocholine]}(\mathrm{d}t)^{-1}[\mathrm{AcSCh}]^{-1} = k_n[\mathrm{OX}] + k_0 \quad (1)$$

the observed zero-order product formation rate, k_0 is the pseudo-first-order rate constant for spontaneous hydrolysis of AcSCh, k_n is the bimolecular rate constant for attack by oximate on AcSCh, and [OX] is the concentration of added test compound, [HOX], present in the dissociated (oximate) form. Equation 2 governs the [HOX]/[OX] equilibrium.

$$[\mathrm{OX}] = [\mathrm{HOX}] \times [1 + \text{antilog}\,(pK_a - \mathrm{pH})]^{-1} \quad (2)$$

Table II summarizes k_n values for AcSCh reaction with the type **2** and type **3** compounds and k_0 values for reaction with aqueous buffer.

Linear least-squares regression of the data in Table II gave the following Brønsted relationship:

$$\log (k_n) = -3.02\ (\pm 0.38) + 0.58\ (\pm 0.05)\ pK_a \quad (3)$$

For comparison, we previously[15] determined that type **1** compounds react with *p*-nitrophenyl acetate according to the following Brønsted relationship:

$$\log (k_n) = -3.04\ (\pm 0.34) + 0.69\ (\pm 0.08)\ pK_a \quad (4)$$

We also wished to demonstrate that the reactivity order of type **2** and type **3** compounds toward trigonal carbon extends to nucleophilic reactivity toward tetrahedral phosphorus. To do so we measured bimolecular rate constant (k_n') values for reaction of **1a**, **2a**, **3e**, and 2-PAM with ethyl *p*-nitrophenyl methylphosphonate (EPMP).

Table III. Biomolecular Rate Constants (k_n') for Reaction of Ethyl *p*-Nitrophenyl Methylphosphonate with Selected Reactivators at pH 7.6, 25 °C

compd[a]	pK_a	k_n',[b] M^{-1} min^{-1}	compd[a]	pK_a	k_n',[b] M^{-1} min^{-1}
1a	7.44	1.12	**3e**	6.32	0.293
2a	9.15	13.5	2-PAM	7.99[c]	5.70

[a] See Table I for structures. Compound **1a** is *p*-$BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2$. [b] k_n' is the bimolecular rate constant for reaction with 7.5×10^{-4} M EPMP under pseudo-zero-order conditions; see text. EPMP spontaneous hydrolysis was negligible under these conditions. [c] Data of ref 15.

The reactions with EPMP were performed under pseudo-zero-order conditions (as described above for reactions with AcSCh) and followed by the spectrophotometric (402 nm) determination of liberated *p*-nitrophenylate. Table III summarizes the experimental results.

Least-squares regression of the data in Table III gave the following Brønsted relationship:

$$\log (k_n') = -4.38\ (\pm 0.78) + 0.61\ (\pm 0.10)\ pK_a \quad (5)$$

Thus (hydroxyimino)methyl group pK_a controls oximate reactivity toward tetrahedral phosphorus as well as toward trigonal carbon. The slope (β) values of the above Brønsted relationships are typical[27-29] for reactions of (hydroxyimino)methyl compounds with various esters and demonstrate that the type **2** and **3** compounds behave as nucleophiles in the anticipated fashion.

Finally, we determined octanol–buffer partition coefficients for several of the nonquaternary compounds (Table I). All of the compounds tested partitioned primarily into the organic phase, whereas 2-PAM partitions almost entirely into the aqueous phase (log P = −3.24).[18]

Reversible AChE Inhibition. We directly determined the degree to which type **2** and type **3** compounds reversibly inhibit AChE activity for two reasons: first, to correct for reversible inhibition in our AChE assay, and second, to probe possible correlations between test compound affinity for the enzyme active site and reactivity toward phosphylated AChE.

We incubated AChE with three or more concentrations of each test compound and assayed for activity at least three times between 30 min and 4 h. In all cases, observed activities were invariant with time and we calculated activities as the mean values (±SD) for all three time points. For some compounds, inhibition was negligible at assay solution concentrations up to 1.0 mM, and in these cases we averaged activities over the range of concentrations tested.

From the observed activities, we calculated the percentage enzyme inhibition, I, according to eq 6, where A_0

$$I = 100(A_0 - A_I)/A_0 \quad (6)$$

and A_I, respectively, are AChE activities in the absence and presence of added test compounds. For compounds that showed a strong dependence of AChE activity on inhibitor concentration, we calculated the inhibitor concentration giving 50% enzyme inhibition (I_{50} values) by linear least-squares regression of I (for I between 10% and 90%) vs. log [HOX] data. Table IV summarizes the inhibition data.

From the table, it is clear that the two classes of compounds differed substantially with respect to their abilities to reversibly inhibit the enzyme: each of the type **3** com-

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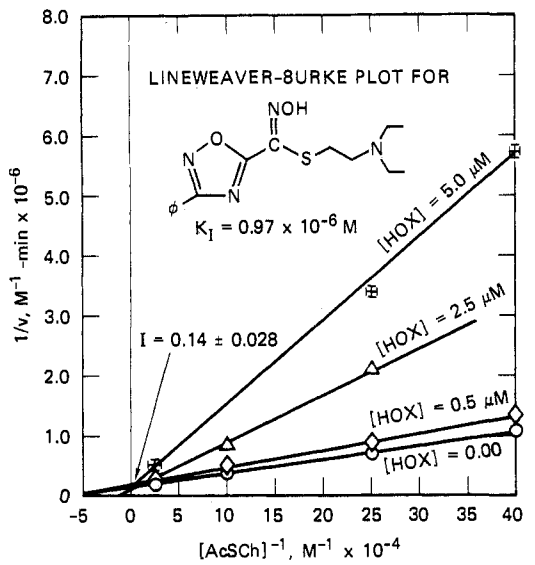

Figure 1. Lineweaver–Burk plot of reciprocal enzyme activity ($1/v$) vs. reciprocal substrate concentration ([AcSCh]$^{-1}$) for AChE inhibition by compound **3e** at 25 °C, pH 8; (○) 0.1 M phosphate buffer. [HOX] indicates the concentration of added **3e**.

pounds inhibited more strongly than the corresponding type **2** compounds. As previously[15] observed for the type **1** compounds, type **2** compounds did not appreciably inhibit AChE at the concentrations used in our assays: I_{50} values were >1 mM for each of the type **2** compounds tested. By comparison, several of the type **3** compounds were rather potent AChE inhibitors. Compound **3e**, in particular, was a powerful inhibitor with I_{50} = 7.5 μM.

To further characterize AChE inhibition by **3e**, we determined AChE activities as a function of the concentration of added **3e** at various substrate (AcSCh) concentrations.

Figure 1, a Lineweaver–Burk[30] plot for enzyme inhibition by **3e**, indicates that the y intercept ($1/v$) was independent of inhibitor concentration; thus, **3e** acts as a *competitive*, reversible inhibitor. Linear least-squares regression analysis of the data shown in the figure gave a y intercept value of 0.139 (±0.028) × 10^6 M^{-1} min.

To calculate the equilibrium constant, K_i, for reversible dissociation of the enzyme–inhibitor complex, we used the relationship shown in equation 7,[30] where $(\text{slope})_{HOX}$ and

$$(\text{slope})_{HOX}/(\text{slope})_{HOX=0} = 1 + [HOX]K_i^{-1} \quad (7)$$

$(\text{slope})_{HOX=0}$ are, respectively, the slopes of the reciprocal enzyme activity vs. [AcSCh]$^{-1}$ lines in the presence and absence of added **3e**. From the data of Figure 1 and eq 7, K_i = 0.97 (±0.03) μM.

Reactivation of Phosphonylated AChE. We began by surveying interactions of type **1**, **2**, and **3** nonquaternary reactivators with AChE inhibited by EPMP. Suitable control experiments demonstrated that potentially complicating side reactions, such as AChE denaturation, dealkylation ("aging") of ethyl methylphosphonylated AChE, and enzyme reinhibition by phosphonyl oxime, proceed at negligibly slow rates compared with rates of oximate-induced reactivation under the conditions chosen for this investigation (see Experimental Section).

Thus, the chemistry of the AChE/ethyl *p*-nitrophenyl methylphosphonate/oximate system that we report here can be satisfactorily described by eq 8–13.

$$(C_2H_5O)CH_3P(O)OC_6H_4NO_2 + EOH \xrightarrow{k_i} EOP(O)CH_3(OC_2H_5) \quad (8)$$

$$EOP(O)CH_3(OC_2H_5) + OX \rightleftharpoons [EOP(O)CH_3(OC_2H_5)\cdot OX] \quad (9)$$

$$[EOP(O)CH_3(OC_2H_5)\cdot OX] \xrightarrow{k_r} EOH \quad (10)$$

$$EOH + HOX \rightleftharpoons [EOH\cdot HOX] \quad (11)$$

$$EOP(O)CH_3(OC_2H_5) + H_2O \xrightarrow{k_{sp}} EOH \quad (12)$$

$$HOX \rightleftharpoons OX + H^+ \quad (13)$$

For this scheme, the following definitions apply: EOH = active enzyme, $EOP(O)CH_3(OC_2H_5)$ = phosphonylated enzyme, $[EOP(O)CH_3(OC_2H_5)\cdot OX]$ = [phosphonyl enzyme/oximate] complex, [EOH·HOX] = [enzyme/oxime] complex, K_r = [phosphonyl enzyme/oximate] dissociation constant, k_r = [phosphonyl enzyme/oximate] displacement rate constant, K_i = [enzyme/oxime] complex dissociation constant, k_{sp} = rate constant for spontaneous reactivation of inhibited enzyme, K_a = oxime/oximate acid dissociation constant.

To determine reactivation kinetics (see Experimental Section for a complete description), we inhibited AChE to approximately 90% of control activity and then incubated the phosphonylated enzyme with various concentrations of the compounds to be tested. At timed intervals, we withdrew aliquots, assayed for activity, and then made appropriate corrections for spontaneous and oximate-induced hydrolysis of the substrate. With reactivator present in large excess over ethyl methylphosphonyl–AChE, restoration of enzyme activity followed pseudo-first-order kinetics according to eq 14. In eq 14, R is the observed

$$\ln (100 - R) = k_{obsd}t \quad (14)$$

percent reactivation at time t given by eq 15, where A_c,

$$R = 100\,\frac{[A_t[100/(100 - I)] - A_i]}{A_c - A_i} \quad (15)$$

A_i, and A_t are observed activities for uninhibited (control) enzyme, enzyme after reaction with ethyl *p*-nitrophenyl methylphosphonate, and ethyl methylphosphonyl–AChE after incubation for t min with reactivator, respectively. In eq 15, observed A_t values are multiplied by the factor $100/(100 - I)$ to correct for AChE inhibition by added test compound in the assay solution (see eq 6).

Spontaneous reactivation (reaction 12) proceeded slowly but at a nonnegligible rate in our system, and we corrected observed reactivation rate constants as in eq 16, where k_{sp}

$$(k_{obsd})_c = k_{obsd} - k_{sp} \quad (16)$$

was calculated according to eq 17.

$$\ln [(A_c - A_i)/(A_c - A_i^0)] = -k_{sp}t \quad (17)$$

We screened all 14 compounds in Table I for activity as reactivators of ethyl methylphosphonyl–AChE, but only 11 of the compounds restored enzyme activity at a significant rate. For these 11 compounds, we determined reactivation kinetics.

To demonstrate structure–activity relationships among the various reactivators, we examined the dependence of reactivation rate on oximate concentration. For the re-

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Table IV. Percentage Reversible Inhibition (I) of AChE Activity by Type 2 and Type 3 Compounds at pH 8.0, 25 °C, in the Presence of 7.5×10^{-4} M Acetylthiocholine

compd[a] no.	[HOX],[b] μM	I[c]	I_{50},[d] mM	compd[a] no.	[HOX],[b] μM	I[e]	I_{50},[d] mM
2a	10.0	8.7 ± 2	>1	**3a**	10.0	8.4 ± 1	0.25
	50.0				50.0	26.9 ± 1	
	100.0				100.0	39.3 ± 1	
2b	10.0	5.7 ± 0.5	>1	**3b**	2.0	4.00 ± 0.4	>1
	50.0				5.0	13.4 ± 0.7	
	100.0				10.0	10.0 ± 4	
					25.0	17.4 ± 3	
					50.0	9.1 ± 2	
2c	10.0	7.5 ± 2	>1	**3c**	10.0	9.97 ± 0.5	1.1
	50.0				50.0	15.4 ± 1	
	100.0				100.0	25.9 ± 0.4	
2d	10.0	10.4 ± 0.3	>1	**3d**	2.0	2.53 ± 0.9	0.022
	25.0				5.0	16.9 ± 3	
	50.0				10.0	25.5 ± 2	
					25.0	54.2 ± 2	
2e	10.0	1.7 ± 3	>1	**3e**	1.50	20.3 ± 4	0.0075
	50.0				5.0	64.3 ± 12	
	100.0				15.0	62.1 ± 5	
					40.0	80.6 ± 2	
					50.0	84.0 ± 2	
2f	2.0	5.6 ± 4.8	>1	**3f**	10.0	19.0 ± 2	0.31
	5.0				25.0	22.2 ± 3	
	10.0				50.0	34.9 ± 2	
2g	2.0	−1.1 ± 3	>1				
	5.0						
	10.0						
2h	2.0	−4.0 ± 2.5					
	5.0						
	10.0						

[a] See Table I for structures. [b] [HOX] is the concentration of added test compound in the assay solution. [c] Mean value for all concentrations. [d] I_{50} is the concentration of HOX that inhibits 50% of AChE activity. [e] Mean value for one concentration at three incubation intervals.

action set given by eq 8–13, it can be shown[15,31–36] that eq 18 follows:

$$(k_{obsd})_c^{-1} = (1/k_r) + [OX]^{-1}(K_r/k_r) \quad (18)$$

The constants K_r and k_r define the reactivation process with respect to dissociation of [inhibited enzyme/oximate] complex and transformation of the complex to active enzyme. In the limit of low reactivator concentration, eq 19 follows:

$$k_{OX} = k_{obsd}/[OX] = k_r/K_r \quad (19)$$

where k_{OX} is a bimolecular rate constant for reactivation and a measure of the inherent reactivity of the oximate form of the reactivator. To account for the degree of dissociation of added test compound to oximate, we define the "effective" bimolecular rate constant, k_{HOX}, as

$$k_{HOX} = k_{OX}[1 + \text{antilog}\,(pK_a - pH)]^{-1} \quad (20)$$

Table V summarizes kinetic constants for reactivation of ethyl methylphosphonyl–AChE by type **2** and type **3** compounds. For comparison, the table also gives data obtained previously for **1a** and 2-PAM.

The table demonstrates the range of activities for the compounds investigated: k_{HOX} values varied from a low of 5.34 M^{-1} min^{-1} (**3f**) to a high of 2780 M^{-1} min^{-1} (2-PAM). Of the nonquaternary reactivators, **2e** was the most effective compound. Relative k_{HOX} values for **1a**–**2e**–2-PAM were 1:3.3:51.

Interestingly, the thiadiazolyl oxime **2g** (which shares the 1,2,4-ring substitution pattern with **2e** and **2f**) also proved to be the most reactive of the three heteroaromatic aldoximes studied by Benschop et al.[18] Apparently, the 1,2,4-substitution pattern offers significant advantages for reactivation of phosphonylated AChE. The following section examines nonquaternary reactivator structure–activity relationships in greater detail.

Structure–Activity Relationships. The extremely low activities of compounds **2a–c** as cholinesterase reactivators relate to the very high pK_a values exhibited by these oximes. At pH 7.6, these reactivators were less than 1% dissociated into the nucleophilic oximate species. Similarly, the pK_a value (6.97) for thiadiazole **2g** explains the low reactivation potency (k_{HOX} = 14.3) of the compound relative to the analogous oxadiazole **2f** for which pK_a = 8.10 and k_{HOX} = 151. The highly acidic thiadiazole yields a conjugate base oximate ion that is simply a poor nucleophile (see eq 3).

More informative structure–activity relationships apply to the other compounds in Table I. Several of these compounds exhibited acid dissociation constants near the optimal value of pK_a = 8. Nevertheless, reactivation potencies varied substantially: in terms of k_{OX} values, **2e** and **2f** were 8–34 times more reactive than **2d**, **3a**, or **3b** toward phosphonylated AChE. Because the inherent nucleophilicities for **2d–f** and **3a** and **3b** were essentially identical (see Table II and eq 13), AChE binding forces and geometries must dictate the relative reactivities of these five compounds.

For type **2** compounds, hydrophobic forces must play an important role in reversibly forming the phosphonyl enzyme/reactivator complex. Type **2** reactivators feature no cationic moieties and should not effectively bind to AChE via Coulombic interactions with anionic centers at

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Table V. Kinetic Constants for Reactivation of Ethyl Methylphosphonyl–AChE by Various Test Compounds at 25 °C, pH 7.6[a]

compd[a] no.	[HOX][b] mM	$[OX]^{-1}$,[c] M^{-1}	$(k_{obsd})_c^{-1}$,[d] min	slope[e] M min × 10^2	intercept[f] min	$10^{-3}k_r$, min^{-1}	$10^{-4}K_r$, M	k_{OX},[g] M^{-1} min^{-1}	k_{HOX},[h] M^{-1} min^{-1}
2d	1.00	7460	149	1.10 +	205 ±	4.88	0.537	91.0	12.2
	0.500	14900	436	0.14	114				
	0.100	74600	1160						
	0.050	149000	1770						
2e	0.500	5990	23.0	0.179 ±	9.32 ±	107	1.92	557	175
	0.200	15000	28.6	0.098	5.27				
	0.0800	37400	82.2						
	0.0300	99800	187						
2f	1.00	4170	82.6	0.125 ±	62.9 ±	15.9	0.199	799	192
	0.500	8330	195	0.0050	23.5				
	0.100	41700	598						
	0.0500	83000	110						
2g	0.790	1560	135	5.66 ±	88.5 ±	11.3	6.40	17.7	14.3
	0.395	3130	258	0.33	57				
	0.0790	15600	1,060						
	0.0395	31300	1,820						
2h	0.767	2730	229	6.90 ±	57.5 ±	17.4	12.0	14.5	7.02
	0.384	5430	393	0.20	62				
	0.0767	27300	2,050						
	0.0384	54300	3,760						
3a	1.00	1930	364	5.40 ±	360 ±	2.78	1.54	18.5	10.6
	0.300	6440	671	0.32	107				
	0.100	19300	1,590						
	0.0300	64400	3,790						
3b	1.00	2230	74.6	1.96 ±	79.9 ±	12.6	2.45	51.4	23.0
	0.300	7430	139	0.26	101				
	0.100	22300	695						
	0.0300	74300	1,490						
3c	1.00	12200	456	1.51 ±	75.0 ±	13.4	2.01	66.1	5.41
	0.300	40700	697	0.080	17				
	0.100	122000	1,630						
	0.0300	407000	6,240						
3d	1.00	1020	490	15.2 ±	265 ±	3.77	5.74	6.57	6.42
	0.500	2040	546	0.54	62				
	0.100	10200	1,730						
	0.0500	20400	3,400						
3e	1.00	1050	132	4.57 ±	102 ±	9.80	4.48	21.9	20.8
		2110	1,100	2.7	27				
	0.100	10500	2,880						
	0.0500	21100	3,970						
3f	1.00	1050	495	17.8 ±	457 ±	2.59	3.89	5.62	5.34
	0.300	3500	353	2.7	27				
	0.100	10500	490						
	0.0300	35000	3,920						
1a	*i*	*i*	*i*	105 ±	36.8 ±	27.2	2.85	95.3	56.2
				0.12	29				
2-PAM	*i*	*i*	*i*	0.0104 ±	27.8 ±	36.0	0.0374	9630	2780
				0.00064	24				

[a] See Table I for structures. 1a is p-$BrC_6H_4C(=NOH)SCH_2CH_2N(C_2H_5)_2HCl$. [b] [HOX] is concentration of added test compound. [c] Calculated according to eq 2. [d] Calculated according to eq 16. [e] Linear least-squares regression slope (±SD) according to eq 18. [f] Linear least-squares regression intercept (±SD) according to eq 18. [g] From eq 19. [h] From eq 20. [i] Data from ref 16.

the enzyme active site. It is well-established that hydrophobic regions near the active site influence binding of organophosphorus[37,38] and other[39-41] inhibitors as well as binding of *N*-alkylpyridinium reactivators.[35,36] Because type **2** compounds are highly lipophilic (log *P* values > 1, see Table I), it is reasonable that hydrophobic interactions should principally govern binding to phosphonylated AChE.

Still hydrophobic forces and inherent nucleophilicities alone do not control overall activity toward ethyl methylphosphonyl–AChE. Consider compounds **2d** and **2e** that feature comparable pK_a and log *P* values but that differ sixfold with respect to k_{OX} values. Both **2d** and **2e** feature phenyl-substituted oxadiazolyl aldoxime functionalities but differ with respect to the relative orientations of the phenyl and (hydroxyimino)methyl groups. As seen in Table V, relative K_r values indicate that **2d** binds to ethyl methylphosphonyl–AChE more tightly than **2e** by a factor of 3.6. However, the relative k_r values show that **2e** displaces inhibitor from the phosphonyl enzyme/reactivator complex more rapidly than **2d** by a factor of 22. Thus **2e** binds to phosphonylated AChE slightly less strongly than **2d**, but the former binds with an orientation that greatly facilitates attack at tetrahedral phosphorus from within the phosphonyl enzyme/reactivator complex. Similar geometric requirements are well-known for 2-PAM and the isomeric 3- and 4-[(hydroxyimino)methyl]-1-methylpyridinium reactivators.

A final point concerns binding interactions of the type **3** compounds. Compound **3e** proved to be a remarkably potent reversible inhibitor of AChE. This unusually high potency as a competitive inhibitor suggests multiple

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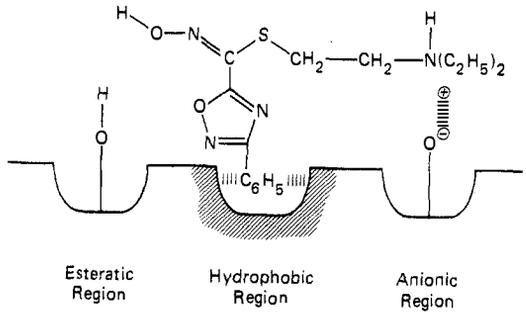

Figure 2. Possible AChE binding interactions for **3e**.

binding interactions. Specifically we propose (Figure 2) that **3e** binds to AChE both via hydrophobic interactions and via electrostatic interactions between the protonated diethylamino group and the AChE anionic site. If so, **3e** resembles several adiphenine derivatives [e.g., $(C_6H_5)_2CHC(:O)OCH_2CH_2N(C_2H_5)_2$·HCl] that also exhibit "two-site" binding to AChE.[42]

From Table V, however, it is clear that the remarkably high affinity of **3e** for AChE does not confer a high affinity towards ethyl methylphosphonyl–AChE. Neither **3e** nor any of the other **3** compounds tested exhibited K_r values below 1×10^{-5} M. Indeed several type **2** compounds exhibited lower K_r values than those of the type **3** compounds. Clearly, phosphonylating AChE drastically influences binding interactions of reactivators with the enzyme active surface. Jarv[38] has shown that organophosphorus ester alkyl groups effectively shield the enzyme anionic region(s) in phosphylated cholinesterases. Presumably, the anionic sites of ethyl methylphosphonyl–AChE are so blocked and prevent interaction with protonated (dialkylamino)alkyl groups of type **3** compounds. Regardless of the precise mechanism(s), one fact is clear: incorporating a (dialkylamino)alkyl group into a nonquaternary reactivator is neither a necessary nor sufficient condition for providing high reactivity toward phosphonylated enzyme.

Conclusions

We prepared a series of α-heteroaromatic aldoximes and thiohydroximates and evaluated the compounds as reactivators of ethyl methylphosphonylated AChE. This work and earlier investigations by ourselves[15,16] and others[18] illustrate some of the molecular parameters that govern the activity of nonquaternary cholinesterase reactivators.

(Hydroxyimino)methyl acid dissociation constants primarily dictate the inherent reactivity of nonquaternary reactivators. Nonquaternary reactivators do not differ from pyridinium aldoximes in this regard: compounds for which $7 > pK_a > 9$ simply do not significantly reactivate phosphylated AChE at near physiological pH. Our work does elucidate substituent types and patterns that can provide reactivators with pK_a values in the useful range. For example, the 1,2,4-oxadiazolyl and 2-aryl-1,3,4-oxadiazolyl ring systems are highly electron withdrawing. Also, converting an α-heteroaromatic aldoxime to the corresponding (dialkylamino)alkyl thiohydroximate lowers the (hydroxyimino)methyl pK_a value by approximately 1.0–1.5 units.

We also observe strict geometrical requirements for α-heteroaromatic aldoximes. Of the ring systems investigated so far, the 3-substituted-5-[(hydroxyimino)-methyl]-1,2,4-oxadiazole derivatives provide the highest reactivity toward phosphonylated AChE. For these heteroaromatic aldoximes, it appears that hydrophobic forces control binding of the compounds to inhibited enzyme.

Converting α-heteroaromatic aldoximes to thiohydroximic acid (dialkylamino)alkyl *S*-esters can enhance affinity of the compounds for the enzyme active site, as evidenced by the relative abilities of the compounds to reversibly inhibit AChE. However, this enhanced affinity does not necessarily increase binding to phosphonylated enzyme.

Of the 12 heteroaromatic compounds that we have reported, only two (3-phenyl- and 3-methyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole, **2e** and **2f**) exhibit pronounced activity as reactivators of ethyl methylphosphonyl–AChE. Compounds **2e** and **2f** are 10 times more reactive than any of the other heteroaromatics we have studied and at least 3 times more reactive than any of the α-keto thiohydroximates reported earlier.[15,16]

In absolute terms, **2e** and **2f** are only modest in vitro reactivators; 2-PAM is 17–18 times more reactive toward ethyl methylphosphonylated AChE. More important than the absolute activity of **2e** and **2f** is the fact that we have developed clearer guidelines for the design of improved nonquaternary reactivators. Specifically, we consider that modifications of the basic 1,2,4-oxadiazolyl system (for example, to optimize hydrophobicity) should lead to new compounds that have enhanced activity and appropriate in vivo pharmacokinetics for efficacy in reactivating inhibited AChE in both the peripheral and central nervous systems.

Experimental Section

Materials. Melting points were determined in glass capillaries with a Laboratory Devices Mel-Temp apparatus or on a Fisher-Johns melting point apparatus; the melting points are uncorrected. Infrared (IR) spectra were obtained on Perkin-Elmer Model 281 and 735B spectrophotometers. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associates EM-360 spectrometer, and chemical shifts are reported in parts per million (δ) relative to an internal tetramethylsilane reference. Microanalyses were performed with a Perkin-Elmer 240 elemental analyzer (C, H, N) by the Stanford University analytical laboratory and by Galbraith Laboratories, Inc., Knoxville, TN.

Tetrahydrofuran (THF) was distilled from calcium hydride and stored under nitrogen over 4A molecular sieve. Metalation reactions were performed under a positive pressure of dry nitrogen. 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 silica gel reagent (90–200 mesh) obtained from Accurate Chemical and Scientific Corp. 5-[(Hydroxyimino)methyl]-1,2,3-thiadiazole (**2h**) and 3-methyl-5-[(hydroxyimino)methyl]-1,2,4-thiadiazole (**2g**) were kindly provided by Dr. L. P. A. De Jong of TNO, The Netherlands. Both compounds exhibited sharp melting points near the literature[18] values and neither showed any impurities on TLC analysis.

Literature methods were used to prepare the following starting materials: 3,4-dimethyl-1,2,5-oxadiazole (**4a**),[43] 3-methyl-1,2,5-thiadiazole (**4b**),[44] 2-methyl-5-phenyl-1,3,4-oxadiazole (**4d**),[45] 3-phenyl-5-methyl-1,2,4-oxadiazole (**4e**),[45,46] 3,5-dimethyl-1,2,4-oxadiazole (**4f**).[47,48] As described in the Results and Discussion, we prepared the heteroaromatic aldoximes **2** and converted these

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to the thiohydroximates 3 via the hydroximoyl chlorides 8. The synthesis of each type 2, 3, and 8 compound is detailed below and is given in the order of the individual ring substitution patterns a–f (see Table I).

3-(Bromomethyl)-4-methyl-1,2,5-oxadiazole (5a) [CAUTION: 3-(Bromomethyl)-4-methyl-1,2,5-thiadiazole is a severe lachrymator and should be handled accordingly]. A mixture of 24.5 g (0.25 mol) of freshly distilled 3,4-dimethyl-1,2,5-oxadiazole (4a), 44.5 g (0.25 mol) of *N*-bromosuccinimide, 1.5 g of benzoyl peroxide, and 600 mL of carbon tetrachloride was heated at reflux overnight. The hot reaction mixture was filtered and the filtrate was washed with three 250-mL portions of water. The organic layer was dried ($MgSO_4$), filtered, and concentrated, yielding 33.5 g of crude product. The NMR of the crude material showed approximately 60% conversion. The crude **5a** was used in subsequent reactions without further purification: NMR ($CDCl_3$) δ 4.59 (s, 2 H, CH_2Br), 2.50 ppm (s, 3 H, CH_3).

3-(Hydroxymethyl)-4-methyl-1,2,5-oxadiazole (6a). A mixture of 33.5 g of crude **5a**, 25 g of potassium carbonate, and 350 mL of water was heated to 90 °C and stirred for 90 min. The resulting aqueous solution was decanted from a small amount of residual oil, saturated with sodium chloride, and extracted with three 75-mL portions of ether. The combined ether extracts were dried ($MgSO_4$), filtered, and concentrated, yielding 11.95 g of the crude alcohol **6a**, which was used without further purification: NMR ($CDCl_3$) δ 4.88 (s, 2 H, CH_2), 4.55 (br s, 1 H, OH), 2.45 (s, 3 H, CH_3).

3-Formyl-4-methyl-1,2,5-oxadiazole (7a).[49,50] To 100 mL of 3.5 N nitric acid was added 11 g of crude **6a**. A solution of 132 g of ceric ammonium nitrate in 300 mL of 3.5 N nitric acid was added at 50 °C and the mixture was stirred at 50 °C for 6 h. The cooled reaction mixture was extracted with three 75-mL portions of methylene chloride; the combined extracts were washed with water (2 × 100 mL), 1 N $NaHCO_3$ (100 mL), and brine (100 mL) and dried ($MgSO_4$). Evaporation of solvent yielded 5.0 g of crude **7a**: NMR ($CDCl_3$) δ 10.40 [s, 1 H, C(O)H], 2.62 (s, 3 H, CH_3).

3-[(Hydroxyimino)methyl]-4-methyl-1,2,5-oxadiazole (2a). A mixture of **7a** (1.12 g, 10 mmol), 0.70 g (10 mmol) of hydroxylamine hydrochloride, and 0.79 g (10 mmol) of pyridine in 100 mL of ethanol was refluxed for 2 h. The ethanol was removed in vacuo and the residue was partitioned between ether (100 mL) and 1 N HCl (2 × 50 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 0.75 g of a white amorphous solid that proved to be a mixture of the desired oxime contaminated with 3,4-dimethyl-1,2,5-oxadiazole (**4a**). Recrystallization from petroleum ether yielded 0.30 g (24%) of pure **2a**: mp 54–56 °C; NMR (Me_2SO-d_6) δ 12.42 (s, 1 H, NOH), 8.40 (s, 1 H, CHNO), 2.50 (s, 3 H, CH_3). Anal. Calcd for $C_4H_5N_3O_2$: C, 37.79; H, 3.97; N, 33.07. Found: C, 37.48; H, 4.19; N, 32.60.

4-Methyl-1,2,5-oxadiazole-3-hydroximoyl Chloride (8a).[51] To a solution of **2a** (230 mg, 1.8 mmol) in 15 mL of dimethylformamide (DMF) was added with stirring 240 mg (1.8 mmol) of *N*-chlorosuccinimide (NCS). The resulting mixture was stirred at room temperature for 20 min, and 5 mL of HCl gas was introduced. After 30 min, the reaction mixture was heated to 50 °C and stirred at 50 °C for 40 min. The cooled mixture was poured into 60 mL of water, and the aqueous solution was extracted with two 50-mL portions of ether. The combined ether extracts were washed with three 50-mL portions of water, dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 280 mg (96%) of the crude hydroximoyl chloride **8a**. NMR showed approximately 80% conversion: NMR ($CDCl_3$) δ 12.10 (s, 1 H, NOH), 2.50 (s, 3 H, CH_3). This material was used without further purification.

4-Methyl-1,2,5-oxadiazole-3-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester Hydrochloride (3a). To a solution of 230 mg (1.4 mmol) of **8a** in 50 mL of chloroform were added 240 mg (1.4 mmol) of (diethylamino)ethanethiol hydrochloride and 290 mg (2.9 mmol) of triethylamine. The mixture was stirred overnight and then washed with water (2 × 50 mL) and dried ($MgSO_4$). Removal of solvent yielded 350 mg of viscous yellow oil, which was chromatographed on 125 g of silica gel with use of ether as eluent, to afford 150 mg (41%) of **3a** as a clear oil. A solution of **3a** in 25 mL of ether was treated with an ether solution saturated with HCl gas to provide 170 mg of hydrochloride salt: mp 121–122 °C; NMR (Me_2SO-d_6) δ 13.14 (s, 1 H, NOH), 3.20 (m, 8 H, CH_2), 2.44 (s, 3 H, CH_3), 1.22 (t, 6 H, CH_3). Anal. Calcd for $C_{10}H_{19}N_4O_2SCl$: C, 40.74; H, 6.50; N, 19.01; S, 10.88; Cl, 12.02. Found: C, 40.54; H, 6.46; N, 18.72; S, 10.68; Cl, 12.29.

3-(Bromomethyl)-1,2,5-thiadiazole (5b). A mixture of 15.30 g (0.1 mol) of 3-methyl-1,2,5-thiadiazole, 29.96 g (0.17 mol) of *N*-bromosuccinimide, and 0.4 g of benzoyl peroxide in 150 mL of carbon tetrachloride was heated at reflux overnight. The crude reaction mixture was washed with two 75-mL portions of 1 N sodium thiosulfate and two 50-mL portions of water. The organic solution was dried ($MgSO_4$), and the solvent was evaporated to yield 20.57 g of crude product, containing about 50% **5b**: NMR ($CDCl_3$) δ 8.63 (s, 1 H, arom), 4.72 (s, 2 H, CH_2Br).

3-(Hydroxymethyl)-1,2,5-thiadiazole (6b). The crude **5b** (prepared above) was treated with 20 g of potassium carbonate in 150 mL of water. The procedure and workup used for **6a** was followed to afford 5.25 g of crude **6b**: NMR ($CDCl_3$) δ 8.60 (s, 1 H, arom), 4.92 (br s, 2 H, CH_2), 4.95 (br s, 1 H, OH).

3-Formyl-1,2,5-thiadiazole (7b). A solution of **6b** (5.25 g, 45 mmol) and 52 g of ceric ammonium nitrate in 190 mL of water was heated to 75 °C for 45 min. The crude reaction mixture was extracted with two 50-mL portions of ether. The combined ether extracts were washed with 1 M $NaHCO_3$ solution (2 × 50 mL) and dried ($MgSO_4$), and the solvent was distilled, yielding the desired aldehyde: NMR ($CDCl_3$) δ 10.25 [s, 1 H, C(O)H], 9.10 (s, 1 H, arom). Isolation of **7b** was difficult because the material was volatile and codistilled with ether. The ether extract of **7b** obtained in a subsequent preparation was used, without isolation, for conversion to the oxime.

3-[(Hydroxyimino)methyl]-1,2,5-thiadiazole (2b). In a 1-L flask were placed approximately 5.7 g (50 mmol) of crude **7b** in 200 mL of diethyl ether, 3.5 g (50 mmol) of hydroxylamine hydrochloride, 3.9 g (50 mmol) of pyridine, and 500 mL of absolute ethanol. The resulting mixture was refluxed overnight, concentrated, and then taken up in 200 mL of ether. The ether solution was washed with 1 N HCl (2 × 150 mL) and water (2 × 150 mL), dried ($MgSO_4$), filtered, and concentrated, yielding 1.05 g (16%) of **2b** as a white crystalline material. The NMR of the crude product was consistent with an assigned structure of the *E* and *Z* aldoximes (see Results and Discussion).

Recrystallization from 25% diethyl ether/75% petroleum ether (30–60 °C) yielded an analytically pure sample of the mixed *E* and *Z* aldoximes: mp 148–149 °C; NMR (Me_2SO-d_6) for *Z* aldoxime (major component) δ 12.10 (s, 1 H, NOH), 9.12 (s, 1 H, CHNO), 8.43 (s, 1 H, arom); *E* aldoxime (minor component) δ 12.53 (s, 1 H, NOH), 9.50 (s, 1 H, CHNO), 7.95 (s, 1 H, arom). Anal. Calcd for $C_3H_3N_3OS$: C, 27.90; H, 2.34; N, 32.54; S, 24.83. Found: C, 27.94; H, 2.61; N, 32.33; S, 24.61.

1,2,5-Thiadiazole-3-hydroximoyl Chloride (8b). A solution of **2b** (150 mg, 1.2 mmol) in 15 mL of DMF was treated with NCS (160 mg, 1.1 mmol). The procedure and workup used for **8a** yielded 190 mg (100%) of crude **8b**: NMR (acetone-d_6) δ 12.07 (s, 1 H, NOH), 9.00 (s, 1 H, arom).

1,2,5-Thiadiazole-3-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester (3b). A 190-mg (1.2 mmol) sample of **8b** was converted to a yellow oil, using the procedure for **3a**. The oil slowly solidified, was triturated with ether, and was filtered, yielding 55 mg (18%) of **3b** as white crystalline material, mp 122–124 °C. An analytical sample of **3b** was obtained by column chromatography on silica gel, using ether as eluent: NMR (acetone-d_6) δ 9.07 (s, 1 H, arom), 3.10 (m, 2 H, SCH_2), 2.50 (m, 6 H, CH_2), 0.95 (t, 6 H, CH_3). Anal. Calcd for $C_9H_{16}N_4OS_2$: C, 41.5; H, 6.19; N, 21.52. Found: C, 41.44; H, 6.30; N, 21.69.

Compounds **5c**, **6c**, and **7c** were prepared by the procedures used for the corresponding compounds **5b**, **6b**, and **7b**.

3-(Bromomethyl)-4-methyl-1,2,5-thiadiazole (5c):* NMR ($CDCl_3$) δ 4.55 (s, 2 H, CH_2Br), 2.50 (s, 3 H, CH_3).

3-(Hydroxymethyl)-4-methyl-1,2,5-thiadiazole (6c): NMR ($CDCl_3$) δ 4.80 (br, s, 2 H, CH_2OH), 2.50 (s, 3 H, CH_3).

3-Formyl-4-methyl-1,2,5-thiadiazole (7c): NMR ($CDCl_3$) δ 10.27 [s, 1 H, C(O)H], 2.80 (s, 3 H, CH_3).

3-[(Hydroxyimino)methyl]-4-methyl-1,2,5-thiadiazole (2c). Following the procedure for **2b**, 6.4 g (50 mmol) of **7c** was con-

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verted to 5.2 g (73%) of **2c**. An analytical sample was obtained by repeated recrystallization from a 1:1 petroleum ether (30–60 °C)/diethyl ether mixture: mp 131–134 °C; NMR (Me_2SO-d_6) δ 12.05 (s, 1 H, NOH), 8.48 (s, 1 H, CH), 2.73 (s, 3 H, CH_3). Anal. Calcd for $C_4H_5N_3OS$: C, 33.55; H, 3.52; N, 29.35; S, 22.40. Found: C, 33.69; H, 3.83; N, 29.01; S, 22.07.

4-Methyl-1,2,5-thiadiazole-3-hydroximoyl Chloride (8c). A solution of 1.73 g (12 mmol) of **2c** in 60 mL of DMF was treated with 1.62 g (12 mmol) of NCS. The procedure and workup employed for **8a** afforded 1.78 g of crude **8c**: NMR (acetone-d_6) δ 12.88 (s, 1 H, NOH), 2.80 (s, 3 H, CH_3).

4-Methyl-1,2,5-thiadiazole-3-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester Hydrochloride (3c). Following the procedure for **3a**, 1.55 g (8.7 mmol) of **8c** was converted to a mixture that was chromatographed on 125 g of silica gel, using ether as eluent. The first fraction proved to be **2c**. Continued elution yielded 0.93 g (39%) of **3c** as a yellow oil. The oil was dissolved in 25 mL of ether and an ether solution saturated with HCl gas was added dropwise to provide 0.98 g of hydrochloride salt: mp 179–182 °C; NMR (Me_2SO-d_6) δ 12.72 (s, 1 H, NOH), 3.18 (m, 8 H, CH_2), 2.63 (s, 3 H, CH_3), 1.10 (t, 6 H, CH_3). Anal. Calcd for $C_{10}H_{19}N_4OS_2Cl$: C, 38.63; H, 6.16; N, 18.03; S, 20.63; Cl, 11.40. Found: C, 38.69; H, 6.16; N, 17.84; S, 20.23; Cl, 11.49.

2-[(Hydroxyimino)methyl]-5-phenyl-1,3,4-oxadiazole (2d). *n*-Butyllithium (7.5 mL, 18 mmol of 2.4 M solution) was added to a solution of 2.41 g (15 mmol) of 2-methyl-5-phenyl-1,3,4-oxadiazole in 40 mL of THF at −60 °C (temperature rising to −30 °C). The dark red solution was allowed to warm to −20 °C over a 5-min period and then cooled to −60 °C. After 10 min, 3.02 mL (22.5 mmol) of isoamyl nitrite was added. After 5 min at −60 °C, the solution was allowed to warm to 20 °C over a 45-min period. Then the solution was cooled to 0 °C and 25 mL of 3 M HCl was added, turning the solution yellow.

After 1 h of stirring, the layers were separated, and the aqueous layer was extracted with ether (2 × 40 mL). Combined organic layers were washed with water (50 mL) and brine (50 mL) and dried (Na_2SO_4). Solvent removal yielded 2.35 g of material that was partitioned between 100 mL of saturated sodium carbonate solution and dichloromethane (3 × 50 mL). The dark red aqueous solution was cooled to 0 °C, treated with 3 M HCl to pH 6, and extracted with dichloromethane (3 × 50 mL). The organic layer was washed with 50 mL of brine and then dried (Na_2SO_4).

Solvent removal yielded 0.59 g of material, 0.49 g of which was placed on three silica gel preparative TLC plates and developed with 3% methanol/dichloromethane (three times). The higher R_f major band was extracted with acetone to furnish 0.16 g of solid.

This material was placed on two silica gel preparative TLC plates, and the plates were developed with ether. Extraction of the uppermost band with acetone yielded 82.6 mg (3%) of oxime. Recrystallization from acetone/hexanes gave the analytical sample: mp 189–190 °C; IR (KBr) 3210, 3150, 3070, 2985, 2885, 1610, 1550, 1480, 1455, 1040, 995, 850, 685 cm^{-1}; NMR (acetone-d_6) δ 7.4–8.3 (m, 5 H, arom), 8.38 (s, 1 H, *HC*=NOH), 11.37 (br s, 1 H, HC=NO*H*). Anal. Calcd for $C_9H_7N_3O_2$: C, 57.14; H, 3.73; N, 22.22. Found: C, 56.94; H, 3.76; N, 21.88.

5-Phenyl-1,3,4-oxadiazole-1-hydroximoyl Chloride (8d). A mixture of 65.0 mg (0.344 mmol) of **2d**, 46.0 mg (0.344 mol) of NCS, and a trace of HCl gas in 5 mL of DMF was heated at 50 °C for 0.5 h. After cooling, the mixture was poured into 50 mL of water and extracted with ether (2 × 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 yielded 62.7 mg (82%) of crude **8d** as a white solid. The material was used without further purification.

5-Phenyl-1,3,4-oxadiazole-2-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester Hydrochloride (3d). To a stirred solution of 62.7 mg (0.28 mmol) of **8d** and 47.6 mg (0.28 mmol) of 2-(diethylamino)ethanethiol hydrochloride in 10 mL of dichloromethane was added 0.3 mL of triethylamine. The solution immediately turned yellow. The solution was stirred 1.5 h, diluted to 50 mL with dichloromethane, washed with 50-mL portions of 1 M sodium bicarbonate solution and brine, and dried (Na_2SO_4). Removal of solvent yielded 94 mg of material, which was placed on one preparative silica gel TLC plate.

After the plate was developed with ether, the lower R_f band was extracted with acetone to provide 46.4 mg (52%) of **3d** as an oil: NMR ($CDCl_3$) δ 1.11 (t, 6 H, $2CH_3$), 2.50–3.63 (m, 8 H, 4 CH_2), 7.55 (m, 3 H, arom), 8.10 (m, 2 H, arom), 10.95 (br, s, 1 H, C=NOH). The thiohydroximate was dissolved in 5 mL of ether and ether saturated with HCl gas was added until no further precipitation resulted. Collection of the precipitate afforded 42.1 mg of the hydrochloride salt, mp 214–215 °C. Anal. Calcd for $C_{15}H_{21}N_4O_2SCl$: C, 50.48; H, 5.93; N, 15.70; S, 8.99. Found: C, 50.57; H, 6.02; N, 15.63; S, 9.07.

3-Phenyl-5-[(hydroximino)methyl]-1,2,4-oxadiazole (2e). *tert*-Butyllithium (0.012 mol, 6 mL of 2.0 M solution) was added slowly to a solution of 3-phenyl-5-methyl-1,2,4-oxadiazole (1.6 g, 0.010 mol) in 40 mL of THF at −78 °C. After 30 min, isopropyl nitrite (1.1 g, 0.012 mol) was added at −78 °C. The reaction mixture was allowed to warm to room temperature over a 30-min period. Acidification with 30 mL of 4 N HCl, extraction with three 20-mL portions of ether, drying the combined ether extracts over $MgSO_4$, and removal of solvent yielded 1.2 g of **2e** contaminated with starting material. Sublimation of the crude product gave 0.150 g (7.9%) of **2e**: mp 166–168 °C; IR (KBr) 3225 (s), 2960 (m), 1540 (m), 1430 (s), 1345 (s), 1035 (s), 990 (s) cm^{-1}; NMR (Me_2SO-d_6) δ 12.87 (br s, 1 H, NOH), 8.37 (s, 1 H, C*H*=NOH), 8.00 (m, 2 H, arom), 7.56 (m, 3 H, arom). Anal. Calcd for $C_9H_7N_3O_2$: C, 57.14; H, 3.73; N, 22.22. Found: C, 57.17; H, 3.53; N, 21.97.

3-Phenyl-1,2,4-oxadiazole-5-hydroximoyl Chloride (8e). A 0.94-g (5.0 mmol) sample of **2e** was converted to 0.94 g (85%) of crude **8e** by using the procedure for **8a**: mp 155–159 °C; NMR (acetone-d_6) δ 12.70 (s, 1 H, NOH), 8.15 (m, 2 H, phenyl), 7.57 (m, 3 H, phenyl).

3-Phenyl-1,2,4-oxadiazole-5-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester (3e). A 0.90-g (4.0 mmol) sample of **8e** was converted to 1.21 g of crude product by using the procedure for **3a**. The crude product was purified by column chromatography (silica gel, ether), yielding 0.35 g (27%) of **3e** as a yellow oil. Anal. Calcd for $C_{15}H_{20}N_4O_2S$: C, 56.22; H, 6.29; N, 17.49. Found: C, 56.16; H, 6.25; N, 17.63.

Purified **3e** was converted to its hydrochloride salt by the usual method: mp 199–201 °C dec; NMR (Me_2SO-d_6) δ 13.88 (br s, 1 H, NOH), 8.10 (m, 2 H, phenyl), 7.69 (m, 3H, phenyl), 3.63 (m, 2H, SCH_2), 3.17 (m, 6H, CH_2), and 1.17 ppm (t, 6H, CH_3).

3-Methyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole (2f). *n*-Butyllithium (38.2 mL, 92 mmol of 2.4 M solution) was added to a solution of 3,5-dimethyl-1,2,4-oxadiazole (7.50 g, 76.4 mmol) in 70 mL of THF at −70 to −50 °C over a 15-min period. The solution turned yellow and a white precipitate formed. The mixture was warmed to −35 °C over a 5-min period and then recooled to −70 °C. Isopropyl nitrite (12.1 mL, 0.115 mol) was added over a 10-min period. After 5 min at −70 °C, the mixture was warmed to 20 °C; 50 mL of 3 M HCl was added, and the mixture was stirred. Water (50 mL) was added, and the mixture was extracted with ether (3 × 100 mL). The combined ether layers were washed with brine (100 ml) and dried (Na_2SO_4). Removal of solvent gave 10.0 g of material, which was partitioned between 50 mL of dichloromethane and 0.5 N NaOH (2 × 50 mL). The aqueous layer was washed with ether (50 mL) and the pH was adjusted to 6 with 3 M HCl. Extraction with dichloromethane (3 × 50 mL), washing with brine (50 mL), drying (Na_2SO_4), and removal of solvent gave 2.05 g of crude acidic product, which was placed on a column containing 100 g of silica gel. Elution with 20% ether/hexane provided 0.67 g (6.9%) of **2f**. Two recrystallizations from acetone/hexane afforded the analytical sample: mp 163.5–164 °C; IR (KBr) 3215, 3150, 3040, 2995, 2900, 1575, 1480, 1395, 1355, 1305, 1010, 855 cm^{-1}; NMR (CD_3COCD_3) δ 2.42 (s, 3 H, CH_3), 8.34 (s, 1 H, HC=NOH), 13.02 (br, s, 1 H, NOH). Anal. Calcd for $C_4H_5N_3O_2$: C, 37.79; H, 3.97; N, 33.07. Found: C, 37.61; H, 4.05; N, 33.40.

3-Methyl-1,2,4-oxadiazole-5-hydroximoyl Chloride (8f). Oxime **2f** (114 mg, 0.90 mmol) was converted to 139 mg (96%) of **8f** by the method used for the preparation of **8d**.

3-Methyl-1,2,4-oxadiazole-5-thiohydroximic Acid 2-(Diethylamino)ethyl *S*-Ester (3f). Compound **3f** was prepared in the same manner as **3d** using 128 mg (0.79 mmol) of **8f**. Preparative TLC (silica gel, ether) of the crude product furnished 128.5 mg (63%) of **3f**, mp 87–89 °C. Recrystallization from acetone/hexane afforded the analytical sample: mp 92–93.5 °C; NMR (CD_3COCD_3) δ 0.97 (t, 6 H, J = 7 Hz, CH_2CH_3), 2.45 (s,

3 H, CH_3), 2.54 (q, 4 H, J = 7 Hz, CH_2CH_3), 2.54–3.47 (m, 4 H, CH_2CH_2), 6.73 (br, 1 H, NOH). Anal. Calcd for $C_{10}H_{18}N_4O_2S$: C, 46.49; H, 7.02; N, 21.69; S, 12.41. Found: C, 46.81; H, 7.14; N, 21.82; S, 12.26.

Methods. General Procedures. Reactivator pK_a values were determined spectrophotometrically as described by Albert and Sargeant.[27] Octanol–buffer partition coefficients were against pH 7.6, 0.1 M phosphate buffer aqueous phase per the general method of Fujita et al.[52]

Acetylcholinesterase Determinations in Vitro. Unless otherwise noted, all experiments were conducted at 25.0 ± 0.1 °C in pH 7.6, 0.1 M 3-morpholinopropanesulfonic acid (MOPS) buffer plus NaN_3 (0.002%), $MgCl_2$ (0.01 M), and bovine serum albumin (0.1%). Enzyme activities were assayed by the Ellman method[53] on a Gilford-modified DU Spectrophotometer coupled to a Hewlett-Packard Model HP 85 laboratory computer for automatic rate determination. All rate constants were determined by least-squares linear regression analysis with error limits reported as standard deviation from the mean.

In general, eel AChE (Worthington) 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. To determine reactivation rates, we incubated the inhibited enzyme with reactivators for timed intervals and assayed (in duplicate) for activity. Parallel control experiments included incubation of the following: uninhibited enzyme alone (to determine enzyme denaturation rate); inhibited enzyme in the absence of reactivator (to determine spontaneous reactivation rate); uninhibited enzyme in the presence of reactivator (to determine reversible inhibition of AChE activity by reactivator, see below); and substrate (acetylthiocholine) in the absence and presence of reactivator (to determine, respectively, spontaneous and reactivator-induced substrate hydrolysis rates). The spontaneous and reactivator-induced substrate hydrolysis rates were subtracted from observed overall reaction rates to provide values for net observed enzyme activities.

Dilution factors and aliquot volumes were determined experimentally for the various transfers involved in the experiments. An exact procedure giving good precision in replicate assays is as follows.

Dilute 110 μL of (nominally) 500 AChE units/mL of enzyme solution with 110 μL of MOPS buffer to give enzyme "stock" solution. For determining uninhibited AChE activity, dilute 25 μL of stock solution to 20 mL with MOPS buffer and withdraw 50 μL for assay (see below).

To inhibit the AChE, dilute 140 μL of stock solution in 132 μL of MOPS plus 8 μL of EPMP (1×10^{-5} M in C_2H_5OH). To determine activity of the inhibited AChE, incubate 20 min, withdraw 10 μL, dilute to 4.0 mL in MOPS, and assay 50 μL.

For reactivation studies, dilute 100 μL of inhibited AChE solution to 1.0 mL with MOPS buffer, remove 25 μL (for each incubation), and dilute to 1.0 mL with MOPS plus reactivator at known concentrations. Incubate at 25 °C for up to 6 h, removing duplicate 50-μL aliquots for assay at several time points.

For control studies, use the same aliquot volumes as for reactivation studies, but add MOPS buffer without active enzyme, inhibited enzyme, or reactivator as required.

For assay of AChE activity, add 50-μL aliquots of solutions to be assayed to 910 μL of pH 8.0, 0.1 M phosphate buffer, plus 30 μL of 0.10 M 5,5′-dithiobis(2-nitrobenzoic acid), plus 10 μL of 0.075 M acetylthiocholine, and monitor increased absorbance at 412 nm vs. time.

Reversible inhibition of AChE by test compounds was measured as above, except that phosphonylation of the enzyme was omitted. Enzyme was incubated with test compounds at three concentrations in MOPS buffer at 25 °C and assayed at least three times between 30 and 240 min. In all cases, the degree of inhibition was found to be independent of incubation time, and reported activities for each concentration of test compound are mean values (±SD) for all time points.

Kinetics of reactions of test compounds with AcSCh were determined under the conditions used for assaying AChE and, therefore, served also as correction factors in our reactivation experiments. The experiments were performed under pseudo-zero-order conditions, and rate constants were calculated as described in the Results and Discussion. Control studies demonstrated that the test compounds were stable to hydrolysis or other degradation pathways under the experimental conditions.

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Registry No. **1a**·HCl, 90507-14-5; **2a**, 90507-15-6; (*E*)-**2b**, 90507-16-7; (*Z*)-**2b**, 90507-17-8; **2c**, 90507-18-9; **2d**, 90507-19-0; **2e**, 90507-20-3; **2f**, 90507-21-4; **2g**, 61444-96-0; **2h**, 61444-94-8; **3a**, 90507-22-5; **3a**·HCl, 90507-23-6; **3b**, 90507-24-7; **3c**, 90507-25-8; **3c**·HCl, 90507-26-9; **3d**, 90507-27-0; **3d**·HCl, 90507-28-1; **3e**, 90507-29-2; **3e**·HCl, 90507-30-5; **3f**, 90507-31-6; **4a**, 4975-21-7; **4b**, 5728-06-3; **4c**, 5728-21-2; **4d**, 4046-03-1; **4e**, 1198-98-7; **4f**, 10403-80-2; **5a**, 90507-32-7; **5b**, 53012-70-7; **5c**, 77127-79-8; **6a**, 78441-74-4; **6b**, 90507-33-8; **6c**, 90507-34-9; **7a**, 90507-35-0; **7b**, 75238-60-7; **7c**, 90507-36-1; **8a**, 90507-37-2; **8b**, 90507-38-3; **8c**, 90507-39-4; **8d**, 70390-97-5; **8e**, 90507-40-7; **8f**, 90507-41-8; $Et_2NCH_2CH_2SH$·HCl, 1942-52-5; isoamyl nitrite, 110-46-3; ethyl *p*-nitrophenyl methylphosphonate, 3735-98-6; AChE, 9000-81-1; AcSCh, 4468-05-7.

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