AD-A157 214 NONQUATERNARY CHOLINESTERASE REACTIVATORS(U) SRI INTERNATIONAL MENLO PARK CA R A KENLEY ET AL. 27 JAN 82 DAMD17-79-C-9178 1/1

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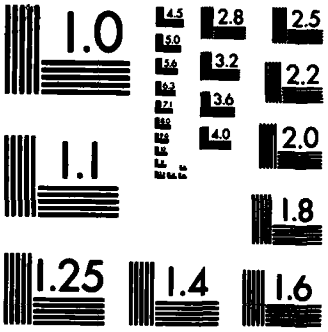

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NONQUATERNARY CHOLINESTERASE REACTIVATORS

Annual Report

Richard A. Kenley, Robert A. Howd, Carol W. Mosher
Clifford D. Bedford

January 27, 1982

Supported By
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-79-C-9178

SRI International
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO. AD-A157 214

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)

NONQUATERNARY CHOLINESTERASE REACTIVATORS

5. TYPE OF REPORT & PERIOD COVERED
Annual
October 1980 through
October 1981

6. PERFORMING ORG. REPORT NUMBER
PYU-1043

7. AUTHOR(s)
Richard A. Kenley, Robert A. Howd, Carol W. Mosher,
and Clifford D. Bedford

8. CONTRACT OR GRANT NUMBER(s)
DAMD17-79-C-9178

9. PERFORMING ORGANIZATION NAME AND ADDRESS
SRI International
333 Ravenswood Avenue
Menlo Park, CA 94025

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
62734A.3M162734A875.AC.421

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development
Command
Ft. Detrick, MD 21701-5012

12. REPORT DATE
Jan. 27, 1982

13. NUMBER OF PAGES
81

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Part I submitted for publication in Journal of Medicinal Chemistry

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

acetylcholinesterase	pKa
ethyl p-nitrophenyl methylphosphonate	in vitro reactivation
α-ketothiohydroximates	kinetics
α-heteroaromatic thiohydroximates	mechanism
α-heteroaromatic aldoximes	alpha

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We prepared a series of dialkylaminoalkyl α-ketothiohydroximates and evaluated them *in vitro* as reactivators of acetylcholinesterase (AChE) inhibited by ethyl p-nitrophenyl methylphosphonate (EPMP). The compounds conformed to the general formula 4-$RH_6H_4C(O)C(NOH)S(CH_2)_nNH_2\cdot HCl$ where: R = MeO, n = 2, 3; and R' = CH_3 or C_2H_5. For comparison we also examined 2-hydroxyiminomethyl-1-methylpyridinium iodide (2PAM), p-$BrC_6H_4C(O)C(NOH)SCH_2CH_2CH_3$, and the related α-oximino thiohydroximate $CH(NOH)C(NOH)SCH_2CH_2NMe\cdot HCl$.

The thiohydroximates reactivate ethyl methylphosphonyl-AChE via a

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mechanism involving equilibrium binding of reactivator to inhibited enzyme followed by displacement of the inhibitor by reactivator. 2PAM is at least 50 times more reactive toward ethyl methylphosphonyl-AChE than any of the above nonquaternary reactivators. The differences in reactivity apparently relate to entropic factors associated with formation of the reactivator/phosphonylated enzyme complex.

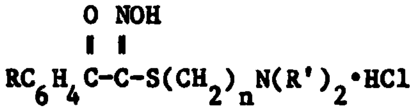
alpha

We also prepared a series of oxadiazoles, thiadiazoles, and triazoles with aldoxime or diethylaminoethyl thiohydroximate functional groups. These compounds were characterized with respect to structure, acidity, and activity as reactivators of ethyl methylphosphonyl-AChE. Of seven α-heteroaromatic aldoximes, only one, 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole, exhibits an oxime acidity (pKa = 7.9) near the optimal value of pKa = 8. This compound is the best nonquaternary AChE reactivator studied to date. 5-Hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole is three times more active than 4-$BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2{\cdot}HCl$ toward ethyl methylphosphonyl-AChE. Several of the α-oxadiazolyl and α-thiadiazolyl thiohydroximic acid 2-(diethylamino)ethyl S-esters exhibit acidities near pKa = 8. The α-heteroaromatic thiohydroximates are poorer reactivators of ethyl methylphosphonyl-AChE than 5-hydroxyiminomethyl-e-phenyl-1,2,4-oxadiazole. Again entropic factors apparently limit the activity of the thiohydroximates.

SUMMARY

Pyridinium aldoximes such as 2PAM are effective therapy in poisoning by toxic organophosphorus compounds. However, as quaternary ammonium salts, the pyridinium compounds penetrate poorly from the serum into tissue regions (e.g., the brain) where organophosphorus anticholinesterase elicit pronounced physiological effects. In an effort to develop therapeutics that should surpass the pyridinium aldoximes with respect to tissue distribution, we have synthesized and evaluated various nonquaternary hydroxyimino compounds as reactivators of phosphonylated acetylcholinesterase (AChE).

Our efforts have focused on three general classes of compounds: α-ketothiohydroximic acid dialkylaminoalkyl S-esters, 1, heteroaromatic aldoximes, 2, and α-heteroaromatic thiohydroximates, 3.



$$RC_6H_4\overset{\overset{O}{\|}}{C}-\overset{\overset{NOH}{\|}}{C}-S(CH_2)_nN(R')_2\cdot HCl$$

1

In each case we characterized the compounds with respect to oxime acid dissociation constant (pKa), structure, and activity *in vitro* as reactivators of eel AChE inhibited by ethyl p-nitrophenyl methylphosphonate (EPMP).

The *in vitro* studies on type 1 compounds are essentially complete, but our investigations on types 2 and 3 are continuing. For this reason we have divided the current report into two major sections: Part I describes the preparation and characterization of type 1 compounds and the development of an *in vitro* model for elucidating structure-activity relationships among novel AChE reactivators. Part II describes progress on type 2 and type 3 compounds.

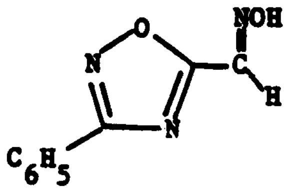
Part I discusses in detail the use of EPMP-inhibited eel AChE for determining of kinetic parameters for AChE reactivation by 2PAM and type 1 compounds. We demonstrated that under appropriate reaction conditions ethyl methylphosphonyl-AChE does not undergo complicating side reactions (e.g., dealkylation, spontaneous reactivation, enzyme denaturation, enzyme reinhibition by phosphonyl oximes) that would otherwise interfere with determining meaningful reactivation kinetics. Of the inhibitor/enzyme combinations tried and reported, we believe the EPMP/eel AChE system to be superior with respect to accurate and convenient determination of kinetics parameters.

We determined kinetic parameters for reactivation of ethyl methylphosphonyl-AChE by type 1 compounds and 2PAM. The kinetics require a mechanism involving reversible formation of a reactivator/inhibited enzyme complex followed by displacement of ethyl methylphosphonate from the enzyme active surface. In terms of inherent activity toward ethyl methylphosphonyl-AChE, 2PAM is 50 to 1000 times more reactive than any of the type 1 compounds investigated. We have interpreted the kinetic data to mean that type 1 compounds and 2PAM bind equally well to the inhibited enzyme but that reactivator/inhibited enzyme complexes derived from 1 have many available conformations, only a small fraction of which feature a geometry suitable for displacing the organophosphorus moiety from the active surface. 2PAM appears to be superior to type 1 compounds not because it is more inherently reactive, but rather because it is more rigid and exhibits a geometry well-suited for reactivation.

We designed our investigation of type 2 and 3 compounds in part to further probe the relationships between reactivator rigidity and activity. We found that the thiohydroximate derivatives, 3, as a class are poorer than type 2 compounds as reactivators of ethyl methylphosphonyl-AChE. This reinforces our hypothesis concerning the need for incorporating structural rigidity into candidate AChE reactivators.

Although none of the nonquaternary reactivators investigated to date equals 2PAM with respect to rate of reactivation of phosphonylated-AChE, several of the new compounds that we have prepared do exhibit reasonable activity as reactivators. Among the most active of the nonquaternary reactivators are:

$BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$, $(HON:)CHC(:NOH)SCH_2CH_2N(CH_3)_2 \cdot HCl$, and,



Each of these compounds, at concentrations of 10^{-4} to 10^{-3} M, completely restores activity to ethyl methylphosphonyl-AChE within a few hours. We believe that the currently available nonquaternary reactivators have considerable promise as research tools for probing central nervous system activity of toxic organophosphorus compounds. We further postulate that through appropriate structural modifications it will be possible to obtain nonquaternary reactivators that actually surpass 2PAM in terms of inherent reactivity toward phosphonylated AChE.

In developing new therapeutics it is necessary at some point to evaluate candidate drugs *in vitro* and *in vivo* against the specific toxic agents that the drugs are designed to treat. We believe that we have reached this juncture with nonquaternary reactivators. Future work will involve synthesis of new compounds plus evaluation of compounds as reactivators *in vitro* of AChE inhibited by 3,3-dimethyl-2-butyl methylphosphonofluoridate. Furthermore, we recommend that an evaluation of the nonquaternary compounds *in vivo* versus various toxic organophosphonates be undertaken.

CONTENTS

Part I. α-Ketothiohydroximates as Reactivators of Ethyl Methylphosphonyl-Acetylcholinesterase

Part II. Heteroaromatic-Oximes and Thiohydroximates as Reactivators of Ethyl Methylphosphonyl-AChE *In Vitro*

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PART II

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PART I

PART II

Part I α-Ketothiohydroximates as Reactivators of Ethyl Methylphosphonyl-Acetylcholinesterase

INTRODUCTION

Biologically-active organophosphorus compounds are widely used in agriculture and medicine.[1-7] Certain highly toxic organophosphonates are also stockpiled for use in war.[8-10] The potential for deliberate or accidental poisoning by organophosphorus esters dictates a requirement for safe and effective therapeutics.

Most toxic organophosphorus esters are irreversible inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) AChE.[1,11-13] The inhibition proceeds via phosphylation* of a serine hydroxyl at the "esteratic" region of the enzyme active site. Conventional therapy of organophosphorus ester intoxication entails coadministration of atropine [to antagonize the effects of accumulated acetylcholine (ACh)] and AChE "reactivators" that restore activity to the enzyme.[14-17] Currently, pyridinium oximes are the only clinically used reactivators. Examples are 2-hydroxyiminomethyl-1-methylpyridinium iodide (2PAM) and 1,3-bis(4-hydroxyiminomethyl-1-pyridinium)propane dibromide (TMB4).

Pyridinium oximes can reactivate AChE because of two factors: an oxime acid dissociation constant (pKa) equal to approximately 8; and a cationic moiety (the n-alkyl pyridinium ion) at the appropriate distance from the oxime to give a structural similarity to ACh. The oxime acid dissociation constant contributes to a high proportion of the anionic (oximate) form of the reactivator at physiological pH. The oximate acts as a nucleophile to displace the organophosphorus moiety from the serine hydroxyl. The cationic moiety interacts electrostatically with an aspartic acid carboxylate at the "anionic" region of the enzyme active site and affords reversible binding of reactivator to the inhibited enzyme. The geometry of the pyridinium oximes places the oximate close to the phosphylated serine so that the nucleophilic displacement reaction proceeds readily.

Although the pyridinium oximes are useful therapeutics, the quaternary ammonium functional group common to this class of compounds limits their antidotal activity insofar as the hydrophilic pyridinium cations penetrate

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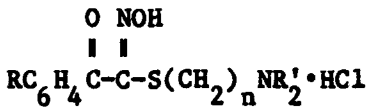
*We use the term "phosphylation" when we do not distinguish between "phosphonylation" and "phosphorylation."

membranes poorly. This causes a disproportionately high serum concentration of the pyridinium reactivators, rapid renal elimination, and marginal antidotal activity in regions (such as the central nervous system) where organophosphorus esters elicit pronounced physiological responses.

In principle, it should be possible to develop nonquaternary AChE reactivators that would not only equal the pyridinium oximes inherent activity toward phosphylated AChE but that would exhibit a tissue distribution more like that of hydrophobic organophosphorus esters. We earlier[8] investigated series of α-ketothiohydroximic acid S-esters, given by the general formula, 1:



$$\mathrm{RC_6H_4\overset{\overset{O}{\|}}{C}-\overset{\overset{NOH}{\|}}{C}-S(CH_2)_nNR'_2\cdot HCl}$$

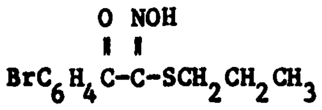
1

where: R was chosen to "fine-tune" the oxime pK_a; the tertiary amine functional group was incorporated to facilitate penetration into hydrophobic tissues while also providing coulombic interaction (via the protonated ammonium salt) with the anionic region of the enzyme active site; and n was varied to optimally separate the cationic and nucleophilic moieties of the reactivator.

In the previous communication we reported the synthesis of nine structurally related α-ketothiohydroximates and characterized them with respect to structure, acidity, nucleophilicity, and activity *in vitro* as reactivators of diisopropyl phosphoryl-AChE. These were moderately active reactivators and we tried to quantitatively compare their activity by determining reactivation kinetics. Unfortunately, diisopropyl phosphoryl-AChE undergoes a side reaction (dealkylation, see below) with a rate comparable to the reactivation reaction. This introduced uncertainty into our determination of reactivation kinetics and thwarted further attempts to establish quantitative structure-activity relationships.

To resolve these issues we have now examined the activity of five thiohydroximates as reactivators of ethyl methylphosphonyl-AChE *in vitro*. Our objectives were to develop a useful *in vitro* model to accurately determine kinetics of reactivation, and to establish structure-activity relationships for the thiohydroximates as reactivators.

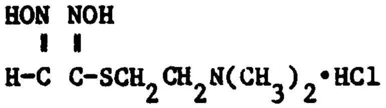
This report describes the synthesis of: 2-(diethylamino)ethyl *p*-bromobenzoylthiohydroximate (Code SR 3018), 1a ($R = Br$, $n = 2$, $R' = C_2H_5$); 3-(dimethylamino)propyl p-bromobenzoylthiohydroximate, 1d ($R = Br$, $n = 3$, $R' = CH_3$; the related compounds n-propyl p-bromobenzoylthiohydroximate, 2:



$$\mathrm{BrC_6H_4\overset{\overset{O}{\|}}{C}-\overset{\overset{NOH}{\|}}{C}-SCH_2CH_2CH_3}$$

2

and 2-(dimethylamino)ethyl thioglyoximate, 3:



$$\mathrm{H{-}\overset{\displaystyle HON}{\overset{\|}{C}}\;\overset{\displaystyle NOH}{\overset{\|}{C}}{-}SCH_2CH_2N(CH_3)_2\cdot HCl}$$

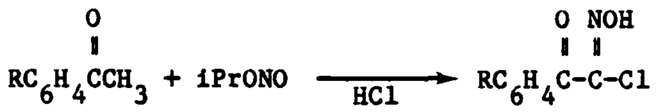
3

We also detail our evaluation of selected thiohydroximates as reactivators *in vitro* of AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate (EPMP). For comparison, we examined the activity of 2-hydroxyiminomethyl-1-methylpyridinium iodide, 4 as a reactivator of EPMP-inhibited AChE.

RESULTS AND DISCUSSION

Synthesis and Structure

Table 1 shows structures and pertinent data for compounds used in the current investigation. We prepared the new compounds 1a, 1d, and 2 as previously described[18] for 1b and 1c via reaction (1) and (2):

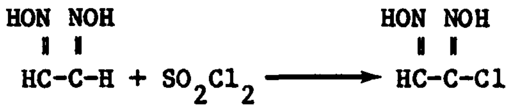


$$RC_6H_4\overset{O}{\overset{\|}{C}}CH_3 + iPrONO \xrightarrow[HCl]{} RC_6H_4\overset{O}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-Cl \tag{1}$$

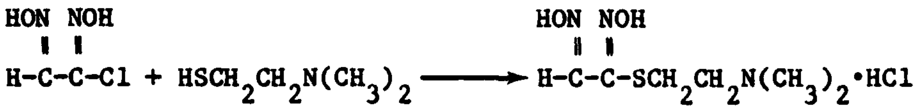
$$RC_6H_4\overset{O}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-Cl + HS(CH_2)_nR' \longrightarrow RC_6H_4\overset{O}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-S(CH_2)_nR' \tag{2}$$

1 and 2

We obtained the thioglyoximate, 3, by chlorination of glyoxime followed by esterification with 2-(dimethylamino)ethane thiol, reactions (3) and (4):



$$H\overset{HON}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-H + SO_2Cl_2 \longrightarrow H\overset{HON}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-Cl \tag{3}$$



$$H-\overset{HON}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-Cl + HSCH_2CH_2N(CH_3)_2 \longrightarrow H-\overset{HON}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-SCH_2CH_2N(CH_3)_2\cdot HCl \tag{4}$$

3

We previously showed that for various 1, oxime acidity correlated well with =NO-H proton NMR chemical shift. Combining data for 1a, 1d, 2, 3, and glyoxime (pK_a = 9.9, NMR = 11.62 δ) with data previously obtained[18] for α-ketothiohydroximates gives equation (5):

$$pK_a = (28.6 \pm 1.2) - (1.62 \pm 0.096)\delta \tag{5}$$

Table 1

SELECTED DATA FOR THIOHYDROXIMATES

Compound	Structure	pK_a	NMR[b] (NO-H) δ, ppm	Reference
1a	$4\text{-}BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$	7.44	13.01	this work
1b	$4\text{-}MeOC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$	7.82	12.69	19
1c	$4\text{-}MeOC_6H_4C(O)C(NOH)SCH_2CH_2CH_2N(CH_3)_2 \cdot HCl$	8.42	12.50	19
3	$(HON)CHC(NOH)SCH_2CH_2N(CH_3)_2 \cdot HCl$	8.37	12.47(br)	this work

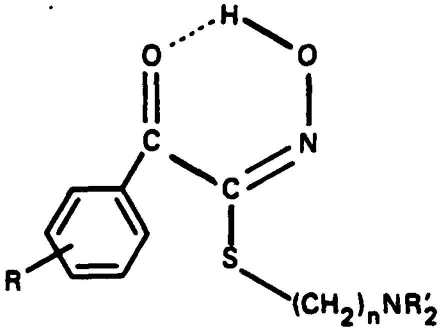
---

[a] Determined spectrophotometrically in 0.1 M phosphate buffer.
[b] Relative to TMS in DMSO-d_6.

Similarly for 1 with n = 2, R' = C_2H_5, and R = NO_2, H, MeO, and Br, the pK_a data of Table 1 and reference 18 correlate with the Hammett substituent constant σ_p[19] to give equation (6):

$$pK_a = (7.61 \pm 0.005) - (0.78 \pm 0.01)\sigma_p \qquad (6)$$

On the basis of IR spectral data, we previously proposed an E-configuration for α-ketothiohydroximates with six-center intramolecular hydrogen bonding of the hydroxyimino proton to the α-carbonyl group. Compounds (1a) and (2) should also exist in the E-configuration.



E-α-Ketothiohydroximate

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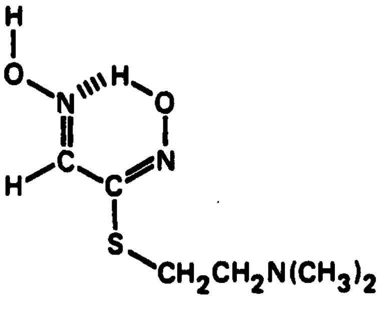
The NMR spectrum of 3 exhibited two hydroxyimino proton signals: a broad resonance centered at 12.4 ppm and a sharp peak at 11.84 ppm. The methine =C-H proton resonance for 3 was centered at 7.80 ppm. By comparison, the NMR of glyoxime in DMSO-d_6[20] shows hydroxyimino protons at 11.62 and methine protons at 7.83 ppm. These data suggest that one of the hydroxyimino protons in 3 is internally hydrogen bonded and that the other retains the same configuration as in glyoxime. A structure consistent with these data is the E, E-thiohydroximate:



E,E-Configuration for 3

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Control Experiments

The inhibition of AChE by EPMP and subsequent reactions of ethyl methylphosphonyl-AChE in the presence or absence of oximate reactivators are described by equations (7) through (15):

$$\mathrm{EOH + R_p{-}C_2H_5O(Me)P(O)OC_6H_4NO_2 \xrightarrow{k_7} EOP(O)(Me)OC_2H_5{-}Sp + HOC_6H_4NO_2} \qquad (7)$$

$$\mathrm{EOH + S_p{-}C_2H_5O(Me)P(O)C_6H_4NO_2 \xrightarrow{k_8} EOP(O)(Me)OC_2H_5{-}R_p + HOC_6H_4NO_2} \qquad (8)$$

$$\mathrm{C_2H_5O(Me)P(O)OC_6H_4NO_2 + OX + H^+ \xrightarrow{k_9} C_2H_5O(Me)P(O)OX + HOC_6H_4NO_2} \qquad (9)$$

$$\mathrm{EOP(O)(Me)OC_2H_5 + H_2O \xrightarrow{k_{10}} EOH + HOP(O)(Me)OC_2H_5} \qquad (10)$$

$$\mathrm{EOP(O)(Me)OC_2H_5\ (or\ EOH) + H_2O \xrightarrow{k_{11}} denatured\ enzyme} \qquad (11)$$

$$\mathrm{EOP(O)(Me)OC_2H_5 + H_2O \xrightarrow{k_{12}} EOP(O)(Me)OH + HOC_2H_5} \qquad (12)$$

$$\mathrm{EOP(O)(Me)OC_2H_5 + OX + H^+ \overset{K_r}{\rightleftharpoons} [EOP(O)(Me)OC_2H_5{\cdot}OX] \xrightarrow{k_r} EOH + C_2H_5O(Me)P(O)OX} \qquad (13)$$

$$\mathrm{C_2H_5O(Me)P(O)OX + EOH \xrightarrow{k_{14}} EOP(O)(Me)OC_2H_5 + OX + H^+} \qquad (14)$$

$$C_2H_5O(Me)P(O)OX + H_2O \xrightarrow{k_{15}} \text{products} \qquad (15)$$

$$HOX \underset{}{\overset{K_a}{\rightleftharpoons}} OX + H^+ \qquad (16)$$

Equations (7) and (8) show the reaction of enzyme (EOH) with EPMP to yield ethyl methylphosphonyl-AChE, $EOP(O)Me(OC_2H_5)$. The designations R_p- and S_p- in equations (7) and (8) refer to the stereochemical configuration about the chiral phosphorus atom in EPMP. By analogy with other chiral phosphonate esters[20-23], one isomer of EPMP (probably the R_p- enantiomer) should inhibit AChE more rapidly than the other isomer. For clarity, in reactions (9) through (15) we have omitted designations for the stereoisomers of EPMP and its derivatives.

Reaction (9) shows formation of phosphonyl oxime, $C_2H_5O(Me)P(O)OX$, by direct reaction of EPMP with the oximate form of a reactivator (OX).

Reactions (10) and (11) are unimolecular reactions of inhibited (or uninhibited) enzyme. Reaction (10) is the spontaneous reactivation process and reaction (11) includes any nonspecific reactions that lead to uncoverable loss of enzyme activity. Reaction (12) shows dealkylation of ethyl methylphosphonyl-AChE to a species, EOP(O)(Me)OH, that cannot be reactivated by oximates.

As shown in reaction (13), the reaction of oximate with inhibited enzyme proceeds via reversible formation of an oximate/inhibited enzyme complex, $[EOP(O)(Me)OC_2H_5 \bullet OX]$, followed by nucleophilic attack on phosphorus to yield active enzyme and phosphonyl oxime. Phosphonyl oxime, in turn, partitions between two pathways: reinhibition of AChE [reaction (14)] or hydrolytic decomposition to noninhibitory products [reaction (15)].

Finally, reaction (16) is the acid-base equilibrium between oximate and oxime (HOX).

The scheme given by reactions (7) through (16) demonstrates the complexity of the AChE-inhibitor-reactivator system. Reactivation kinetics can be accurately determined and meaningfully interpreted only when reaction (13) greatly predominates reactions (9) through (12), (14) and (15).

We originally chose to investigate EPMP as an inhibitor because ethyl methylphosphonyl-AChE reportedly[24] undergoes reactions (10) and (12) very slowly. We planned to circumvent problems associated with reaction (7) and the presence of unreacted inhibitor by adding EPMP to a slight excess of the enzyme. We recognized that reactions (8), (9), and (14) could introduce complicating factors but anticipated that reaction (13) would predominate under conditions of high reactivator concentration and low enzyme concentration. Under such conditions, reaction (13) is first-order in [AChE] and pseudo-zero-order in [OX]. Reaction (8) and (14), however, are second-order overall, i.e., first-order in [AChE] and also first order in [S_p-$C_2H_5O(Me)P(O)C_6H_4NO_2$] or in [$C_2H_5O(Me)P(O)OX$], respectively. Thus low enzyme concentrations should favor reaction (13).

To determine the accuracy of these expectations we first measured enzyme activities under different reaction conditions and at different time points. The values determined were:

A_o = uninhibited AChE activity

A_t = AChE activity at time t

A_{max}(t min) = maximal AChE activity observed (t = duration of experiment)

A_I = AChE activity after addition of EPMP

The first step was to determine the concentration of EPMP that would inhibit various levels of purified eel AChE to approximately 5% of original activity. For a solution of AChE at a nominal concentration of 65 ACh units-mL^{-1}, incubation with 0.40 µM of EPMP yielded maximal inhibition (12% of original activity) within 30 to 60 min. A rough estimate of the rate of the inhibition reaction gave $k_7 \approx 3 \times 10^5\ M^{-1}\ min^{-1}$ at 25°C.

We next examined spontaneous reactivation of inhibited enzyme, reaction (10), as a function of enzyme concentration at 37°C. We inhibited a stock solution of 62.5 ACh U.-mL^{-1} AChE to approximately 5% of original activity. The stock solution of inhibited enzyme was then diluted to three different concentrations (3.13, 0.625, and 0.125 ACh U.-mL^{-1} of total AChE, and incubated for time intervals. We withdrew aliquots from each incubation solution and diluted the aliquots into substrate solution for assay of activity, choosing the final aliquot volumes and dilution factors to give identical concentrations of total enzyme in the assay solution for all three incubation solutions. In this experiment we determined rate constants for spontaneous reactivation according to equation (17):

$$\ln(A_I) = k_{10} \cdot t \qquad (17)$$

The results in Table 2 show that both the rate of reaction (10) and the maximal activity observed after 1440 min incubation of inhibited enzyme varied inversely with the concentration of total enzyme in the incubation solution. This indicates that some EPMP remained in solution (probably the less reactive S_p- enantiomer) after the initial rapid inhibition of the stock enzyme solution and that at long reaction times additional enzyme inhibition [reaction (8)] competed with reaction (10).

To probe the effects of enzyme level on reactivation of ethyl methylphosphonyl-AChE by oximates, we repeated the above experiment, diluting the stock solution of inhibited enzyme to three concentrations into 0.010 mM <u>4</u>.

Table 2

SPONTANEOUS REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT THREE CONCENTRATIONS OF TOTAL ENZYME

Total [AChE][a] ACh-U.-mL^{-1}	A_o[b,c] µM-min^{-1}	A_I µM-min^{-1}	A_{max} (t = 1440 min) µM-min^{-1}	k_{10}[d] min^{-1} x 10^3
0.125	12.5	0.676	3.31	0.85 ± 0.1
0.625	12.5	0.875	2.53	0.45 ± 0.69
3.13	12.5	0.548	1.43	0.35 ± 0.21

---

[a] Total [AChE] in incubation solution.

[b] AChE activity in assay solution. Not equal to AChE activity in incubation solution.

[c] For definition of symbols, see text.

[d] Calculated according to Equation (17), k_{10} is the observed rate constant for spontaneous reactivation.

As before, we withdrew aliquots at timed intervals for assay, choosing aliquot volumes to give the same level of total enzyme in all three assay solutions.

Figure 1 shows the results of this experiment, and illustrates two important points. First, the rates of oximate-induced reactivation varied inversely with the level of enzyme in the incubation solution. Second, at the lowest AChE concentration investigated, 4 completely restored AChE activity to control (uninhibited) values.

The effect of AChE level on reactivation rates indicates that reinhibition of the enzyme by phosphonyl oxime [reaction (14)] was significant only at the two higher AChE concentrations. At the 0.125-ACh U.-mL^{-1} level of AChE, enzyme reactivation [reactions (13) and (15)] predominated over the reinhibition reaction, and for this reason we performed all subsequent reactions at the 0.125-ACh U.mL^{-1} level. The complete restoration of AChE activity by 4 also demonstrated that dealkylation [reaction (12)] was slow compared with reaction (13). We further proved the unimportance of reaction (12) in a separate experiment by incubating ethyl methylphosphonyl-AChE for timed intervals at 25°C before adding the inhibited enzyme to 1.0 mM 4 and assaying for activity after reactivating the enzyme. In this experiment, 4 reactivated AChE to 106% and 93% of uninhibited enzyme activity after pre-incubation periods, respectively of 6 and 28 h.

As a final control, we studied the oximate-induced reactivation of ethyl methylphosphonyl-AChE as a function of time and concentration of added 3. For comparison we also monitored spontaneous reactivation in the absence of added 3 and the loss of activity of uninhibited enzyme.

Figure 2 shows the results of this experiment and demonstrates that rates of uninhibited enzyme denaturation and spontaneous reactivation of inhibited AChE were slow compared with oximate-induced reactivation. As with 4, reactivation with high concentrations of 3 totally restored AChE activity to control values.

In summary, appropriate control experiments have demonstrated that under suitable reaction conditions the oximate-induced reactivation of ethyl methylphosphonyl-AChE proceeds to the virtual exclusion of complicating side reactions. The EPMP-inhibited eel AChE system appears to be ideally suited to determination of reactivation kinetics. Accordingly, we examined rates of reactivation of ethyl methylphosphonyl-AChE as a function of concentration of added 1, 2, 3, and 4. The following section describes the results of this investigation.

Reactivation Kinetics

Incubation of ethyl methylphosphonyl-AChE with a large excess of reactivator restores enzyme activity according to pseudo-first-order kinetics, equation (18):

$$\ln(100-R) = k_{obs} \cdot t \qquad (18)$$

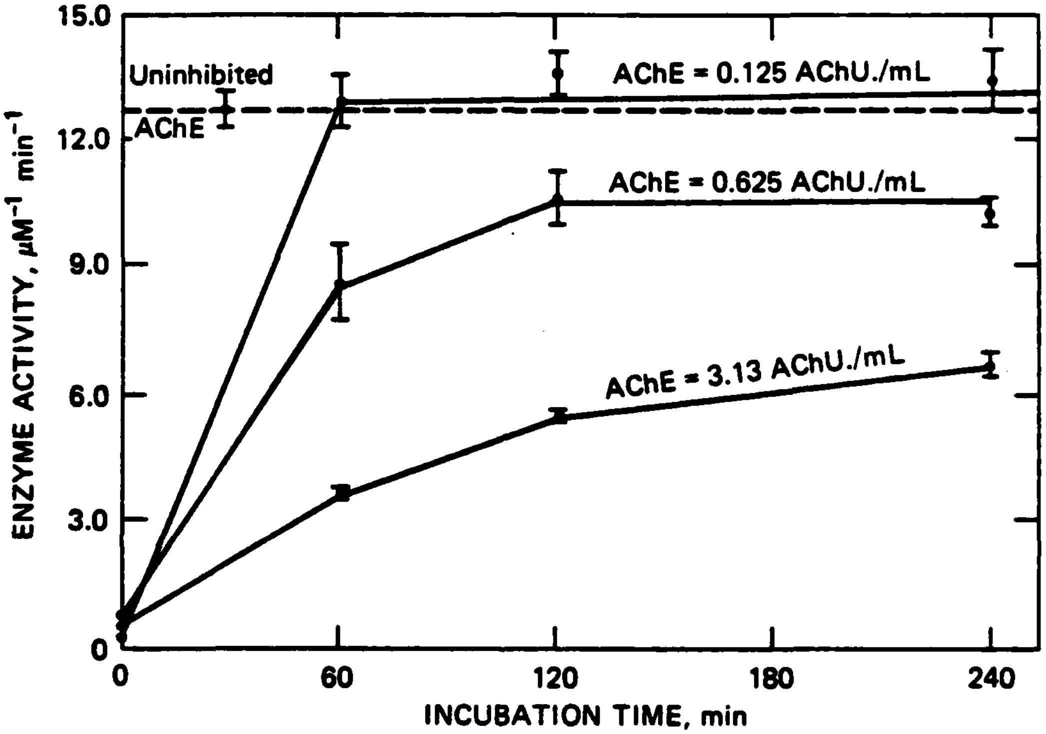

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FIGURE 1 REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY 0.010 mM 4 AT 37°C AS A FUNCTION OF TOTAL ENZYME CONCENTRATION IN THE INCUBATION SOLUTION

(Error bars indicate means ± S.E. for n = 4 determinations.)

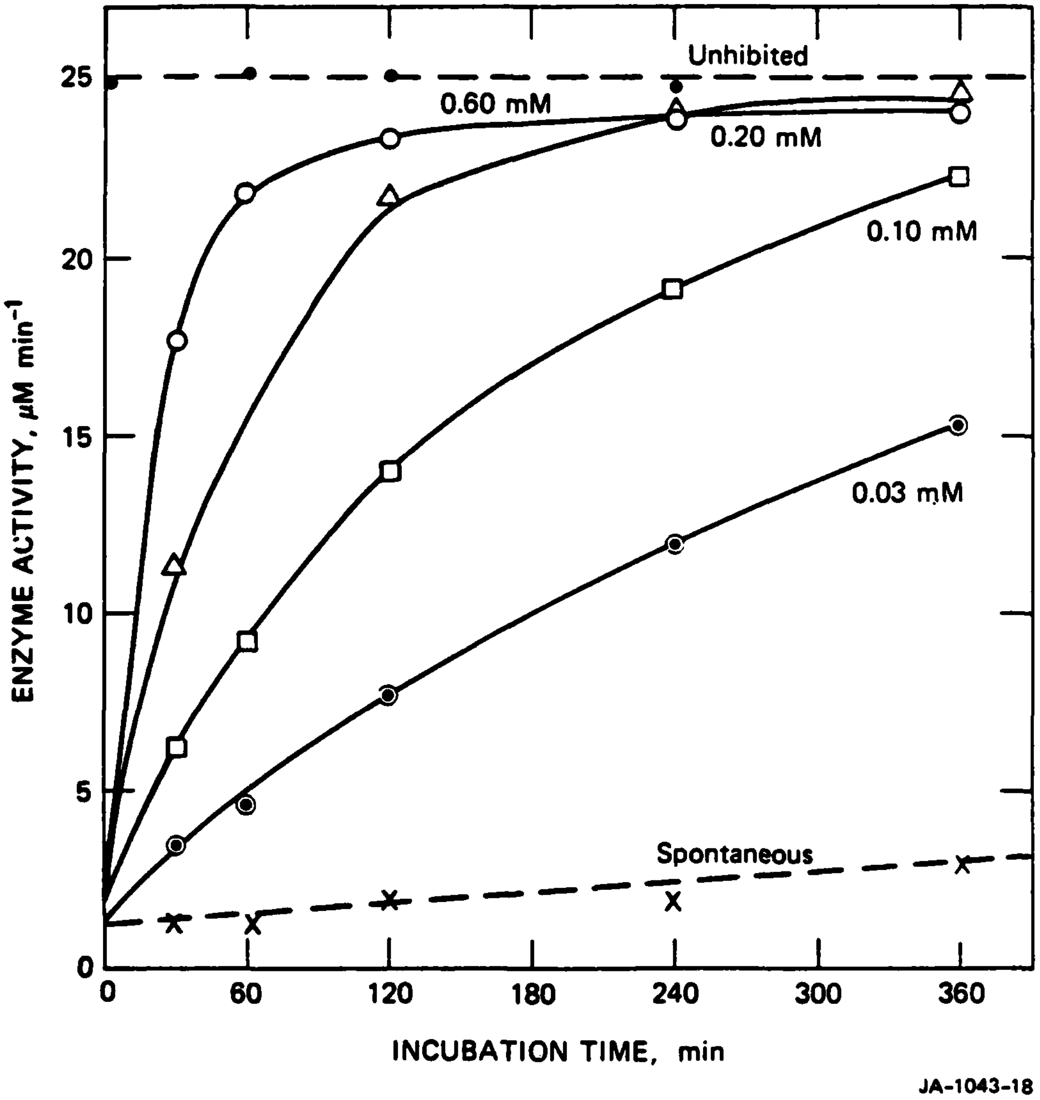

FIGURE 2 REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS CONCENTRATIONS OF 3: 0.030 mM 3,
◉——◉ ; 0.10 mM 3, □——□ ; 0.20 mM 3.
△——△ ; 0.60 mM 3, ○——○ ; NO ADDED 3,
×– – –× ; UNINHIBITED AChE, ●– – – –● .

where:

k_{obs} = observed first-order rate constant for reactivation

R = percent reactivation
= $100(A_t - A_I)/(A_c - A_I)$

A_c = control AChE activity
= A_o or 1/2 $A_o + A_{max}$ [for <u>4</u>] ,

and A_t and A_I are as defined in the preceding section.

We determined reactivation kinetics for <u>4</u> and for the compounds listed in Table 1. The exact procedure is described in the experimental details section. Briefly, we incubated EPMP with a slight excess of AChE for 20 min to produce ethyl methylphosphonyl-AChE. The inhibited enzyme was then diluted into solutions containing various concentrations of reactivators, and aliquots were withdrawn at timed intervals for assay of activity. In each experiment involving a nonquaternary reactivator, we included at least one concentration of <u>4</u> to serve as a standard. In experiments where A_{max} for reactivation by <u>4</u> was nearly identical to A_o, we defined control activity, A_c, as the mean value of A_o and A_{max}. In cases where reactivation by <u>4</u> did not go to completion within the duration of the experiment we set A_c equal to A_o. Spontaneous denaturation of AChE was negligible in all cases studied. In calculating values for R, we corrected for any contribution to observed activity due to spontaneous reactivation. To do this, we directly determined A_I values at each time point for the experiment and used these values to calculate R.

Table 3 summarizes k_{obs} values and other pertinent data for the kinetic experiments. For most runs, the reaction kinetics adhered well to equation (18); semi-logarithmic plots of (100 - R) versus time were typically linear to 90% reactivation for experiments that were followed to high conversion. Figure 3 shows typical data plotted according to equation (18) for several of the test compounds. For clarity, not all of the kinetic runs are plotted in Figure 3. In most runs spontaneous reactivation was negligible compared with oximate-induced reactivation. For some relatively poor reactivators at low concentrations, spontaneous and induced reactivation proceeded at comparable rates, but the contribution due to spontaneous hydrolysis could be accurately subtracted.

To relate observed reactivation kinetics to the mechanism for reactivation shown in reaction (13) we treated the data of Table 3 according to equation (19):

$$(k_{obs})^{-1} = \frac{K_r}{k_r}[OX]^{-1} + \frac{1}{k_r} \qquad (19)$$

Table 3

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) AND RELATED DATA[a] FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS

Experiment	Test Compound[b]	[HOX],[c] nM	A_o, μM min^{-1}	A_I, μM min^{-1}	$A_{max}(t)$[e] μM min^{-1}	A_c, $min^{-1} \times 10^3$	k_{10},[f] $min^{-1} \times 10^3$	k_{11},[g] $min^{-1} \times 10^3$	k_{obs},[h] $min^{-1} \times 10^3$
1	1a	0	9.19	1.99	--	9.19	0.34	0.064	--
		0.0300			4.31(20)				1.63 ± 0.039
		0.100			8.68				7.04 ± 0.081
		0.300			10.7				17.8 ± 0.81
		1.00			9.34				21.6 ± 0.41
	4	0.00150			6.16				3.58 ± 0.41
2	1a	0	11.9	0.397	--	11.7	1.2	< 0.05	--
		0.0600			6.63(300				2.48 ± 0.11
		0.200			8.97				4.43 ± 0.29
		0.500			11.0				11.8 ± 1.0
	4	0.0100			11.5				18.2 ± 1.8
3	1b	0	12.3	1.94	--	12.3	0.39	0.11	--
		0.0300			1.44(300)				0.398 ± 0.026
		0.100			3.25				1.22 ± 0.046
		0.300			5.84				1.22 ± 0.18
		1.00			7.00				4.15 ± 0.45
	4	0.00150			6.75				3.43 ± 0.40
4	1c	0	10.5	0.243	--	10.7	2.2	< 0.02	--
		0.0300			2.13(360)				0.507 ± 0.024
		0.100			1.69				0.380 ± 0.0082
		0.300			4.04				1.23 ± 0.037
		1.00			5.66				2.07 ± 0.045
	4	0.0100			10.8				23.7 ± 4.3
5	1d	0	9.27	0.191	--	9.27	1.8	--	--
		0.00300			0.323(300)				0.0680 ± 0.0086
		0.0100			0.640				0.223 ± 0.018
		0.100			4.83				2.21 ± 0.018
6	2	0	12.4	0.265	--	12.4	2.9	0.16	--
		0.500			1.78(300)				0.362 ± 0.028
		1.00			2.43				0.581 ± 0.044
		2.00			2.74				0.664 ± 0.035
		3.00[i]			1.51				0.728 ± 0.082
	4	0.00150			7.71				2.98 ± 0.20

Table 3 (concluded)

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) AND RELATED DATA[a] FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS

Experiment	Test Compound[b]	[HOX],[c] mM	A_o, μM min^{-1}	A_I, μM min^{-1}	$A_{max}(t)$[e] μM min^{-1}	A_c, min^{-1} x 10^3	k_{10},[f] min^{-1} x 10^3	k_{11},[g] min^{-1} x 10^3	k_{obs},[h] min^{-1} x 10^3
7[j]	3	0	25.3	1.21	--	25.0	2.3	0.046	--
		0.0300			15.4(360)				2.26 ± 0.0345
		0.100			22.2				5.60 ± 0.12
		0.200			24.5				15.9 ± 0.41
		0.600			23.4				33.4 ± 3.3
	4	0.0100			24.7				18.3 ± 4.4
8	4	0	11.5	0.195	--	9.56	0.77	--	--
		0.00050			3.67(360)				1.36 ± 0.14
		0.00150			6.60				3.06 ± 0.25
		0.00400			8.53				9.15 ± 0.91
		0.0100			8.68				23.7 ± 3.4

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[a] k_{obs} = observed first-order rate constants for reactivation
= percent reaction
= $100(A_t - A_I)/A_c - A_I)$
A_c = control AChE activity
= A_o or 1/2 {A_o + A_{max}[for 4]}.

[b] See Table 1 for structures.

[c] [HOX] = concentration of added test compound.

[d] Reported value of A_I calculated at t = 0 from the intercept of least-squares linear regression of A_I data according to equation (17).

[e] All A_{max} values determined at time, t, in minutes, given parenthetically for first A_{max} value in an individual experiment.

[f] Calculated according to equation (17).

[g] Calculated according to the expression $\ln(A_o) = k_{11} \cdot t$.

[h] Calculated acording to equation (18).

[i] Control experiment showed that 2 at 3 mM inhibits AChE to 92% control activity. Corrected A_t values for 3 mM 2 were calculated.

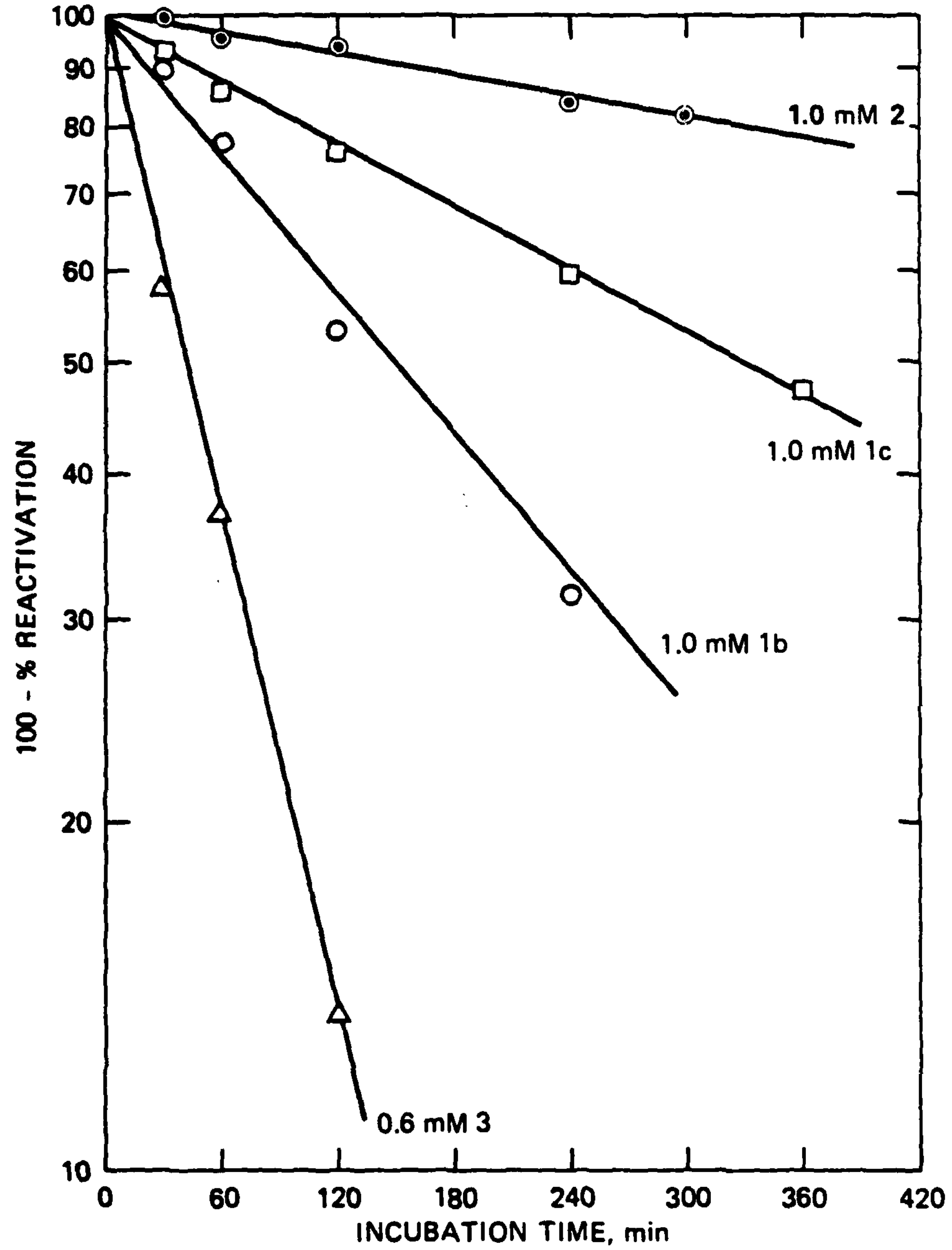

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FIGURE 3 SEMILOGARITHMIC PLOT OF (100-% REACTIVATION) VERSUS INCUBATION TIME FOR REACTION OF ETHYL METHYLPHOSPHONYL-AChE WITH COMPOUND: 1b, ○——○; 1c, □——□; 2, ⊙——⊙; AND 3, △——△

where [OX] is the concentration of oximate ion at pH 7.6, calculated from the concentration of added test compound, [HOX] as in equation (20)

$$[OX] = [HOX]\ \{1 + \text{antilog}[pK_a - 7.6]\}^{-1} \tag{20}$$

The derivation of equation (19) is straightforward and has been described elsewhere.[25-28] We used the concentration of oximate (as opposed to [HOX]) in equation (20) because the protonated form of the oxime is essentially unreactive as a nucleophile or as a reactivator.

According to equation (19), a plot of $(k_{obs})^{-1}$ versus $[OX]^{-1}$ is linear with slope = K_r/k_r and intercept = $1/k_r$. Such plots therefore permit the calculation of the individual rate constants for reactivation, k_r and K_r. It can also be shown,[27-28] that in the limit of low reactivator concentration ($[OX] << K_r$) the ratio of k_r to K_r is equivalent to an apparent bimolecular rate constant for reactivation, i.e.:

$$k_r/K_r = k_b \tag{21}$$

The bimolecular reactivation rate constant, k_b, is a measure of the <u>inherent</u> activity of an oximate as a reactivator of the inhibited enzyme. Because various oximes and thiohydroximates ionize to different extents at pH 7.6, we define an effective rate constant for reactivation of inhibited AChE as the product of k_b and the fraction of added test compound present as oximate at pH 7.6, i.e.,

$$k_{eff} = k_b[1 + \text{antilog}(pK_a - 7.6)]^{-1} \tag{22}$$

Table 4 summarizes values for reactivation rate constants calculated from the data of Table 3 using equations (19) through (22). Figure 4 is a double-reciprocal plot of the data according to equation (19) for <u>1a</u> through <u>1c</u>, <u>2</u>, and <u>3</u>. Figure 4 does not show data for <u>1d</u> and <u>4</u> because of the differences in scale. In examining Table 4, it may be helpful to recall that K_r, by definition, equals $[OX][EOP(O)(Me)OC_2H_5]/[EOP(O)(Me)OC_2H_5 \bullet OX]$ and that a low value for K_r reflects a high proportion of oximate present as the oximate/inhibited enzyme complex.

Table 4 and Figure 4 demonstrate that the kinetic data conformed well to equation (19). Least-squares linear regression of the data for experiments 1 through 8 gave slopes with reasonably small relative uncertainty. Relative uncertainties were larger in calculations of intercept values, but least-squares correlation coefficients indicated a good fit of the data to equation (19).

Table 4

RATE CONSTANTS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS AT 25°C, AT pH 7.6

Cmpd[a]	[HOX] mM	$[OX]^{-1}$[b] $M^{-1} \times 10^{-3}$	$(k_{obs})^{-1}$ min	Slope[c] M-min x 10^2	Intercept[c] min	Correlation[c] Coefficient	k_r[d] $min^{-1} \times 10^3$	K_r[d] $M \times 10^4$	k_b[e] M^{-1} min^1	k_{eff}[f] M^{-1} min^1
1a	0.0300	56.4	613.0	1.05 ± 0.14	41.5 ± 35	0.958	24.1	2.53	95.3	56.3
	0.0600	28.2	403.0							
	0.100	16.9	142.0							
	0.200	8.46	226.0							
	0.300	5.64	56.2							
	5.00	3.38	84.7							
	10.0	1.69	30.3							
1b	0.0300	88.5	2060							
	0.100	26.6	763	2.68 ± 0.052	133 ± 24	0.999	7.52	2.02	37.2	14.0
	0.300	8.85	362							
	1.00	2.66	212							
1c	0.100	76.1	2630	3.23 ± 0.36	133 ± 170	0.994	7.52	2.43	30.9	4.06
	0.300	25.4	813							
	1.00	7.61	483							
1d	0.00300	715	14700	2.05 ± 0.0082	34.4 ± 35.4	0.999	29.1	5.96	48.8	22.8
	0.0100	215	4480							
	0.100	21.5	452							
2	0.500	14.6	2760							
	1.00	7.31	1720	11.4 ± 1.3	1050 ± 113	0.986	0.952	1.09	8.77	1.26
	2.00	3.65	1510							
	3.00	2.44	1370							

Table 4 (concluded)

RATE CONSTANTS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS AT 25°C, AT pH 7.6

Cmpd[a]	[HOX] mM	$[OX]^{-1}$[b] $M^{-1} \times 10^{-3}$	$(k_{obs})^{-1}$ min	Slope[c] M-min x 10^2	Intercept[c] min	Correlation[c] Coefficient	k_r[d] $min^{-1} \times 10^3$	K_r[d] $M \times 10^4$	k_b[e] $M^{-1}\ min^1$	k_{eff}[f] $M^{-1}\ min^1$
3	0.0300	230	442	0.188 ± 0.0162	16.4 ± 19.7	0.998	61.2	1.15	534	77.4
	0.100	68.9	179							
	0.200	34.4	62.9							
	0.600	11.5	29.9							
4	0.000500	6910.0	735	0.0104 ± 0.00064	27.8 ± 24	0.996	36.0	0.0374	9630	2780
	0.00150	2300.0	307[g]							
	0.00400	864.0	109							
	0.0100	345.0	43.5[h]							

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[a] See Table 1 for structures and pK_a data.
[b] Calculated from equation (20).
[c] From linear least-squares linear regression of data according to equation (19).
[d] From equation (19).
[e] From equation (21).
[f] From equation (22).

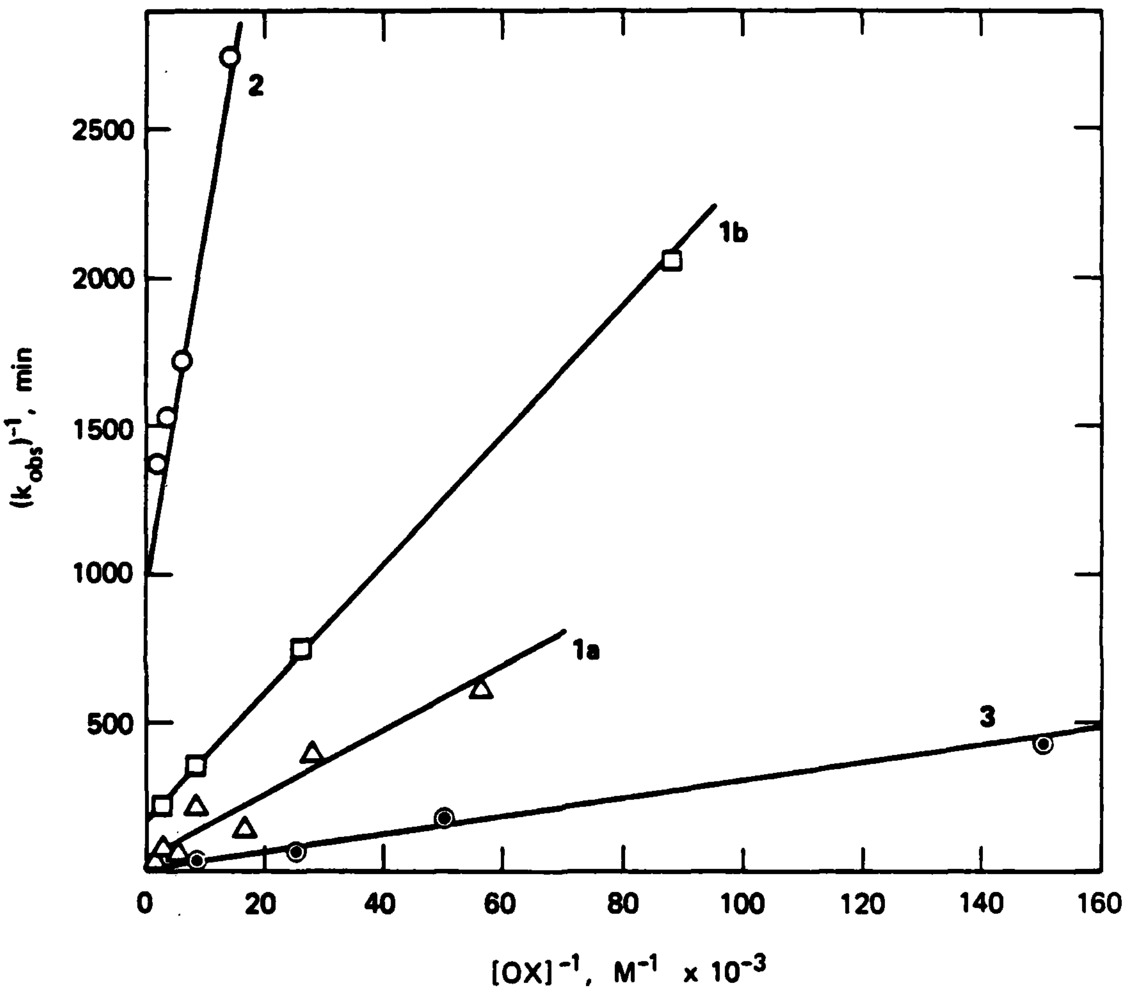

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FIGURE 4 RECIPROCAL OBSERVED RATE CONSTANT FOR REACTIVATION, $(k_{obs})^{-1}$, VERSUS RECIPROCAL OXIMATE CONCENTRATION FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY: 1a,△——△; 1b,□——□; 2,○——○; AND 3,◉——◉

The precision of the kinetic data demonstrates the general utility of ethyl methylphosphonyl-AChE as an *in vitro* model for evaluating AChE reactivators. Other inhibitors have been investigated as *in vitro* models for reactivation but most of the literature models suffer important disadvantages. Examples of phosphylated AChE's and complicating factors are 3,3-dimethyl-2-butyl methylphosphonyl-AChE (rapid dealkylation[28,29], incomplete reactivation[30]), cyclopentyl methylphosphonyl-AChE (rapid dealkylation,[31] reinhibition by phosphonyl-oxime[32]), diisopropyl phosphoryl-AChE (rapid dealkylation[20]), ethyl dimethylphosphoramido-AChE (dealkylation[33], low rates of reactivation[34]) and diethyl phosphoryl-AChE (reinhibition[35,36]).

Similarly, for any *in vitro* model system, different approaches to determining reactivation kinetics may or may not provide meaningful data. For example, determining percent activity restored to inhibited AChE after incubation with one concentration of reactivator for a single time period is convenient for preliminary screening of novel reactivators,[37,38] but cannot be used to calculate reactivation rates. Determination of observed pseudo-first-order rate constants for reactivation at a single concentration of reactivator is sometimes used to calculate bimolecular rate constants, assuming a simple second-order kinetic expression for the reactivation reaction.[39-41] However this assumption is clearly invalid in view of the well-established[25-28] mechanism for reactivation shown in reaction (13). It is also invalid to calculate rate constants for reactivation according to equation (19) if reinhibition of the enzyme by phosphonyl oxime is demonstrably important under the experimental conditions used.[42]

In summary there is a definite need for a well-characterized *in vitro* model system for determining kinetics of reactivation of phosphylated-AChE. Of the many systems described in the literature few, if any, are wholly satisfactory. By contrast, ethyl methylphosphonyl-AChE proves to be exceptionally useful for kinetic determinations. Under the conditions that we have chosen, reactivation of ethyl methylphosphonyl-AChE by oximates proceeds without substantial interference from complicating side reactions. The system conforms well to a simple rate expression and accurate reactivation kinetics can be conveniently determined. The inhibitor (EPMP), enzyme (eel AChE), and standard reactivator (4) reported herein are all obtainable conveniently and in high purity and this should facilitate comparison of data obtained in different laboratories using the single model system.

Structure-Activity Relationships

Having developed a useful *in vitro* model, we wished to examine structure-activity relationships for the thiohydroximates. The goal was to relate specific chemical and structural properties of the nonquaternary reactivators to the kinetic constants k_r, K_r, and k_b. We anticipated, for example, that k_r values might correlate with the inherent nucleophilicity (and hence to the basicity)[18] of the oximates and that K_r values would vary with structural changes (such as replacing the diethylamino moiety in 1a with a methyl group, as found in 2).

Surprisingly, however, the individual values of k_r and K_r did not vary in a systematic fashion with oximate structure or inherent reactivity. Compounds 2 and 3 for example exhibited identical pK_a values and the two oximates should feature similar Bronsted nucleophilicities. However, compounds 2 and 3 showed, respectively, the lowest and highest k_r values of all the reactivators tested.

Similarly we expected 2 to exhibit a distinctly low affinity for inhibited enzyme (and hence an unusually high value for K_r) because of all the reactivators reported herein only 2 does not feature a protonated or quaternary amine functionality to provide coulombic interactions with the enzyme anionic region. We found, however, the K_r value for 2 to be identical (within a factor of two) with K_r values for 1 and 3.

Thus we conclude that k_r and K_r values cannot be considered independently in elucidating structure-activity relationships for AChE reactivators, that k_r and K_r are coupled, and that only k_b values genuinely reflect the reactivity of oximate reactivators.

To date, the literature has not adequately addressed the interdependence of k_r and K_r values and the mechanistic implications of our findings deserve further comment. In this regard, it is important to recall that K_r is not truly a direct measure of the fraction of total oximate bound to the anionic region of the inhibited enzyme. Rather, K_r derives from observed rate constants for restoration of enzymatic activity, and the value of K_r therefore only reflects the fraction of bound oximate that contributes to reactivation.

Any molecule (including any oximate/inhibited enzyme complex) exists in a finite number of low energy conformations that are accessible at ambient temperature. For a particular oximate/inhibited enzyme complex only those conformations that feature the $= NO^-$ moiety suitably positioned to approach phosphylated serine will result in reactivation. Also, the inherent nucleophilicity of the oximate will determine the rate at which nucleophilic attack on phosphylated serine proceeds for any conformer of reactivator/inhibited enzyme complex. Thus k_r and K_r values depend not only on oximate reactivity but also on geometry of the oximate/inhibited enzyme complex. This interdependence between oximate reactivity and structure necessarily couples k_r and K_r values and precludes interpretation of individual values of these constants.

Differences in oximate/inhibited enzyme complex conformations may also explain the generally lower activity of the nonquaternary reactivators compared with 4. Table 4 shows that K_r for 4 is lower by a factor of approximately 10^2 than K_r values for 1, 2, and 3. In view of the foregoing discussion we do not believe that the low K_r value for 4 indicates a particularly high affinity of the pyridinium oxime for the inhibited enzyme. Rather, we consider that, as a first approximation, 1, 3 and 4 form oximate/inhibited enzyme complexes equally well. Two observations support this view. First, we observed previously[18] that 1b and its methyl quaternary analog, $MeOC_6H_4C(O)C(NOH)CH_2CH_2N(C_2H_5)\cdot CH_3I$, do not differ greatly in reactivity toward phosphylated AChE. Second, Table 4 demonstrates that 2, which is devoid of an amine functionality, is markedly inferior to 1 and 3 as a reactivator. Thus the protonated dialkylaminoalkyl moiety common to 1 and 3 does provide electrostatic binding of oximate to the inhibited enzyme. Because simple coulombic interactions between the AChE aspartate and the (quaternary or protonated) ammonium functionalities of 1, 3, and 4 should be roughly equivalent, it seems unreasonable to attribute the comparatively high reactivity of 4 to enhanced affinity toward inhibited AChE. Rather, it is likely that the nonquaternary reactivators differ from 4 primarily with respect to conformational degrees of freedom. Because 1 and 3 are rather flexible, we suggest that the nonquaternary reactivators combine with phosphylated AChE to yield an oximate/inhibited enzyme complex featuring many low-energy conformations, only a small fraction of which have the oximate moiety positioned close to phosphylated serine. In the case of the more rigid molecule, 4, a higher proportion of oximate/inhibited enzyme complex conformations have geometries that favor nucleophilic attack on phosphylated serine.

Thus in terms of free energy of activation for reactivation of phosphylated-AChE, we propose that the nonquaternary reactivators with dialkylaminoalkyl groups and 4 do not exhibit appreciably different enthalpies of activation; the binding affinities are approximately equal in both cases. Rather, the difference in reactivity apparently relate to entropic factors, i.e., to degrees of freedom for the oximate/inhibited enzyme complexes.

CONCLUSIONS

The search for AChE reactivators for treating organophosphorus ester poisoning has continued unabated for the last 25 years. Despite considerable research in this field surprisingly few investigators have examined details of the *in vitro* activity of candidate drugs using a well-characterized model system. We believe that accurate determination of reactivation kinetics for structurally related compounds is critical to the rational development of improved therapeutics. Of the many enzyme/inhibitor combinations reported in the literature, we believe the ethyl *p*-nitrophenyl methylphosphonate/eel AChE model system described herein to be the only combination that has been demonstrated to be substantially free of complicating side reactions (such as dealkylation, reinhibition, and spontaneous reactivation) that otherwise interfere with measurement of reactivation kinetics. EPMP is easily synthesized and, though quite toxic, can be safely and conveniently used to prepare inhibited AChE samples for kinetic experiments. We strongly recommend that the ethyl methylphosphonylated eel AChE be considered as a model system for characterization of any experimental AChE reactivators.

The thiohydroximates reported herein proved to be modest AChE reactivators compared with 2-hydroxyiminomethyl-1-methylpyridinium iodide: the thiohydroximates are less reactive towards ethyl methylphosphonyl-AChE by factors of 10^1 to 10^3. An examination of structure-activity relationships for the nonquaternary reactivators suggests that entropic factors (as opposed to low affinity for inhibited AChE) limit the activity of the thiohydroximates. We have argued that the thiohydroximates bind strongly to the anionic region of inhibited AchE but that only a relatively small fraction of the low-energy conformations of the reactivator/inhibited enzyme complex feature an oximate/phosphonyl serine geometry suitable for reactivation of the enzyme. Thus we include rigidity with other important factors (such as nucleophilicity, geometry, and cationic functionality) as a requirement for oximate activity in reactivating inhibited AChE.

EXPERIMENTAL DETAILS

The materials and methods were generally as previously described.[18] In the following we provide information on new compounds and procedures.

Diethyl methylphosphonate (Specially Organics, Inc.), p-bromobenzophenone (Aldrich Chemical Co.), and glyoxime (Aldrich) were used as supplied by the manufacturer. Ethyl p-nitrophenyl methylphosphonate (EPMP) was prepared by reaction of p-nitrophenol with diethyl methylphosphonate and purified by vacuum distillation.[43] 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.

2-(Diethylamino)ethyl p-bromobenzoylthiohydroximate hydrochloride (1a) was prepared per our previously described method[18] by conversion of p-bromoacetophenone to α-chloro-α-hydroxyimino-p-bromoacetophenone followed by esterification with 2-(diethylamino)ethanethiol and treatment with HCl. Yield, 29%; m.p. 161-165°C; TLC, R_f = 0.58 ($CHCl_3$:MeOH, 6:1). NMR (DMSO-d_6), δ 13.00 (br. s, 1H, =NOH), 10.64 (br, 1H, NH^+), 7.80 (m, 4H, Ar), 3.4-2.9 (m, 8H, CH_2), 1.18 (t, 6H, CH_3). IR(nujol), ν, 2640(s), 1660(s), 1600(s), 985(S) cm^{-1}. Anal. C,H,N,Cl.

3-(Dimethylamino)propyl p-bromobenzoylthiohydroximate (1d) was prepared as described for 1a, substituting 3-(dimethylamino)propanethiol in the esterification step. Yield, 32%; m.p. 104-106°C; TLC, R_f = 0.45 ($CHCl_3$:MeOH, 6:1). The NMR of 1d in DMSO-d_6 showed no separate resonances for hydroxyimino or oxalic acid protons. However D_2O exchange removed three proton signals occurring near the aromatic protons at 7.7 ppm. To show the presence of the =NOH proton in 1d, the NMR of the free base of 1d was also determined in $CDCl_3$. NMR data in both solvents follow. DMSO-d_6, δ 7.80 (m, 7H, Ar, =NOH, oxalate), 2.98 (m, 4H, $2CH_2$), 2.65 (s, 6H, $2CH_3$), 1.7 to 2.0 (m, 2H, CH_2). $CDCl_3$, δ 11.40 (s, br, 1H, =NOH), 7.73 (q, 4H, Ar), 2.93 (m, 2H, CH_2), 2.52 (m, 2H, CH_2), 2.18 (s, 6H, CH_3), 1.6 to 1.9 (m, 2H, CH_2). Anal. C,H,N.

n-Propyl p-bromobenzoylthiohydroximate (2) was prepared by esterification of α-chloro-α-hydroxyimino-p-bromoacetophenone with propanethiol after purification by flash and thick layer chromatography in silica gel using $CHCl_3$:MeOH (20:1). Yield 15%; m.p. 67-69°C; TLC, R_f = 0.66 ($CHCl_3$:MeOH, 20:1). NMR (DMSO-d_6) δ 12.60 (s, 1H, =NOH), 7.81 (m, 4H, Ar), 2.83 (t, 2H, CH_2), 1.47 (m, 2H, CH_2), 0.86 (t, 3H, CH_3); IR ($CHCl_3$) ν,3400 (br, m), 1690, (m), 1590, (m), 1220, (s), cm^{-1}. Anal. C,H,N,Cl.

2-(Dimethylamino)ethyl thioglyoximate hydrochloride (3) was prepared from monochloroglyoxime.[44] To a solution of crude monochloroglyoxime (2.5 g, ~ 2 x 10^{-2} mol) and triethylamine (4.0 g, 4.0 x 10^{-2} mol) in 75 mL of $CHCl_3$ was added 2-(dimethylamino)ethanethiol hydrochloride (2.8 g, 2.0 x 10^{-3} mol). A slight exotherm was noted and the mixture was stirred 2 hours at ambient temperature. The reaction mixture was then concentrated and triturated with two 50 mL portions of diethyl ether. The ether triturations were combined, dried over anhydrous $MgSO_4$, and concentrated to yield 2.0 g of oil, containing oxime as indicated by IR. The residue from the trituration was dissolved in water and extracted repeatedly with CH_2Cl_2. No oxime was evident (IR) in the CH_2Cl_2 extract. The ether triturations were column chromatographed (20% CH_3OH:80% acetone), and oxime-containing fractions were combined and concentrated, affording an oil insoluble in $CHCl_3$ and acetone but soluble in CH_3OH and dimethyl sulfoxide (DMSO). The oil was dissolved in 10 mL DMSO, and 100 mL $CHCl_3$ was added. The solution was cooled to -20°C and scratched to yield 0.150 g (10%) of a white crystalline precipitate. The solid material exhibited a broad melting point range (55-134°C). This suggests that the compound readily loses water on heating in the melting point apparatus. Consistent with this, the field ionization mass spectrum of 1c shows no molecular ion, but a parent peak at mass 141 corresponding to 4-(2-dimethylamino)ethyl-1,2,5-oxadiazole, the product resulting from elimination of HCl and H_2O from 1c. NMR (DMSO-d_6), δ 12.47 (br, s, 1H, =NOH); 11.84 (s, 1H, CHNOH) 7.80 (s, 1H, CHNOH); 3.33 (m, 4H, CH_2) and 2.73 ppm (s, 6H, CH_3). IR (KBr), 3300 (s), 2940 (s), 2700 (s), 1460 (s), 1390 (s) and 955 (s) cm^{-1}. Analysis for $C_6H_{14}N_3O_2SCl$. Calc'd: C, 31.65; H, 6.20; N, 18.45; S, 14.08; Cl, 15.56. Found: C, 31.64; H, 6.23; N, 18.44; S, 14.08; Cl, 15.56.

Acetylcholinesterase determinations in vitro. Unless otherwise noted, all experiments were conducted at 25.0 ± 0.1°C in pH 7.6 0.1 M morpholinopropanesulfonic acid (MOPS) buffer plus NaN_3 (0.002%), $MgCl_2$ (0.01 M) and bovine serum albuin (0.1%). Enzyme activities were assayed by the Ellman method[45] on a Gilford-modified DU Spectrophotometer coupled to a Digital Electronics Corp. MINC-11 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. The inhibited enzyme was incubated with reactivators for timed 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. Control experiments demonstrated that inhibition of AChE by reactivators in the assay solutions and decomposition of reactivators were negligible under the reaction conditions. Observed activities were corrected to net activities by subtracting rates of spontaneous and reactivator-induced substrate hydrolysis.

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

To inhibit the AChE, dilute 140 µL stock solution in 132 µL MOPS plus 8 µL of EPMP (1 x 10^{-5} M in C_2H_5OH). To determine activity of the inhibited AChE, incubate 20 min, withdraw 10 µL, and 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 (including zero) concentrations. Remove 50 µL for assay.

For assay of AChE activity, add 50 µL aliquots of solution to be assayed to 910 µL pH 8.0, 0.1 M phosphate buffer plus 30 µL of 0.10 M bis-dithionitrobenzoic acid, plus 10 µL of 0.075 M acetylthiocholine, and monitor increased absorbance at 412 nm versus time.

ACKNOWLEDGEMENT

This work was supported by the US Army Medical Research and Development Command contract DAMD17-79-C-9178. The authors gratefully acknowledge Dr. Brennie Hackley of the US Army Medical Research Institute for Chemical Defense and Dr. Edward Acton of SRI International for many helpful suggestions. We also thank Dorris Taylor of SRI for pK_a determinations, and Dr. Ronald Fleming for developing the MINC program for enzyme kinetics.

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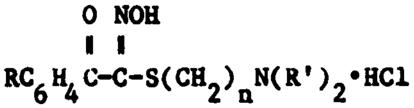
Part II. Heteroaromatic Oximes and Thiohydroximates as Reactivators of Ethyl Methylphosphonyl-AChE In Vitro

INTRODUCTION

Organophosphorus compounds are widely used in agriculture, and cases of accidental or deliberate poisoning by these chemicals are commonplace.[1-6] Certain highly toxic organophosphorus esters have also been developed for use in war.[7-10] In the event of armed conflict involving toxic chemicals, mass casualties among civilians are almost certain.[9] Thus development of effective therapy for organophosphorus ester intoxication remains an important area for research.

Typically, toxic organophosphorus esters are irreversible inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) AChE.[11-13] The inhibition proceeds via phosphylation[14] of a serine hydroxyl at the enzyme active site. Quaternary oximes, such as 2-hydroxyiminomethyl-1-methylpyridinium halide (2PAM), find clinical application as therapeutics against organophosphorus ester intoxication.[15-18] The pyridinium oximes (or more precisely, the conjugate base oximate anions) are nucleophiles that attack phosphylated AChE to liberate active enzyme. Although these "reactivators" of inhibited enzyme constitute effective therapy, the pyridinium oximes as a class suffer from the disadvantage of limited tissue distribution. As charged species the pyridinium oximes penetrate poorly into hydrophobic cell membranes. Penetration of pyridinium oximes into the central nervous system is particularly poor and conventional therapy does little to counteract central effects of organophosphorus inhibitors.[19-21]

In an attempt to improve upon the limited activity of the pyridinium oximes we have undertaken an investigation of nonquaternary cholinesterase reactivators. Previously [22,23] we prepared a series of α-ketothiohydroximates (1)

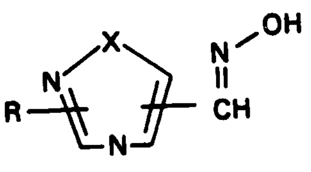


$$RC_6H_4\overset{O}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-S(CH_2)_nN(R')_2\cdot HCl$$

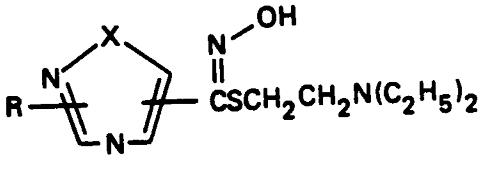
1

and evaluated them in vitro as reactivators of diisopropyl- and ethyl methylphosphonyl-AChE. Although the α-ketothiohydroximates do reactivate phosphylated-AChE, they are inferior in this regard to 2PAM. We attributed the lower reactivity of type 1 compounds, at least in part, to flexibility of the dialkylaminoalkyl side chain. We rationalized that the α-ketothiohydroximates bind reversibly to the inhibited enzyme and that the reactivator/inhibited enzyme complexes feature many configurations, only a small fraction of which have the oxime suitably positioned to react with phosphylated serine at the active site.

In a continuation of our study of nonquaternary cholinesterase reactivators, we have prepared two new series of novel compounds. These are the heteroaromatic aldoximes, 2, and the 2-(diethylamino)ethyl α-heteroaromatic thiohydroximates, 3:



2



3

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where x = O (for oxadiazoles), S (for thiadiazoles), or C_6H_5N (for 1-phenyl substituted triazoles); one ring nitrogen is in the 2-position and the second ring nitrogen is in the 3, 4, or 5 position; R = H, CH_3 or C_6H_5 in the 3, 4 or 5 substituent positions; and the hydroxyiminomethyl or diethylaminoethyl thiohydroximate functionalities are in the 4 or 5 positions.

We predicted that certain heteroaromatic systems would be sufficiently electronegative to lower the pKa's for the oxime/oximate acid base equilibria to approximately pKa = 8 (the empirically known optimal value for AChE reactivators).[12] We further considered that the heterocyclic rings would be approximately isosteric with the N-methyl pyridinium ring of 2PAM and that the compounds conforming to the general structure 2 would be appropriate nonquaternary analogs of the pyridinium aldoximes. However, we also anticipated that many examples of 2 would exhibit oxime acidities in the range pKa = 9 to 10 and that none of the heteroaromatic aldoximes would feature a cationic moiety (such as a quaternary pyridinium or protonated trialkyl amine functionality) to provide coulombic interaction with the aspartic acid carboxylate at the anionic region of the AChE active site.

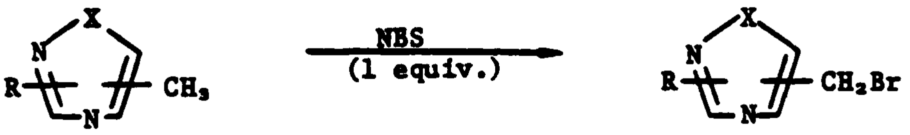
Our previous work demonstrated that the protonated $(C_2H_5)_2NCH_2CH_2S$- functionality contributes to reversible binding of nonquaternary reactivators to the AChE anionic region. Furthermore, for glyoxime, HC(:NOH)C(:NOH)H, and its analogous thioglyoximate, $HC(:NOH)C(:NOH)SCH_2CH_2N(CH_3)_2$, conversion of the aldoxime to the thiohydroximate ester lowers the oxime pKa by nearly two units. Thus we wished to compare analogous type 2 and type 3 compounds with respect to oxime acidity, and reactivity toward inhibited AChE. We also hoped to further clarify the degree to which the flexible $(C_2H_5)_2NCH_2CH_2S$- functionality influences the activity of nonquaternary oximes as reactivators of phosphylated AChE.

In this report we describe the synthesis of seven examples of type 2 and type 3 compounds and their characterization with respect to structure and oxime acidity. We also detail preliminary findings on the activity of the compounds as reactivators of eel AChE inhibited by ethyl p-nitrophenyl methylphosphonate, EPMP.

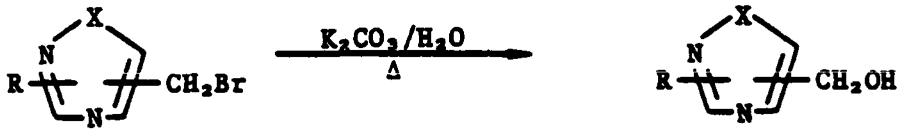
RESULTS AND DISCUSSION

Synthesis and Structure

Table 1 gives structures and pertinent data for the series of heteroaromatic aldoximes 2a-2g. We synthesized the oxadiazole 2b, the triazole 2e, and the thiadiazoles 2f and 2g from the corresponding methyl derivatives using N-bromosuccinimide (NBS) as shown in reactions (1)-(4):



(1)



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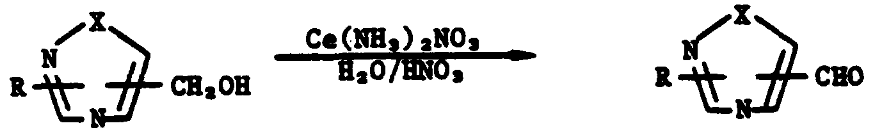
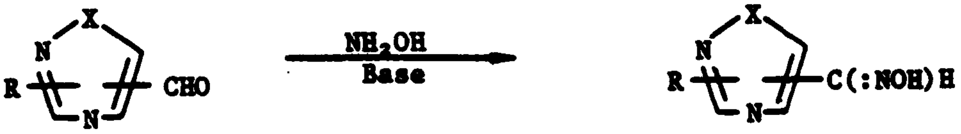


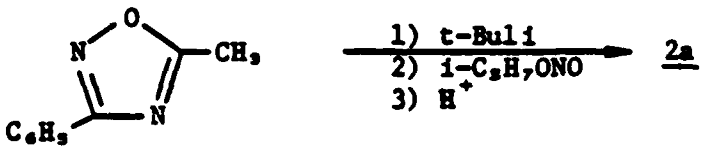
(3)



(4)

2

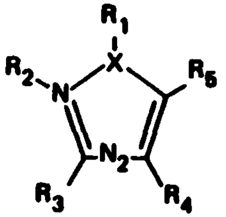
For the oxadiazole, 2a, we converted the methyl derivative directly to the oxime via nitrosation with alkyl nitrite in base, reaction (5)



(5)

Table 1

SELECTED DATA FOR HETEROAROMATIC ALDOXIMES

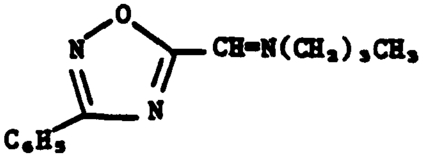


Code No.	Cmpd No.	X	N_2[a]	R_1	R_2	R_3	R_4/R_5	Yield % [b]	m.p. °C	NMR[c] (=NOH)	pKa[d]	Anal.[e]
4185-17	2a	O	4	f	f	C_6H_5	f/CHNOH	30	161-163	12.87(hr)	7.88	C,H,N
4185-94	2b	O	5	f	f	CHNOH	CH_3/f	24	54-56	12.47(s)	9.15	C,H,N
4185-18	2c	N	3	C_6H_5	f	f	C_6H_5/CHNOH	35	163-165	12.10(br)	9.25	C,H,N[h]
4185-19	2d	N	3	C_6H_5	f	f	CHNOH/C_6H_5	70	194-196	11.47(s)	10.2	C,H,N
4185-85	2e	N	3	f	f	f	CHNOH/f	29	241-243	12.17(s)	10.5	C,H,N
11879-10	2f	S	5	f	f	H	CHNOH/f	16	148-149 12.53	12.10[g](s)	9.45	C,H,N,S
11879-7	2g	S	5	f	f	CH_3	CHNOH/f	73	131-134	12.05(s)	9.61	C,H,N,S

---

[a] N_2, indicates ring position of second heterocyclic nitrogen atom.
[b] Based on conversion from starting aldehyde.
[c] In DMSO-d_6.
[d] Determined spectrophotometrically in 0.1 M phosphate or glycine buffer.
[e] Experimental and calculated values for elements shown agree within ± 0.4% unless otherwise noted.
[f] No substituent.
[g] Peak area ratio = 20:80, respectively for 12.53:12.10 singlets.
[h] See experiment details section.

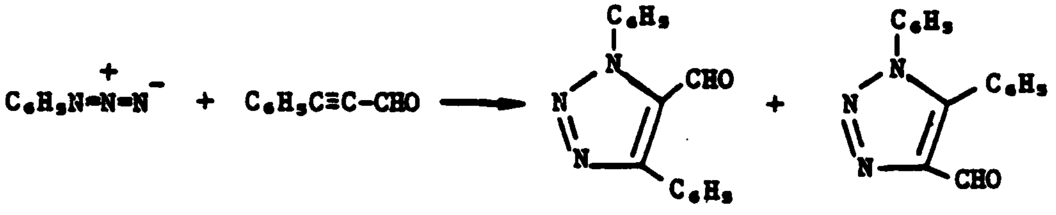
Reaction (5) appeared to be less generally useful than the synthesis route described above. Reaction (5) gave the desired product 2a in poor (30%) yield. Furthermore, substitution of a less hindered base (n-butyl lithium) for t-BuLi yielded the imine below as the only isolable product.



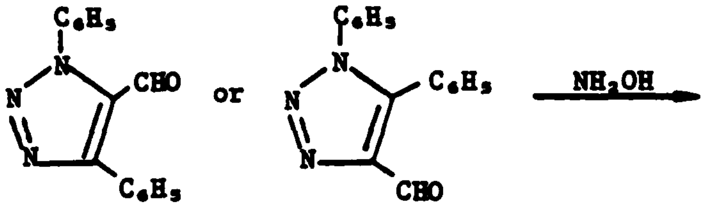
The major limitations to the sequence of reactions (1) through (4) are reluctance of some methyl-substituted heteroaromatics to undergo free-radical bromination and poor or incomplete oxidation of the alcohols to the aldehydes. Barring these limitations, the method does permit the rapid and versatile production of new oximes not readily accessible through alternative synthesis procedures. We found that 3-methyl-5-phenyl-1,2,4-oxadiazole was inert to bromination using NBS plus benzoyl peroxide in refluxing CCl_4. After 24 h, this reaction yielded only starting material. We succeeded in obtaining an approximate 10% yield of 3-bromomethyl-5-phenyl-1,2,4-oxadiazole only after reaction of the methyl compound with NBS, benzoyl peroxide, and Br_2 using heat and light to initiate the reaction. Work is continuing on the preparation of this oxime.

Although we investigated numerous methods for oxidizing the hydroxymethyl-substituted heteroaromatics to the corresponding aldehydes, only reaction (3), oxidation with ceric ammonium nitrate in water or 3 to 5 N HNO_3 generally provided the aldehydes cleanly and rapidly.

We prepared triazole aldoximes 2c and 2d via reactions (6) and (4):

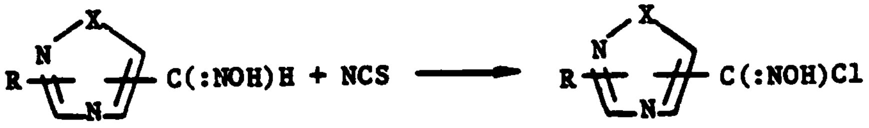


(6)



2c or 2d (4)

Table 2 gives structures and pertinent data for α-heteroaromatic thiohydroximates 3a through 3g. We prepared the thiohydroximates by treating the corresponding oximes with N-chlorosuccinimide (NCS) reaction (7), followed by esterifying the resulting hydroximoyl chlorides (4) with 2-(diethylamino)ethane thiol, reaction (8):



2 4 (7)

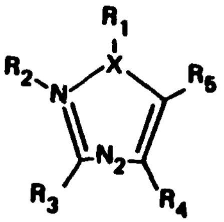
$4 + HSCH_2CH_2N(C_2H_5)_2$ 3 (8)

We determined pKa values for all type 2 compounds listed in Table 1, using a spectrophotometric technique.[24] We obtained pKa values for four of the α-heteroaromatic thioglyoximates listed in Table 2, but were unable to determine accurate pKa's for the triazoles 3c-3e owing to poor aqueous solubility and small spectral changes as a function of pH for these three compounds.

We also recorded the hydroxyimino proton chemical shift for all examples of type 2 and for most examples of type 3 compounds. Thiohydroximates 3e and 3f (which were isolated as the amine free bases) did not show hydroxyimino proton signals. Similarly, the free base 3d exhibited a very broad signal for the hydroxyimino proton. For 3a, 3b and 3g (which we isolated as the amine hydrochloride salts) the hydroxyimino protons appeared as sharp singlets. This dependence of NMR chemical shift on the degree of protonation of the amine functionality suggests zwitterion formation in DMSO solution for thiohydroximates added in the free base form, i.e., reaction (9)

Table 2

SELECTED DATA FOR α-HETEROAROMATIC THIOHYDROXIMATES



Code No.	Cmpd. No.	X	N_2[a]	R_1	R_2	R_3	R_4[b]	R_5[b]	Yield[c] %	m.p. °C	NMR[d] (=NOH) δ	pKa[e]	Analysis
4185-54	3a	0	4	g	g	C_6H_5	g	$C(NOH)SR_6$	27	199-201	13.88(S)	6.32	C,H,N
4185-96	3b	0	5	g	g	$C(NOH)SR_6$	CH_3	g	41	121-122	13.14(s)	7.59	C,H,N,S,Cl
4185-43	3c	N	3	C_6H_5	g	g	C_6H_5	$C(NOH)SR_7$	24	152-155	12.58(br)	h	C,H,N,S
4185-41	3d	N	3	C_6H_5	g	g	$C(NOH)SR_7$	C_6H_5	83	185-187	12.13(s)	h	C,H,N,S
4185-88	3e	N	3	C_6H_5	g	g	$C(NOH)SR_7$	H	44	241-243	h	h	C,H,N,S,[i]
11879-14	3f	S	5	g	g	H	$C(NOH)SR_7$	g	19	122-124	h	7.69	C,H,N,S[i]
11879-12	3g	S	5	g	g	CH_3	$C(NOH)SR_6$	g	39	179-182	12.72(s)	8.65[j]	C,H,N,S,Cl

---

[a] N_2 indicates ring position of second heterocyclic nitrogen atom.

[b] $R_6 = CH_2CH_2N(C_2H_5)_2 \cdot HCl$ and $R_7 = CH_2CH_2N(C_2H_5)_2$.

[c] Based on conversion from starting heteroaromatic hydroximoyl chloride.

[d] In DMSO-d_6.

[e] Determined spectrophotometrically in 0.1 M phosphate or glycine buffer.

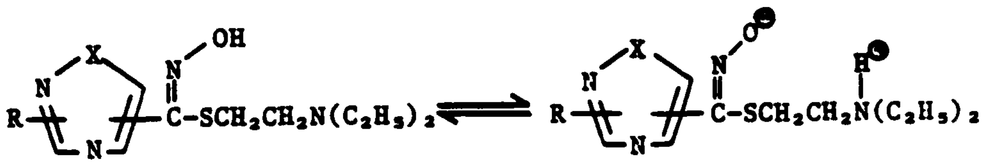
[f] Experimental and calculated values for elements shown agree within ± 0.4% unless otherwise noted.

[g] No substituent.

[h] Not determined.

[i] See experimental details section.

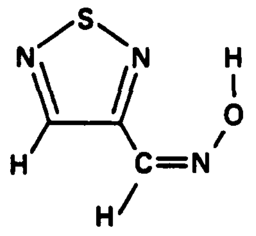
[j] Determined potentiometrically in 0.1 M NACl)$_4$.



(9)

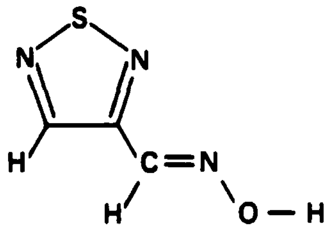
Compound 3c appeared to be anomalous in this regard, the free base form showing a sharp oxime proton signal. The two phenyl substituents that flank the hydroxyimino functionality perhaps limited the ionization shown in reaction (9).

Of the seven heteroaromatic aldoximes shown in Table 1 only one (2f) exhibited two sets of proton signals, indicating a mixture of E- and Z-aldoxime isomers. The major component (80%) of the mixture exhibited proton resonances at δ 12.10 (= NO-H), 9.12 (= C-H), and 8.43 (Ar-H), whereas the minor isomer showed peaks at δ 12.53 (=N-OH), 9.50 (C-H), and 7.95 (Ar-H). For a wide variety of aldoximes, the isomers with hydroxyimino hydroxyl group cis to the trigononal carbon hydrogen exhibit methine proton resonances that are shifted upfield, and ortho-aromatic ring protons that are shifted downfield, from the comparable resonances exhibited by the isomer with hydroxyl group trans to the aldehydic =C-H.[26-29] By analogy, we assign the Z- and E-configurations respectively to the major and minor components of the 2f isomer mixture.



Z-2f

(Major Component)



E-2f

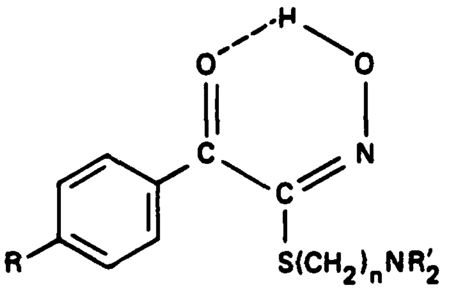
(Minor Component)

JA-1043-138

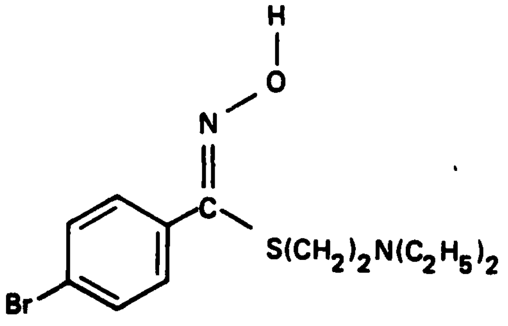
Aldoximes with cis-hydroxyl and aldehydic =C-H groups typically exhibit hydroxyimino proton chemical shifts at higher field than the trans isomers.[26-27] The hydroxyimino resonances for Z- and E-isomers of 2f occurred at high and low field, respectively, opposite to the trend expected by analogy with reported compounds. We consider that the assignment of Z- and E-configurations as shown above for 2f are valid on the basis of NMR data for the methine and ring protons. The anomalous chemical shift observed for the hydroxyimino proton in Z-2f could be a result of intramolecular hydrogen bonding to the ring nitrogen.

We hoped to establish configurations for the other examples of type 2 compounds in Table 1, but such assignments are treacherous in the absence of pairs of E- and Z-isomers or crystallographic data.[28-31] Therefore, at present we do not assign isomeric configurations for heteroaromatic aldoximes other than 2f.

We also wished to determine configurations for the α-heteroaromatic thiohydroximates in Table 2. For the related α-ketothiohydroximates, type 1 compounds we previously[22] suggested an E-configuration favored by intramolecular hydrogen bonding although IR spectral data supported a Z-configuration for 2-(diethylamino)ethyl p-bromophenylthiohydroximate, 5.



E-Arylthiohydroximate

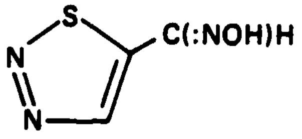


5

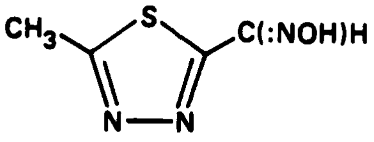
Z-Arylthiohydroximate

JA-1043-136

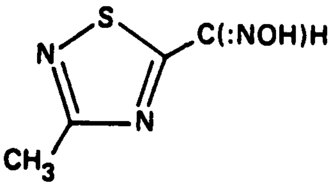
We also previously found a linear correlation between hydroxyimino proton chemical shift and acidity for type 1 compounds. To compare the α-heteroaromatic-aldoximes and -thiohydroximates with related compounds, we plotted =NO—H proton chemical shift versus oxime pKa for 1, 2, 3, 5 and 2PAM. In this plot we also included literature data[25] for thiadiazolyl aldoximes 6a–6c



6a



6b



6c

JA-1043-137

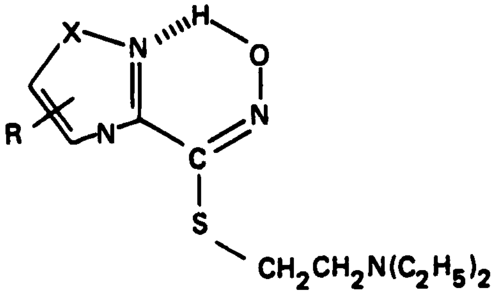
Figure 1 shows two general linear correlations: one for α-keto and α-heteroaromatic thiohydroximates (the dashed line); and a second correlation (solid line) for 2PAM, 5, 6, and most type 2 compounds. Least-squares linear regression of the data for type 1 and type 3 compounds gave equation (10):

$$pKa = (26.2 \pm 1.3) - (1.43 \pm 0.12)\delta \qquad (10)$$

and similar treatment of the data for 5, 6, 2PAM, and type 2 compounds (excluding 2a and 2c) gave equation (11):

$$pKa = (25.2 \pm 1.1) - (1.30 \pm 0.091)\delta \qquad (11)$$

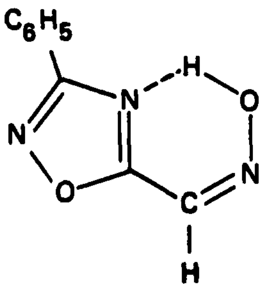
The above correlations were rather imprecise, and the differences between the two general classes of compounds were small but apparently real. We tentatively conclude that the differences relate to the availability (or lack thereof) of intramolecular hydrogen bonding for hydroxyimino protons. Thus data for 3a, 3b and 3g best fit the correlation for type 1 compounds. Because 1 exhibit intramolecular hydrogen bonding, we suggest an E-configuration for the α-heteroaromatic thiohydroximates.



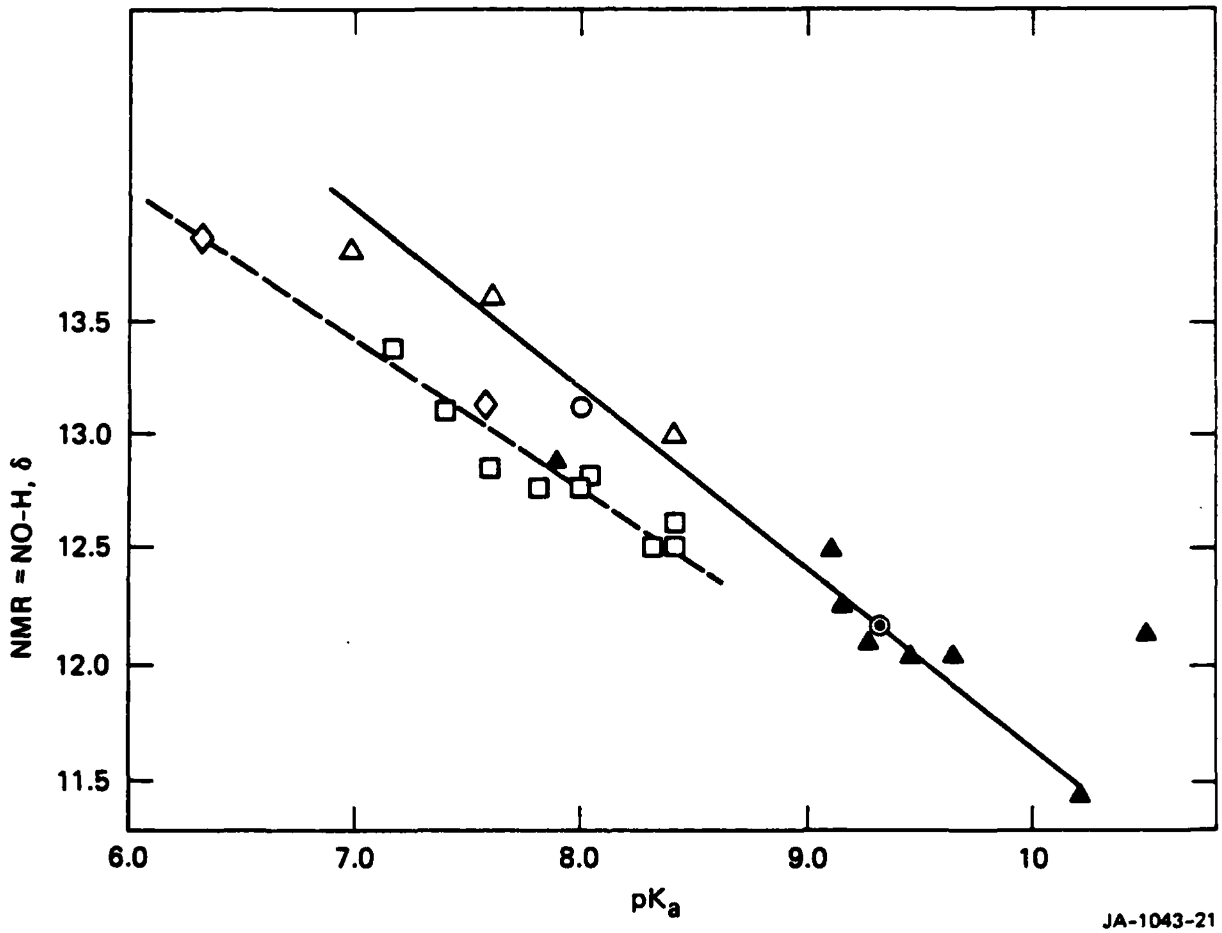
E-3

JA-1043-135

Interestingly, the pKa and NMR data for 2a (the parent compound of 3a) correlated better with equation (10) (the dashed line in Figure 1) than with equation (11). One possible explanation is a Z-configuration for 2a with hydrogen-bonding to the ring nitrogen.



Z-2a

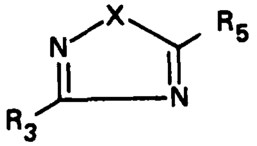


JA-1043-21

FIGURE 1 Oxime proton NMR chemical shift versus pK_a for:
α-ketothiohydroximates, *1*, □–□ (data of references 22, 23);
α-heteroaromatic thiohydroximates, *3*, ◇–◇ (data of Table 2);
α-heteroaromatic aldoximes, *2*, ▲—▲ (data of Table 1);
α-thiadiazolyl aldoximes, *6*, △—△ (data of reference 25);
2-hydroxyiminomethyl-1-methylpyridinium iodide, 2PAM, O; and
$4\text{-BrC}_6H_4C(:NOH)SCH_2CH_2N(C_2H_5)_2$, *5*, ◉ (Data of reference 22).

We emphasize that in the absence of further data our assignments for configurations of 2a and 3a, 3b and 3e are tentative. Nevertheless, the proposed structural relationships are plausible, consistent with the observed data, and illustrative of the types of bonding interactions that can be generally important for hydroxyimino compounds.

To summarize, we find the triazolyl aldoximes, as a class, to be poorly soluble in water and very weak acids. The related triazolyl thiohydroximates were also poorly soluble and this appears to be a major limitation of the triazolyl compounds. Of all the compounds in Table 1 only 2a exhibited an acidity (pKa = 7.88) near the optimal value for reactivators. Interestingly, of the three thiadiazolyl aldoximes reported by Benschop et al.,[25] 6c was the most acidic (pKa = 6.97). This suggests that the general heteroaromatic structure



with a 1,2,4-ring substitution pattern is particularly electron-withdrawing. We are currently uncertain whether inductive and/or resonance effects primarily contribute to the electron-withdrawing properties of these compounds. In either case, it appears that as a class 5-hydroxyiminomethyl 1,2,4-trisubstituted heteroaromatics would feature oxime pKa's in a useful range.

Converting various type 2 compounds to type 3 compounds did lower hydroxyimino proton pKa's by 1.5 to 2 units. This compares favorably with the difference in pKa values (9.9 versus 8.4, respectively) previously[23] observed for glyoxime and 2-(diethylamino)ethyl thioglyoximate. The effect on pKa of substituting an aminoalkyl thioester for an aldehydic hydrogen has practical significance because the substitution in some cases converts hydroxyimino compounds (e.g., aldoximes) with otherwise low acidity to analogs (thiohydroximates) that dissociate appreciably to the reactive oximate anion at physiological pH. The degree to which such substitution actually influences the activity of α-heteroaromatic hydroxyimino compounds as reactivators of phosphylated AChE is discussed in the following section.

Acetylcholinesterase Reactivation

Screening for Activity

We screened type 2 and type 3 compounds for in vitro activity as reactivators of ethyl methylphosphonyl-AChE. The method involved incubating ethyl p-nitrophenyl methylphosphonate with a slight excess of eel AChE followed by incubating of the inhibited enzyme at pH 7.6 and 25°C with various concentrations of test compounds and assaying for restored enzymatic activity. The general method has been described in detail.[23]

For the determining of reactivation data we defined the percentage reactivation after t min incubation according to equation (12)

$$\%R_t = 100\ (A_t - AI_t)/(A_c - AI_t) \tag{12}$$

where A_t is activity of reactivated AChE at time t, AI_t is activity (at time t) of AChE inhibited with EPMP, and A_c is control AChE activity (= activity of uninhibited enzyme or = mean value of uninhibited enzyme activity and enzyme activity after complete reactivation with 2PAM). All activities reported herein are net activities corrected for spontaneous and reactivator-induced hydrolysis of substrate in assay solutions. The data that follow are <u>not</u> corrected for inhibition of AChE by test compounds.

In comparing various reactivators we have calculated reactivator concentrations on the dual basis of the concentration of the protonated hydroxyimino form (HOX) of added test compound and on the concentration of the anionic oximate form (OX), where

$$[OX] = [HOX]\{1 + \text{antilog}[pKa - 7.6]\}^{-1} \tag{13}$$

Table 3 shows the percent restoration of AChE activity after incubating the inhibited enzyme with reactivators for 120 and 240 min.

The table shows pronounced differences in activity for various examples of <u>2</u> and <u>3</u>. Compounds <u>2c</u> and <u>3e</u> were insoluble in water or in 10% EtOH, even at low concentrations, and we were unable to obtain reactivation data for these materials. Compounds <u>2d</u>, <u>2e</u>, <u>3c</u> and <u>3d</u> were sparingly soluble in water but dissolved in 10% EtOH at the indicated concentrations. Reactivation data for <u>2d</u>, <u>2e</u>, <u>3c</u> and <u>3d</u> were obtained in aqueous ethanol using appropriate control solutions. All other data were obtained in water.

The α-heteroaromatic aldoximes <u>2d</u>, <u>2e</u>, <u>2f</u>, and <u>2g</u> showed no significant activity as reactivators at the concentrations tested. We attribute this lack of activity to the low acidities of the aldoximes and the correspondingly low concentrations of the nucleophilic oximate species at pH 7.6.

Two of the aldoximes, <u>2a</u> and <u>2b</u>, did reactivate ethyl methylphosphonyl-AChE: indeed <u>2a</u> was the most active of any of the compounds investigated to date.

All of the α-heteroaromatic thiohydroximates (except <u>3c</u>) reactivated phosphylated-AChE significantly. As a class, the α-triazolyl thiohydroximates (<u>3c</u>, <u>3d</u>, and <u>3e</u>) reactivated poorly. In general, the aldoxime and

Table 3

PERCENT REACTIVATION ($\%R_t$) OF ETHYL METHYLPHOSPHONYL-AChE AFTER INCUBATION WITH REACTIVATORS AT VARIOUS CONCENTRATIONS AT 25°C, pH 7.6

Compound[a] No.	[HOX][b] mM	[OX][c] mM	$\%R_t$[d] After Incubation for t, min	
			t=120	t=240
2a	0.500	0.172	100	98
	0.200	0.0688	78	90
2b	1.00	0.0274	7.0	12
	0.100	0.00274	1.0	3.0
2c	0.100	0.00219	e	e
2d	0.0500	0.00013	f	f
	0.0250	0.00007	f	f
2e	1.00	0.00126	3.5	f
	0.100	0.00013	f	f
2f	1.00	0.0139	f	f
2g	1.00	0.00968	f	f
3a	1.00	0.950	6.0	8.9
	0.100	0.0950	11	21
3b	0.500	0.500	19	34
	0.100	0.0500	11	15
3c	0.100	g	f	1.7
3d	0.100	g	2.8	5.7
3e	0.100	g	e	e
3f	1.00	0.448	47	59
	0.500	0.224	29	37
3g	1.00	g	28	42
	0.500	g	7.7	17
2PAM	0.0100	0.00289	93	97

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[a] See Tables 1 and 2 for structures.

[b] [HOX] is the concentration of added test compound.

[c] [OX] is the concentration of anionic form of added test compounds, calculated according to equation (13).

[d] $\%R_T$ calculated according to equation (12).

[e] Compound was insoluble at indicated concentration. Reactivation data not obtained.

[f] Reactivation not detectable, < 1%.

[g] No pKa available for compound [OX] not calculated.

thiohydroximate derivatives of triazoles appear to be poor candidates for nonquaternary reactivators.

The α-oxadiazolyl thiohydroximates 3a and 3b were modest reactivators of ethyl methyl phosphonyl-AChE. Thiohydroximate 3a was substantially less reactive toward phosphonylated AChE than the corresponding aldoxime 2a, which we attribute at least in part to high acidity (pKa = 6.32) of the thiohydroximate and low nucleophilicity of the conjugate base oximate form of 3a. Compound 3a was not well-behaved in our in vitro assay; higher concentrations of 3a actually reactivated inhibited enzyme more slowly and less completely than low concentrations. This suggests inhibition of AChE by 3a itself at high concentration.

The thiohydroximates 3b, 3f and 3g were more reactive towards phosphonyl AChE than the corresponding aldoximes 2b, 2f and 2g, presumably a result of pKa differences.

None of the type 3 compounds tested surpassed 2a as a reactivator of ethyl methylphosphonyl-AChE and 2a was significantly less reactive than 2PAM toward the inhibited enzyme.

Reactivation Kinetics

To more accurately assess structure-activity relationships among the nonquaternary AChE reactivators, it is necessary to examine reactivation kinetics. The reactivation of ethyl methylphosphonyl-AChE proceeds via the mechanism shown in equation (14):

$$\mathrm{OX} + \mathrm{EOP} \overset{K_r}{\rightleftharpoons} [\mathrm{OX{\bullet}EOP}] \xrightarrow{k_r} \mathrm{EOP} + \text{products} \qquad (14)$$

where OX is the oximate form of the reactivator, EOP is phosphonylated enzyme, [OX•EOP] is a reversibly formed reactivator/inhibited enzyme complex, and EOH is active enzyme.

Under conditions where reactivator is present in large excess over inhibited enzyme, we have shown[22,23] that the following equations can be obtained:

$$\ln(100 - \%R_t) = k_{obs} \cdot t \qquad (15)$$

$$(k_{obs})^{-1} = \frac{K_r}{k_r}[\mathrm{OX}]^{-1} + \frac{1}{k_r} \qquad (16)$$

$$k_b = k_r/K_r \qquad (17)$$

$$k_{eff} = k_b[1 + \text{antilog } (\mathrm{pKa}-7.6)]^{-1} \qquad (18)$$

where k_{obs} is a pseudo-first-order observed rate constant for reactivation, K_r is the equilibrium constant for dissociation of the reactivator/inhibited enzyme complex, k_r is the rate constant for displacement of inhibitor from the reactivator/inhibited enzyme complex, k_b is equivalent to a bimolecuular rate constant for reactivation in the limit of low reactivator concentration, and k_{eff} is an effective rate constant for reactivation that accounts for the fraction of added test compound present as the oximate form at pH = 7.6.

To date we have determined reactivation kinetics at four different oximate concentrations only for 2a. We have determined reactivation kinetics at one or two oximate concentrations for 2b, 3a, 3b, 3f and 3g. Because equation (16) requires a minimum of three different points for valid calculation of K_r and k_r values, and because we wished to make at least preliminary comparisons of reactivator activity, we calculated apparent bimolecular rate constants (k_{app}) for reactivation of phosphonylated AChE by oximate anion according to equation (19):

$$k_{app} = k_{obs} \cdot [OX]^{-1} \qquad (19)$$

Because equation (19) is only an approximation, k_{app} values determined at high reactivator concentration underpredict inherent oximate reactivity. Furthermore, in our calculations we have not corrected for AChE inhibition by reactivators themselves, and at least for 3a, inhibition was apparently significant. With these caveats in mind, we can examine the calculated k_{app} values and use them as a first approximation to rate relative activities for the various reactivators. Table 4 gives k_{app} values calculated as above for six different reactivators.

The table demonstrates the remarkable differences in activity for the reactivators. Both oxadiazolyl aldoximes 2a and 2b exhibited k_{app} values higher than any of the related thiohydroximates. It is especially interesting that, 2a was more reactive than the analogous 3a by approximately two orders of magnitude. We had anticipated that converting the aldoxime to the thiohydroximate form would enhance affinity of reactivator for the anionic region of the enzyme active center and therefore enhance reactivity towards ethyl methylphosphonyl-AChE. Such is not the case, however. We attribute the relatively low activity of 3a, in part, to high oxime acidity (low oximate nucleophilicity) and perhaps also to inhibition of AChE by reactivator.

The relatively low activity of the α-heteroaromatic thiohydroximates as a class seems significant. Poor oximate nucleopholicity seems an unlikely explanation for low activity of 3b, 3f and 3g insofar as all three compounds exhibited pKa values near pKa = 8. We previously demonstrated[22,23] that the 2-(diethylamino)ethyl functionality in 3 significantly contributes toward reversible binding of the reactivator to inhibited-AChE; thus low affinity of oximate for the anionic region of AChE cannot readily explain the observed lower activities.

Table 4

APPARENT BIMOLECULAR RATE CONSTANTS (k_{app}) FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT 25°C, pH 7.6

Compounds[a] No.	[HOX][b] mM	[OX][c] mM	k_{obs}[d] $min^{-1} \times 10^3$	k_{app}[e] M-min-1
2a	0.200	0.0688	36.3 ± 0.17	528
	0.0300	0.0103	5.58 ± 0.29	542
2b	1.00	0.0274	0.375 ± 0.026	13.7
	0.100	0.00274	0.054 ± 0.026	19.7
3a	1.00	0.950	0.834 ± 0.059	0.878
	0.100	0.0950	0.393 ± 0.021	4.14
3f	1.00	0.448	2.44 ± 0.28	2.60
	0.500	0.224	1.67 ± 0.12	7.46
3g	1.00	0.334	2.34 ± 0.12	7.01
	0.100	0.0334	0.758 ± 0.033	22.7

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[a] See Tables 1 and 2 for structures.

[b] [HOX] is concentration of added test compound.

[c] [OX] is concentration of oximate form of reactivator, calculated according to equation (13).

[d] Calculated according to equation (15), error limits are linear-least squares standard deviations.

[e] Calculated according to equation (19).

To better understand the factors that control activity of the reactivators it is helpful to examine reactivation rate constants determined as in equations (15) through (18). Table 5 summarizes these data for 2a, and for comparison gives earlier data[23] for 2PAM, and $BrC_6H_4C(O)C(NOH)-SCH_2CH_2N(C_2H_5)_2 \cdot HCl$, 1a (code SR 3018). The table shows that, in terms of k_b values, 2a was more reactive than 1a toward ethyl methylphosphonyl-AChE by a factor of approximately six and less reactive than 2PAM by a factor of approximately 20. Surprisingly, 2a and 1a exhibited nearly equivalent values for K_r, despite the fact that 1a bears a cationic moiety (protonated diethylaminoethyl group) whereas 2a does not (the ring nitrogens of heteroaromatics such as pyridine are poorly protonated at pH 7.6 and the same should be true for the oxadiazole 2a).

We previously suggested[23] that k_r and K_r values cannot be independently interpreted and that K_r does not necessarily reflect the fraction of reactivator bound to inhibited AChE. Rather K_r relates to the fraction of bound oximate that actually displaces inhibitor from the reactivator/inhibited enzyme complex. We postulated that the dialkylaminoalkyl thiohydroximic acid S-esters, which exhibit many rotational degrees of freedom, bind to inhibited AChE with multiple low-energy conformations and that only a relatively small fraction of the conformations of the oximate/inhibited enzyme complex have a geometry that favors attack by oximate oxygen on phosphorus. We rationalized the powerful reactivity of 2PAM on the basis of strong affinity for inhibited AChE plus a relatively small number of total low energy conformations and a high proportion of available conformations with geometry suitable for reactivation.

In this context 2a seems to afford relatively high activity toward ethyl methylphosphonyl-AChE, at least in part because the molecule is relatively rigid and resembles 2PAM in the number of low energy conformations. The available data do not directly address the relative binding affinities of 2a and 2PAM for inhibited AChE. Because 2a has no cationic functionality to provide electrostatic attraction for the AChE anionic region, hydrophobic forces presumably govern the reversible binding of the reactivator to the inhibited enzyme.

The data of Table 4 show that the aldoximes 2a and 2b were more reactive than the corresponding α-heterocyclic thiohydroximic acid esters. Even for compounds that exhibited similar pKa values and reasonably isosteric heterocycles, incorporation of the 2-(diethylamino ethyl) moiety into the general molecular framework lowered activity towards inhibited enzyme. Because obvious chemical differences in nucleophilicity or steric bulk are not apparent, we consider that the difference in reactivity between α-heteroaromatic aldoximes and thiohydroximates relate to entropic factors and differences in rigidity for the two types of compounds.

Conclusions and Future Work

Nonquaternary reactivators based on heteroaromatic aldoximes seem likely candidates for treatment of central nervous system effects of organophosphorus

Table 5

KINETIC PARAMETERS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT 50°C, pH 7.6

Cmpd[a] No.	[HOX][b] mM	$[OX]^{-1}$ [c] $M^{-1} \times 10^{-3}$	k_{obs} [d] $min^{-1} \times 10^{3}$	$(k_{obs})^{-1}$ min	Slope[e] M-min x 10^2	Intercept[e] min	k_r [e] $min^{-1} \times 10^3$	K_r [e] M x 10^4	k_b [f] $M\text{-}min^{-1}$	k_{eff} [g] $M\text{-}min^{-1}$
2a	0.500	5.99	45.0 ± 5.3	22.2	0.171 ± 0.0099	9.43 ± 5.3	106	1.81	585	195
	0.200	15.0	36.3 ± 0.17	27.6						
	0.0800	37.4	12.4 ± 0.52	80.1						
	0.0300	99.8	5.58 ± 0.79	179						
1a	h	h	h	h	1.05 ± 0.144	41.5 ± 35	24.1	2.53	95.3	56.3
2PAM	h	h	h	h	0.0104 ± 0.00064	27.8 ± 24	36.0	0.0374	9630	2780

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[a] See Table 1 for structure. 1a is $BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$.

[b] [HOX] is concentration of added test compound.

[c] [HOX] is concentration of oximate form of added test compound calculated according to equation (13).

[d] Calculated according to equation (15).

[e] Calculated according to equation (16).

[f] Calculated according to equation (17)

[g] Calculated according to equation (18).

[h] See reference 23 for original data.

ester poisoning. Benschop et al[25] found thiadiazole **6a** to be less reactive than 2PAM toward phosphylated-AChE but nevertheless observed useful therapeutic activity for the nonquaternary aldoxime. We showed that the α-keto-thiohydroximate **1a** was one-fiftieth as potent as 2PAM *in vitro* as a reactivator of ethyl methylphosphonyl-AChE, but equivalent to 2PAM as a reactivator of mouse diaphragm AChE, and superior to 2PAM as reactivator of brain AChE *in vivo*. Oxadiazole **2a** is more reactive toward phosphylated-AChE than **1a** by a factor of approximately three. This raises the possibility that **2a** may also be more effective than **1a** at reversing the toxic effects of organophosphorus inhibitors *in vivo*. This possibility should be evaluated in future work.

As a class, α-heteroaromatic aldoximes appear to be more reactive *in vitro* than analogous α-heteroaromatic thiohydroximates toward ethyl methylphosphonyl-AChE. We interpret this behavior in terms of the structural flexibility that is available to dialkylaminoalkyl thiohydroximic acid esters that contributes to a reduction in the entropy of activation for the reactivation of inhibited AChE. This hypothesis must be fully evaluated and we plan to determine reactivation kinetics for compounds such as **3a** and **3g** to more clearly elucidate the controlling factors.

The 5-hydroxyiminomethyl-1,2,4-oxadiazole or -1,2,4-thiadiazole structure appears to be a useful framework for developing improved reactivators. Compound **2a** is the most powerful nonquaternary reactivator that we have examined so far. **2a** is approximately one-fifteenth as reactive toward phosphonylated-AChE as 2PAM. In general, the nonquaternary reactivators exhibit strong dependence of activity on structure. We will prepare substituted analogs of **2a** in an effort to obtain α-heterocyclic aldoximes with inherent activity comparable with that of 2PAM.

EXPERIMENTAL DETAILS

Materials

Melting points were obtained in glass capillaries with a Laboratory Devices Mel-Temp apparatus and are presented uncorrected. Infrared spectra were determined with Perkin-Elmer Models 281 and 735B spectrophotometers. Nuclear magnetic resonance spectra were obtained with a Varian Associates EM-360 spectrometer, and chemical shifts are reported in parts per million (δ) relative to an internal tetramethylsilane reference. C,H,N, microanalysis was performed with a Perkin-Elmer 240 elemental analyser. Other elements were analyzed at Stanford University analytical laboratory. Elemental analytical results are reported below only for compounds whose observed values were not within ± 0.4% of theoretical.

The following heterocyclics were prepared according to literature methods: 3-phenyl-5-methyl-1,2,4-oxadiazole (7a);[32] 3,4-dimethyl-1,2,5-oxadiazole (7b);[33] 3-methyl-5-phenyl-1,2,4-oxadiazole (7c);[34] 1,4-diphenyl-5-formyl-1,2,3-triazole (7d);[35] 1,5-diphenyl-4-formyl-1,2,3-triazole (7e);[35] 1-pheny-4-formyl-1,2,3-triazole (7f);[36] 3-methyl-1,2,5-thiadiazole (7g);[37] and 3,4-dimethyl-1,2,5-thiadiazole (7h).[37] Other materials were prepared or obtained as previously described.[22,23]

3-Phenyl-5-hydroxyiminomethyl-1,2,4-oxadiazole (2a)

Oxadiazole 7a (1.6 g, 0.01 mol) was dissolved in 40 mL of freshly distilled tetrahydrofuran and cooled to -78°C. To this solution, t-butyllithium (2.0 M, in 6 mL pentane, 0.012 mol) was added slowly. Subsequently addition of $i\text{-}C_3H_7ONO$ (1.1 g, 0.012 mol) followed by acidification of the solution with 30 mL of 4N 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 2a contaminated with starting material 7a. Sublimation of the crude product gave 0.150 g (5.0%) of 2a, m.p. 161-163°C. NMR (DMSO-d_6), δ 12.87 (br, s, 1H, N-OH); 8.37 (s, 1H, CHNOH); 8.00 (m, 2H, phenylaromatic) and 7.56 ppm (m, 3H, phenylaromatic). IR (KBr) 3225 (s), 2960 (m), 1540 (m), 1430 (s), 1345 (s), 1035 (s) and 990 (s) cm^{-1}. Analysis: C,H,N.

3-Phenyl-1,2,4-oxadiazol-5-yl hydroximoyl chloride (4a)

Compound 2a (0.94 g, 5.0 mmol) dissolved in 30 mL dimethylformamide was treated with 0.67 g (5.0 mmol) of N-chlorosuccinimide. The resulting mixture was stirred 15 min followed by bubbling approximately 15 mL of HCl (g) through the solution. The solution was stirred an additional 15 min, and then was heated to 55°C and allowed to cool slowly (approximately 1 h). The resulting mixture was poured into 200 mL water and extracted with two 100-mL portions of ether. The ether extracts were combined, washed with three 100-mL portions water, dried over anhydrous $MgSO_4$, filtered, and solvent evaporated yielding

0.94 g (85%) of a white amorphous solid; m.p. 155-159°C. The crude reaction product was assigned the hydroximoyl chloride structure of 4a, based on the following: NMR (DMK-d_6) δ 12.70 (s, 1H, NOH), 8.15 (m, 2H, phenyl), and 7.57 ppm (m, 3H, phenyl). Analysis for $C_9H_6N_3O_2Cl$; Cal'd: C, 48.32; H, 2.68; N, 18.79. Found: C, 51.03; H, 2.93; N, 18.24.

3-Phenyl-1,2,4-oxadiazol-5-ylthiohydroximic acid 2-(Diethylamino)ethyl S-Ester Hydrochloride (3a).

To the crude hydroximoyl chloride 4a (0.90 g, 4.0 mmol) was added 0.68 g (4.0 mmol) of diethylaminoethanethiol hydrochloride dissolved in 75 mL chloroform which contained 2 mL of freshly distilled triethylamine. The total reaction volume was increased to 100 mL by adding an additinal 25 mL chloroform and the resulting solution was stirred overnight. The reaction mixture was subsequently washed with two 100 mL portions of water, dried over anhydrous $MgSO_4$, filtered, and the solvent removed on the rotary evaporator yielding 1.21 g of crude 3a. The crude material was purified by column chromatography using 100% diethyl ether eluant. The crude product was dissolved in a minimal amount of ether and placed on a silica-gel column. Fractions of 40 mL were collected and examined by TLC, like fractions were combined and concentrated yielding 0.35 g (27%) of an analytically pure yellow oil. Analysis: C,H,N.

Purified 3a was converted to its HCl salt by dissolving the chromatographed product in diethyl ether followed by the dropwise addition of an ethereal solution saturated with HCl gas. White crystals of the hydrochloride precipitated from solution and were filtered and dried in vacuum, m.p. 199-201°C (Dec). NMR (DMSO-d_6), δ 13.88 (br, s, 1H, NOH), 8.10 (m, 2H, 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-Bromomethyl-4-methyl-1,2,5-oxadiazole*

In a 500-mL round bottom flask was placed 24.5 g (0.25 mole) of freshly distilled 3,4-dimethyl-1,2,5-oxadiazole (7b), 44.5 g (0.25 mole) of N-bromosuccinimide, 1.5 g of benzoyl peroxide, and 600 mL of carbon tetrachloride. The resulting slurry was refluxed overnight, filtered hot into 1 L of water and the organic layer subsequently washed with three 250-mL portions of water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 33.5 g of crude product. The NMR of the crude material showed an approximate 60% conversion with the remainder starting material. The monobromide can be distilled at 96-100°C under a water aspirator vacuum; however, the crude material was used in subsequent reactions without further purification; NMR ($CDCl_3$), δ 4.59 (s, 2H, CH_2Br) and 2.50 ppm (s, 3H, CH_3).

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*Caution: 3-Bromomethyl-4-methyl-1,2,5-oxadiazole is a severe lachrymator and should be handled accordingly.

3-Hydroxymethyl-4-methyl-1,2,5-oxadiazole

In a 500-mL round bottom flask was placed 33.5 g of the crude 3-bromomethyl-4-methyl-1,2,5-oxadiazole, prepared as above, 25 g of potassium carbonate and 350 mL of water. The resulting mixture was heated to 90°C and stirred for 90 minutes. 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 diethyl ether. The combined ether extracts were dried over $MgSO_4$, filtered and concentrated, yielding 11.95 g of the crude alcohol. The NMR of the crude alcohol showed a 30% contamination with 3,4-dimethyl-1,2,5-oxadiazole. The 3-hydroxymethyl compound was used without further purification. NMR ($CDCl_3$), δ 4.88 (s, 2H, CH_2), 4.55 (br, s, 1H, OH) and 2.47 ppm (s, 3H, CH_3).

3-Formyl-4-methyl-1,2,5-oxadiazole

To 100-mL of 3.5 N nitric acid was added 11 g of the crude 3-hydroxymethyl-4-methyl-1,2,5-oxadiazole prepared above. To this solution, preheated to 50°C, was added 132 g of ceric ammonium nitrate in 300 mL of 3.5 N nitric acid, and the resulting mixture stirred at this temperature for 6 hr. The resulting mixture was cooled, extracted with three 75-mL portions of methylene chloride, the combined extracts washed with two 100-mL portions of water, then with 100 mL of 1 N $NaHCO_3$ and 100 mL brine. The methylene chloride was dried over anhydrous $MgSO_4$, filtered and solvent evaporated, yielding 5.0 g of crude aldehyde. The NMR of the crude product indicated the presence of three components; methylene chloride, aldehyde and starting material 3,4-dimethyl-1,2,5-oxadiazole. An overall yield of 3.75% from the dimethyl compound, 7b was recorded. NMR ($CDCl_3$), δ 10.40 (s, 1H, C(O)H) and 2.62 ppm (s, 3H, CH_3).

3-Hydroxyiminomethyl-4-methyl-1,2,5-oxadiazole (2b)

3-Formyl-4-methyl-1,2,5-oxadiazole (1.12 g, 10 mmol) dissolved in 100 mL of absolute ethanol was treated with 0.7 g (10 mmol) of hydroxylamine hydrochloride and 0.79 g (10 mmol) of pyridine. The resulting mixture was refluxed for two hr. The ethanol was removed under aspirator vacuum and the residue taken up in 100 mL diethyl ether. The ethereal solution was washed with two 50-mL portions of 1 N HCl, dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 750 mg of a white amorphous solid which proved to be a mixture of the desired oxime contaminated with 3,4-dimethyl-1,2,5-oxadiazole. Recrystallization from 30-60°C petroleum ether yielded 300 mg (23.6%) of analytically pure 2b; m.p. 54-56°C. NMR (DMSO-d_6), δ 12.42 (s, 1H, NOH), 8.40 (s, 1H, CHNO), and 2.50 ppm (s, 3H, CH_3). Analysis: C,H,N.

4-Methyl-1,2,5-oxadiazol-3-yl Hydroxyimoyl Chloride (4b)

To 3-hydroxyiminomethyl-4-methyl-1,2,5-oxadiazole (2b) (230 mg, 1.8 mmol) dissolved in 15 mL of dimethylformamide was added with stirring 240 mg (1.8 mmol) of N-chlorosuccinimide. The resulting mixture was stirred at room temperature for 20 min and 15 mL of HCl gas was bubbled into the reaction mixture. After 30 min the reaction mixture was heated to 50°C and allowed to slowly cool for 40 min. At the end of the 90-min reaction time the mixture was poured into 60 mL of water. The aqueous solution was extracted with two 50-mL portions of diethyl 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. NMR showed an approximate 80% conversion to the desired hydroximoyl chloride product. NMR ($CDCl_3$), δ 12.10 (s, 1H, NOH), and 2.50 ppm (s, 3H, CH_3). This material was used without further purification.

4-Methyl-1,2,5-oxadiazol-3-ylthiohydroximic acid 2-(Diethylaminoethyl S-ester Hydrochliride (3b)

To the 230 mg (1.4 mmol) of hydroximoyl chloride prepared above and dissolved in 50 mL chloroform was added 240 mg (1.4 mmol) of diethylaminoethanethiol hydrochloride and 290 mg (2.8 mmol) of triethylamine. The resulting mixture was stirred overnight, then washed with two 50-mL portions of water, dried over anhydrous $MgSO_4$, filtered, and solvent removed, yielding 350 mg of viscous yellow oil. NMR indicated that the majority of the crude reaction mixture was the desired product 3b contaminated with the starting material 2b. This mixture was chromatographed on 125 g of silica gel using a diethyl ether eluent; like fractions were combined and concentrated after TLC examination, yielding 150 mg (41%) of the free amine 3b as a clear oil. The hydrochloride salt of 3b was prepared by dissolving the above oil in 25 mL diethyl ether and adding dropwise an ethereal solution saturated with HCl gas. White crystals of the hydrochloride precipitated from solution. The crystals were filtered, and dried under vacuum, yielding 170 mg of analytically pure 3b hydrochloride; m.p. 121-122°C. NMR (DMSO-d_6), δ 13.14 (s, 1H, NOH), 3.20 (m, 8H, CH_2) and 1.22 ppm, (t, 6H, CH_3). Analysis: C,H,N,S,Cl.

1,4-Diphenyl-5-hydroxyiminomethyl-1,2,3-triazole (2c)

Phenyl azide[36] (12.0 g, 0.10 mol) and phenylpropargyl aldehyde (12.5 g, 0.1 mol) were added to 65 mL toluene and refluxed overnight. The solvent was removed and the intermediate 1,4-diphenyl-5-formyl-1,2,3-triazole (7d) was separated and purified from the isomeric 1,5-diphenyl-4-formyl-1,2,3-triazole (7e) by silica gel chromatography with 4:1 CH_2Cl_2:30-60°C petroleum ether eluant. Subsequent treatment of 7d with one equivalent of hydroxylamine hydrochloride and recrystallization from CH_3OH per the method of Sheehan and Robinson[35] yielded 1.5 g (5%) of 2c. NMR (DMSO-d_6), δ 12.10 (br, s, 1H, NOH); 8.22 (s, 1H, CHNOH); 7.88 (m, 2H, phenyl) and 7.60 ppm (m, 8H, phenyl). IR (KBr) 3000 (br, s), 1580 (m), 1480 (s), 1440 (s), 970 (s) and 825 (s) cm^{-1}. Analysis for $C_{15}H_{12}N_4O$. Calc'd: C, 68.17; H, 4.58; N, 21.20. Found: C, 68.05; H, 4.53; N, 20.53.

1,4-Diphenyl-1,2,3-triazol-5-yl hydroximoyl chloride (4c)

1,4-Diphenyl-5-hydroxyiminomethyl-1,2,3-triazole, 2c (1.07 g, 4 mmol) dissolved in 30 mL of dimethylformamide was treated with 0.54 g (4 mmol) of N-chlorosuccinimide. Reaction and workups, similar to those described for 4a, yielded 1.07 (90%) of a white crystalline material; m.p. 164-168°C. The crude reaction product was assigned the hydroximoyl chloride structure 4c based upon the following; NMR (DMK-d_6), δ 12.53 (s, 1H, NOH), 7.95 (m, 2H, phenyl) and

7.60 ppm (m, 8H, phenyl). Analysis for $C_{15}H_{11}N_4Cl$; Cal'd: C, 60.30; H, 3.69; N, 18.76; Cl, 11.89. Found: C, 61.50; H, 3.93; N, 18.45; Cl, 10.13.

1,4-Diphenyl-1,2,3-Triazol-5-yl Thiohydroximic Acid 2-(Diethyl amino)ethyl S-Ester (3c)

Treatment of hydroximoyl chloride 4c with diethylaminoethanethiol hydrochloride as described previously for 4a and 4b, yielded 0.50 g of crude 3c. Recrystallization from 95% ethanol yielded 0.38 g (24%) of analytically pure 3c; m.p. 152-155°C; NMR (DMSO-d_6), δ 12.58 (s, 1H, NOH) 7.92 (m, 2H, phenyl), 7.67 (m, 8H, phpenyl), 2.20 (m, 8H, CH_2) and 0.70 ppm (t, 6H, CH_3). Analysis: C,H,N,S.

1,5-Diphenyl-4-hydroxyiminomethyl-1,2,3-triazole (2d)

Compound 2d was prepared from the corresponding aldehyde 7e as described above for 2c. Recrystallization from 95% C_2H_5OH yielded 3.1 g (10%) of the desired material. NMR (DMSO-d_6), δ 11.4 (s, 1H, NOH); 8.16 (s, 1H, CHNOH), and 8.20 ppm (m, 10H, phenyl). IR (KBr), 3175 (s), 1480 (s), 980 (s), 830 (s), 755 (s) and 680 (s) cm^{-1}. Analysis: C,H,N.

1,5-Diphenyl-1,2,3-Triazol-4-yl Hydroximoyl Chloride (4d)

4-Hydroxyiminomethyl-1,5-diphenyl-1,2,3-triazole, 2d, (2.64 g, 10 mmol) was dissolved in 50 ml dimethylformamide with stirring while N-chlorosuccinimide (1.34 g, 10 mmol) was added. The resulting mixture was stirred 15 min followed by bubbling 15 mL of HCl gas through the solution. The solution was stirred an additional 15 min, heated to 55°C and allowed to cool slowly to ambient temperature (about one hr). The resulting mixture was poured into 200 mL water and extracted with two 100-mL portions of ether. The ether extracts were combined, washed with three 100-mL portions water, dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 2.76 g (92%) of a white crystalline compound; m.p. 179-184°C. The solid material was assigned the hydroximoyl chloride structure 4d based upon the following: NMR (DMK-d_6), δ 11.25 (s, 1H, NOH) and 7.38 ppm (m, 10H, C_6H_5). Analysis for $C_{15}H_{11}N_4Cl$: Calc'd: C, 60.30; H, 3.69; N, 18.76; Cl, 11.89. Found: C, 61.18; H, 3.72; N, 18.81; Cl, 10.15.

1,5-Diphenyl-1,2,3-Triazol-4-yl Thiohydroximic Acid 2-(Diethyl amino)ethyl S-Ester (3d)

The hydroximoyl chloride 4d (2.69 g, 9.0 mmol) in 75 mL of chloroform was treated with 1.48 g (9.0 mmol) of diethylaminoethanethiol hydrochloride and 6 mL of triethylamine (excess) and the resulting solution stirred overnight. Concentrating the crude reaction solution on the rotary evaporator yielded 3.18 g of a white crystalline product. The crude precipitate was triturated with a 60% ethanol/40% water solution and filtered. The precipitate was dried over P_2O_5 yielding 2.94 g (83.0%). Two recrystallizations from a 75% chloroform/25% methanol solution yielded analytically pure 3d; NMR (DMSO-d_6), δ 12.13 (br, s, 1H, NOH) 7.48 (m, 10H, phenyl), 2.55 (m, 9H, CH_2) and 0.89 ppm (t, 6H, CH_3). Analysis: C,H,N,S.

4-Formyl-1-phenyl-1,2,3-triazole (7f)

To 5.0 g (28.5 mmol) of 4-hydroxymethyl-1-phenyl-1,2,3-triazole (prepared by the cycloaddition of 2-propyn-1-ol with 1 equivalent of phenyl azide,[38]) was added a 0.5M solution of ceric ammonium nitrate (32.9 g, 2.1 equivalents) in 120 mL water. The reaction mixture was heated to 75°C for 90 min, cooled, and extracted with three 100-mL portions of diethyl ether. The combined ether extracts were washed with two 100-mL portions of a 1 N $NaHCO_3$ solution, dried over anhydrous $MgSO_4$, filtered and the solvent was removed yielding light yellow crystals of the crude aldehyde. Column chromatography of the crude aldehyde on silica-gel using a methylene chloride eluent yielded, after removal of solvent, 1.77 g (35.4%) of analytically pure aldehyde, m.p. 97-99°C (Lit. m.p. 99-100°C).[36] Analysis: C,H,N.

4-Hydroxyiminomethyl-1-phenyl-1,2,3-triazole (2e)

4-Formyl-1-phenyl-1,2,3-triazole (7f) (1.79 g, 10.2 mmol) dissolved in 150 mL of absolute ethanol was treated with 0.77 g, one equivalent, of hydroxyamine hydrochloride and 0.81 g pyridine. The resulting mixture was refluxed for 2 h, the ethanol evaporated, and the residue taken up in 150-mL diethyl ether. The ether was washed with two 100-mL portions water, followed by the addition of 100 mL of diethyl ether saturated with HCl gas to remove residual pyridine. Subsequent washing with two additional 100-mL portions of water, drying over anhydrous $MgSO_4$, filtering and solvent removal yielded 750 mg of a white crystalline product. The material was recrystallized twice from 95% ethanol yielding 550 mg (29%) of an analytically pure sample of 2e; m.p. 171-173°C. NMR (DMSO-d_6), δ 12.17 (s, 1H, NOH), 9.24 (s, 1H, CHNO), 7.98 (m, 2H, phenyl), 7.88 (s, 1H, triazolyl) and 7.55 ppm (m, 3H, phenyl). Analysis: C,H,N.

1-Phenyl-1,2,3-triazol-4-yl Hydroximoyl Chloride (4e)

To 2e (250 mg, 1.3 mmol) dissolved in 15 mL of diemthylformamide, 180 mg (1.3 mmol) of N-chlorosuccinimide was added. After 20 min stirring the solution was heated to 50°C and allowed to cool; then 10 mL of HCl gas, was bubbled into the reaction mixture. After 20 min additional stirring the crude reaction mixture was poured into 60 mL ice-water. Extraction with two 40-mL portions of diethyl ether followed by washing the combined ethereal extracts with three 50-mL portions of water, drying over anhydrous $MgSO_4$, filtering and concentrating, yielded 260 mg (89%) of a white crystalline product. NMR showed essentially quantitative conversion to the desired hydroximoyl chloride product. NMR ($CDCl_3$), δ 11.53 (s, 1H, NOH); 8.8 (s, 1H, CH triazolyl), 7.85 (m, 2H, phenyl) and 7.50 ppm (m, 3H, phenyl). This material was used without further purification.

1-Phenyl-1,2,3-triazol-4-yl Thiohydroximic Acid 2-(Diethyl amino)ethyl S-Ester (3e)

To 260 mg (1.2 mmol) of 4e, prepared above and dissolved in 50 L chloroform, was added 0.20 g diethylaminoethanethiol hydrochloride and 0.5 g (excess) triethylamine. The resulting mixture was stirred for two hours, then washed with two 50-mL portions of water. A solid that remained suspended in the chloroform solution dissolved after the addition of 50 mL of diethyl ether. The mixture was dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 170 mg (44%) of a white crystalline compound. The crude product was recrystallized twice from petroleum ether (30-60°C) yielding off-white needles of 3e; m.p. 241-243°C. NMR (DMSO-d_6), δ 9.15 (s, 1H, CH, triazolyl), 7.95 (m, 2H, phenyl), 7.60 (m, 3H, phenyl), 2.90 (m, 2H, CH_2), 2.33 (m, 6H, CH_2) and 0.80 ppm (t, 6H, CH_3). Analysis for $C_{15}H_{20}N_5OS$. Cal'd: C, 56.60; H, 6.29; N, 22.01; S, 10.06. Found: C, 55.41; H, 6.00; N, 23.02; S, 7.52.

3-Bromomethyl-1,2,5-thiadiazole

In a 250-mL round bottom flask was placed 15.30 g (15.3 mmol) of 3-methyl-1,2,5-thiadiazole (7g) 29.96 g (16.8 mmol) of N-bromosuccinimide, 0.4 g benzoyl peroxide, and 150 mL carbon tetrachloride and the reaction mixture was refluxed overnight. The crude reaction mixture was washed with two 75-mL portions of 1 N sodium thiosulfate and with two 50-mL portions of water. The resulting solution was dried over anhydrous $MgSO_4$, filtered, and solvent evaporated, yielding 20.57 g of crude product. An NMR of the product showed a mixture of starting material, plus mono-, di-, and tribromo-substituted products formed during the reaction in a ratio of 15:50:25:10, respectively. This crude product was used in subsequent reactions without further purufication. NMR ($CDCl_3$), δ 8.63 (s, 1H, CH) and 4.72 ppm (s, 2H, CH_2Br).

3-Hydroxymethyl-1,2,5-thiadiazole

The crude 3-bromomethyl-1,2,5-thiadiazole (prepared above) was treated with 20 g potassium carbonate and 150 mL water. The resulting mixture was heated to 100°C and stirred for 90 min. The crude reaction mixture was then saturated with sodium chloride and extracted with three 100-mL portions of diethyl ether. The combined ethereal extracts were dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 5.25 g of the desired alcohol, contaminated with a small quantity of the dibromo compound. NMR ($CDCl_3$), δ 8.60 (s, 1H, CH), 4.92 (br. s, 2H, CH_2) and 4.95 ppm (br, s, 1H, OH).

3-Formyl-1,2,5-thiadiazole

3-Hydroxymethyl-1,2,5-thiadiazole (5.25 g, 45 mmol) was treated with 52 g of ceric ammonium nitrate dissolved in 190 mL of water. The resulting solution was heated to 75°C for 45 min and the crude reaction mixture extracted with two 50-mL portions of diethyl ether. The combined ether extracts were dried over anhydrous $MgSO_4$, filtered, and solvent removed, yielding the desired aldehyde. Isolation of the aldehyde proved difficult because the material was volatile and codistilled with ether. As a result of this volatility the aldehyde was converted to the desired oxime without isolation. NMR ($CDCl_3$), δ 10.25 (2, 1H, C(O)H) and 9.10 ppm (s, 1H, CH).

3-Hydroxyiminomethyl-1,2,5-thiadiazole (2f)

In a 1 L flask was placed approximately 5.7 g (50 mmol) of crude 3-formyl-1,2,5-thiadiazole in 200 mL diethyl ether, 3.5 g (50 mmol) hydroxylamine hydrochloride, 3.9 g pyridine and 500 mL absolute ethanol. The resulting mixture was refluxed overnight, concentrated, and then taken up in 200 mL diethyl ether. The ethereal solution was washed with two 150-mL portins of 1 H HCl followed by two 150-mL portions of water, dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 1.05 g (16%) of 2f as a white crystalline material. The NMR of the crude product was consistent with an assigned structure of the E- and Z-aldoximes (see above).

Recrystallization from 25% diethyl ether/75% petroleum ether (30-60°C) yielded an analytically pure sample of the mixed E- and Z-aldoximes; m.p. 148-149°C NMR (DMSO-d_6); z-aldoxime (major component), δ 12.10 (s, 1H, NOH), 9.12 (s, 1H, CHNO), and 8.43 ppm (s, 1H, CH); E-aldoxime (minor component), δ 12.53 (s, 1H, NOH), 9.50 (s, 1H, CHNO), and 7.95 ppm (s, 1H, CH). Analysis; C,H,N,S.

1,2,5-Thiadiazol-3-yl Hydroximoyl Chloride (4f)

Aldoxime 2f (160 mg, 1.1 mol) dissolved in 15 mL dimethylformamide was treated with N-chlorosuccinimide (160 mg, 1.1 mmol). Reaction and workup identical to those described previously for 4a yielded 190 mg (100%) of the desired hydroxyimoyl chloride. NMR (DMK-d_6), δ 12.07 (s, 1H, NOH), and 9.00 ppm (s, 1H, CH). This material was converted to 3f without further purification or characterization.

1,2,5-Thiadiazol-3-yl thiohydroximic acid 2-(Diethylamino)ethyl S-Ester (3f)

To 190 mg (1.1 mmol) of 4f prepared above in (50 mL of chloroform) was added 210 mg (1.1 mmol) of diethylaminoethanethiol hydrochloride and 250 mg (2.2 mmol) of triethylamine. The reaction mixture was stirred for 2.5 h then washed with two 100-mL portions of water, dried over $MgSO_4$, filtered and concentrated, yielding a yellow oil.

The oil slowly solidified, was triturated with diethyl ether, and filtered, yielding 55 mg (19%) of 3f as white crystalline material; m.p. 122-124°C. An analytical sample of 3f was obtained by column chromatography of the ethereal residue on silica-gel using a diethyl ether eluant. Like fractions were combined and concentrated after TLC examination. NMR (DMK-d_6), δ 9.07 (s, 1H, CH), 2.10 (m, 2H, SCH_2), 2.50 (m, 6H, CH_2) and 0.95 (t, 6H, CH_3). Analysis for $C_9H_{16}N_4S_2$: Cal'd: C, 41.54; H, 6.15; N, 20.77. Found: C, 41.13; H, 6.26; N, 21.62.

3-Bromomethyl-4-methyl-1,2,5-thiadiazole*

The subject material was prepared in a manner similar to that described above for 3-bromomethyl-1,2,5-thiadiazole. An NMR of the crude reaction product showed a mixture of starting material and monobromide, with an

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*Caution: 3-Bromomethyl-4-methyl-1,2,5-thiadiazole is a severe lachrymator and should be handled accordingly.

approximate 60% conversion. The material was converted to the alcohol without further characterization. NMR ($CDCl_3$), δ 4.55 (2, 2H, CH_2Br) and 2.50 ppm (s, 3H, CH_3).

3-Hydroxymethyl-4-methyl-1,2,5-thiadiazole

The crude monobromide, described above, was treated as described previously for 3-bromomethyl-1,2,5-thiadiazole with potassium carbonate in water at 90°C, yielding the crude monoalcohol contaminated with only small quantities of the dibromide. NMR ($CDCl_3$), δ 4.80 (br, s, 3H, CH_2OH), and 2.50 ppm (s, 3H, CH_3). The material was oxidized to the corresponding aldehyde without further purification.

3-Formyl-4-methyl-1,2,5-thiadiazole

The crude monoalcohol (12.75 g, 0.1 mmol) was treated with 104 g, 2.1 equivalents of ceric ammonium nitrate dissolved in 400 mL of water. Reaction and workup, as described for the previously prepared aldehydes, yield the volatile aldehyde. Because of the aldehyde's volatility it was converted to the desired oxime without isolation. NMR ($CDCl_3$), δ 10.27 (s, 1H, C(O)H) and 2.80 ppm (s, 3H, CH_3).

3-Hydroxyiminoethyl-4-methyl-1,2,5-thiadiazole (2g)

In a 1 L flask was placed 6.4 g (50 mmol) of the freshly prepared aldehyde described above in 225 mL diethyl ether, 3.5 g (50 mmol) hydroxylamine hydrochloride 3.9 g pyridine and 500 mL of absolute ethanol. The resulting mixture was refluxed for two h, and the solvent was evaporated. The residue was dissolved in 75 mL of diethyl ether, the ether washed with two 150-mL portions of 1N HCl followed by 150 mL of water. The ethereal solution was dried over $MgSO_4$, filtered and concentrated to yield crystalline 2g. An analytical sample was obtained by repeated recrystallization from a 1:1 petroleum ether (30-60°C)/diethyl ether mixture; m.p. 131-134°C. NMR (DMSO-d_6), δ 12.05 (s, 1H, NOH), 8.48 (s, 1H, CH), and 2.73 (s, 3H, CH_3). Analysis: C,H,N,S.

4-Methyl-1,2,5-thiadiazol-3-yl Hydroximoyl chloride (4g)

A 1.73 g (12 mmol) sample of 2g, prepared above, dissolved in 60-mL diemthylformamide was treated with 1.62 g (12 mmol) of N-chlorosuccinimide. The hydroximoyl chloride 4g was obtained in a manner similar to that described for the previous hydroximoyl chlorides. The NMR indicated an approximate 85% conversion to the hydroximoyl chloride. NMR (DMK-d_6), δ 12.88 (s, 1H, NOH) and 2.80 ppm (s, 3H, CH_3). The material was converted to 3g without further purification.

4-Methyl-1,2,5-thiadiazol-3-yl Thiohydroximic acid 2-(Diethyl amino)ethyl S-Ester hydrochloride (3g)

To 1.55 g (8.7 mmol) of 4g prepared above, in 75 mL of chloroform was added 1.48 g (8.7 mmol) diethylaminoethanoethiol hydrochloride and 1.76 g

(17.4 mmol) of triethylamine (two equivalents), and the resulting solution was stirred overnight. The crude reaction mixture was then washed with 100 mL of water, dried over anhydrous $MgSO_4$, filtered, and concentrated. TLC examination of the residue (diethyl ether eluent on silica-gel) indicated three components. The crude material was purified on 125 g of silica-gel using a 100% diethyl ether eluant. The first fraction proved to be 2g; NMR and melting point were identical to authentic sample. The second fraction, 0.93 g (39%) of a yellow oil, proved to be the free amine of the desired 3g. The hydrochloride salt of 3g was prepared by dissolving the above oil in 25 mL of diethyl ether and adding dropwise an ethereal solution saturated with HCl gas. Upon addition of the ethereal HCl, white crystals of the hydrochloride salt 3g precipitated. After saturation, the white crystals were filtered and dried under vacuum, yielding 0.98 g of analytically pure 3g; m.p. 179-182°C. NMR (DMSO-d_6), δ 12.72 (s, 1H, NOH), 3.18 (m, 9H, CH_2), 2.63 (s, 3H, CH_3) and 1.10 ppm (t, 6H, CH_3). Analysis: C,H,N,S,Cl.

AChE Reactivation

Ethyl p-nitrophenyl methylphosphonate was added to a slight excess of electric eel AChE in pH 7.6, 0.1 M morpholinopropane sulfonic acid buffer, and NaN_3 (0.002%), $MgCl_2$ (0.01 m), and bovine serum albumin (0.01%). Aliquots of ethyl methylphosphonyl-AChE were diluted into buffer solution containing reactivators at various concentrations, and incubated for timed intervals before withdrawing aliquots and assaying for activity by the method of Ellman.[39] All experiments were performed at 25 ± 0.5°C. Rate constants were determined by linear least-squares regression analysis of the data and error limits reported as standard deviations. Details of the method have been previously described.[23]

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