Final Report

DEFENSE AGAINST NERVE AGENTS: NOVEL ANTIDOTES AND BIOCHEMICAL APPROACHES TO PROPHYLACTICS AND DETOXIFICATION

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Organophosphorus nerve agents inhibit acetylcholinesterase (AChE), an enzyme which is involved in controlling nerve impulses in the central nervous system. The goal of this project is to develop novel reactivators which will restore the activity of "aged" AChE inactivated by Soman. None of the currently available AChE reactivators will reactivate organophosphonate inhibited AChE molecules which have undergone aging. Heterocyclic aromatic derivatives have been designed and synthesized as potential reactivators. Several derivatives of 3-hydroxy-1-methylpyridinium-2-aldoxime are active in animals as reactivators. Dimethylcarbamyl derivatives of 3-hydroxypyridines are active in animals as prophylactic agents. Other compounds are currently being tested in animals.

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SUMMARY

Most chemical warfare nerve agents are organophosphorus esters. Soman is the chief nerve gas produced and stockpiled by the Soviets, and other common agents are Sarin and the persistent nerve gas VX . These nerve agents react covalently with the enzyme acetylcholinesterase (AChE). This enzyme hydrolyzes the neurotransmitter acetylcholine which is released during nerve impulse transmission. In the absence of AChE activity, the acetylcholine concentration builds up at the acetylcholine receptor resulting in severe neurological imbalance, paralysis, and death.

Therapy for anti-AChE intoxication is based on coadministration of anticholinergics (e.g. atropine) and of AChE "reactivators" that displace the phosphonyl residue from the active site and restore enzymatic activity. Such therapy is ineffective with Soman intoxication since the alkyl phosphonate ester formed from Soman undergoes a very rapid loss of the alkoxy side chain after the initial phosphonylation of AChE. This "aging" process results in a negatively charged phosphonyl ester which repels the nucleophilic reactivators. It is thus evident that the development of effective antidotes for Soman and other organophosphorus nerve agents is an important goal.

The goal of this research program was the rational design and synthesis of reactivators which will work even with Soman-inhibited AChE which has undergone aging. Potential reactivators were designed and synthesized which contain not only a positively charged group to bind to the enzyme and a nucleophile to reactivate the enzyme, but also an additional positively charged group or hydrogen bonding group to neutralize the negative charge on the phosphonate, thus making it susceptible to nucleophilic attack and removal from the enzyme. A number of compounds were synthesized which meet the design criteria, but adequate solution reactivation kinetic studies have not yet been performed. Thus the validity of our design hypothesis remains untested.

In the latter portions of this contract, heterocyclic, aromatic, and amino acid reactivators were synthesized and some have been tested in animals. The most active compound were 3-hydroxy-2-pyridinealdehyde semicarbazone (ICD 722). Testing is incomplete on analogs of this semicarbazone and several directly related oximes showed no activity. Thus, it is difficult to derive significant structure-function relationships at present.

Several pyridine dimethyl carbamates have been synthesized and tested as pretreatment agents. Several compounds show very high *in vivo* activity, particularly derivatives with hydrophobic oximes in the 2-position. A number of potentially effective carbamates also have not yet been tested.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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James C. Powers DATE

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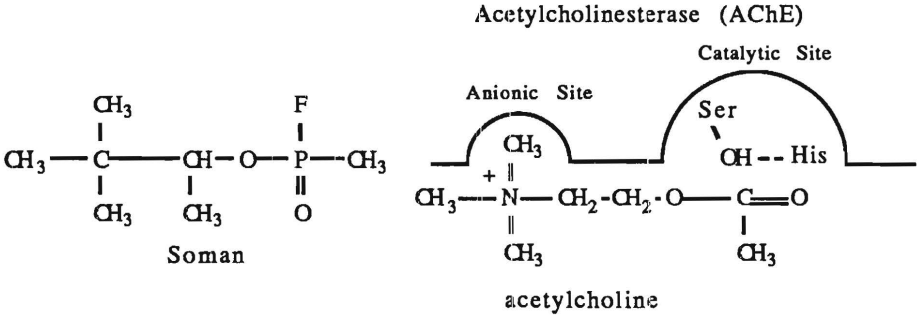
INTRODUCTION

Most chemical warfare nerve agents are organophosphorus esters. Soman (GD, 1,2,2-trimethylpropyl methyl phosphonofluoridate) is the chief nerve gas produced and stockpiled by the Soviets, and other common agents are Sarin (GB, isopropyl methylphosphonofluoridate) and the persistent nerve gas VX (O-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate). These nerve agents react covalently with the enzyme acetylcholinesterase (AChE). This enzyme hydrolyzes the neurotransmitter acetylcholine which is released during nerve impulse transmission. In the absence of AChE activity, the acetylcholine concentration builds up at the acetylcholine receptor resulting in severe neurological imbalance, paralysis, and death.



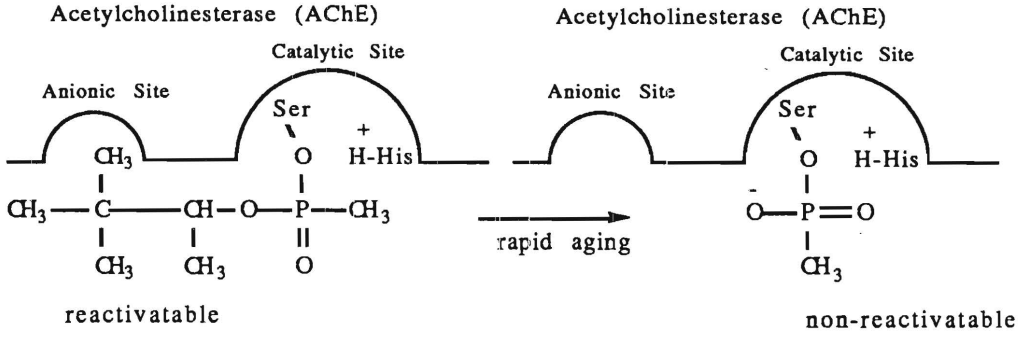
The active site of AChE consists of a negative subsite, which attracts the quaternary group trimethylammonium group of choline through both coulombic and hydrophobic forces, and a catalytic site where nucleophilic attack occurs on the acyl carbon of the substrate. The catalytic mechanism resembles that of serine proteases, where a serine hydroxyl group is rendered highly nucleophilic due to hydrogen bonding with the imidazole ring of an active site histidine residue. Nerve agents inhibit AChE by reacting with the active site serine residue forming stable phosphonyl derivatives.

Acetylcholinesterase Reactivators. The inhibition of AChE by organophosphorus esters is difficult to reverse since the active-site serine phosphonate (or phosphate) esters formed are hydrolyzed extremely slowly by water. Stronger nucleophiles such as hydroxylamine will displace the phosphonate and reactivate the enzyme, but high concentrations are required. One of the most effective antidotes against nerve agents such as Sarin is 2-pyridine aldoxime methiodide or methane sulfonate (PAM). PAM has a good nucleophile (oximino functional group) to reactivate the enzyme by displacing the phosphonate from the active site serine of AChE. In addition, it is cationic and can bind at the anionic site of AChE, an essential feature in useful reactivators. Due to a proximity effect, PAM is 10^6 times more effective at reactivating AChE than nucleophiles such as hydroxylamine. Agents such as PAM have significant pharmacological properties in their

own right and individuals treated with PAM are immobilized for considerable periods of time.

During the last decade, considerable effort has been devoted to the development of other types of AChE reactivators. As a result more effective AChE reactivators have been discovered. Bispyridinium dioximes such as Obidoxime and bispyridinium monooximes such as HS-6 and HI-6 have been shown to be particularly effective (DeJong and Wolring, 1980). Indeed HI-6 is a more powerful reactivator than conventional oximes, is one of the most potent reactivators of Soman-inhibited AChE, and is able to compete with aging (see next section). The fact that bispyridinium compounds are effective reactivators indicates that the active site of AChE contains an additional anionic site and is also quite hydrophobic (Cannon, 1981).

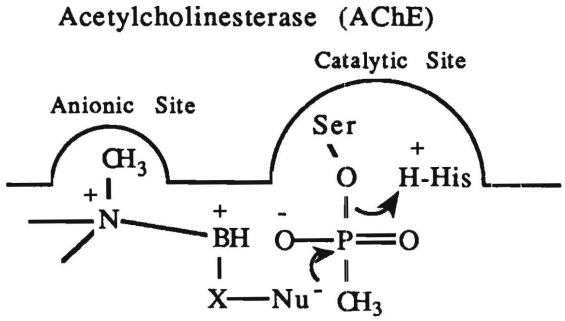
Aging. Soman is an especially insidious nerve agent since it is a powerful AChE inhibitor and the inhibited enzyme is difficult to reactivate. The alkyl phosphonate ester formed from Soman undergoes a very rapid loss of the alkoxy side chain after the initial phosphonylation of AChE, resulting in a negatively charged phosphonyl ester (shown below). The aging reaction occurs with all phosphonylated or phosphorylated AChE molecules, but in the case of Soman-inhibited AChE it is particularly rapid (half-life = 1-2 min). The aging reaction is acid catalyzed, may involve the histidine of the enzyme, and some reactivators actually accelerate the aging process (Berry and Davies, 1970; Sun et al., 1979; Schoene et al., 1980). The negatively charged phosphonyl enzyme formed upon aging repels nucleophiles and even oximes such as PAM are ineffective in dephosphonylating AChE which has been inhibited by Soman. With Sarin and VX, the rate of aging is slower, and thus oximes can compete with aging and effect reactivation of the AChE molecules. Soman-inhibited AChE is essentially impossible to reactivate with currently available reactivators unless a highly efficient reactivator such as HI-6 is present at the time of exposure.



APPROACH TO THE PROBLEM

Initial Approach. The initial goal of this research was the rational design and synthesis of reactivators of Soman-inhibited AChE in order to develop more effective antidotes for Soman poisoning. The AChE reactivators designed up to this point contain both a positively charged functional group to bind to the anionic site of the enzyme and a nucleophile to remove the phosphonate from the enzyme. This type of reactivator does not work with inhibited AChE molecules which have undergone aging since the phosphonate in the enzyme's active site has a negative charge and is poorly susceptible to nucleophilic attack

Our initial rationale was designed to overcome this problem. In particular, we designed and synthesized agents which contain not only a positively charged group to bind to the enzyme and a nucleophile to reactivate the enzyme, but <u>also</u> an additional positively charged group or hydrogen bonding group to neutralize the negative charge on the phosphonate, making it susceptible to nucleophilic attack and removal from the enzyme. Most antidotes developed thus far contain only one cationic group which is designed to bind to the substrate anionic recognition site of AChE. Some of the reactivators contain a second cationic group (e.g. bispyridinium PAM derivatives), but <u>none</u> contain a second cationic group placed in the <u>appropriate</u> structural position so as to facilitate nucleophilic attack on the organophosphonate ester and its subsequent removal. A schematic drawing illustrating the design rationale is shown below. The nucleophile (Nu^-) could be a functional group such as an oxime, hydroxamic acid, thiol, or carboxylate. The cationic group (BH^+) could be a cationic ammonium group or a hydrogen bonding group such as an alcohol. Of course the structure also must contain a cationic group to interact with the anionic binding site of AChE.



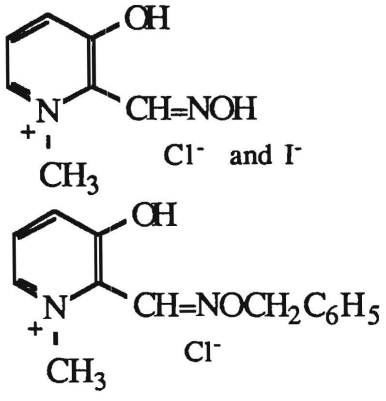
Combine in one molecule a nucleophile, an enzyme recognition moiety, and a group to neutralize the phosphonate negative charge.

Nucleophiles (Nu) could be oximes, hydroxamic acids, thiols, carboxylates, etc.

The cationic group (BH) could be ammonium groups, carboxylic acids, hydrogen bonding groups or many other proton donors.

We investigated a variety of molecules with the structural features described above, but a great portion of our effort was directed at heterocyclic bifunctional reactivators. This approach involves addition of hydrogen bonding groups or acidic groups to

PAM to give structures that fit our design criteria. One such structure is shown below (top left). This is a 3-hydroxy derivative of PAM and should act as a reactivator by the mechanism shown (right). The hydroxyl group can hydrogen bond to or protonate the oxyanion of the phosphonate while the oxime functional group effects dephosphonylation. Other structures investigated will be described in the Results and Discussion section.



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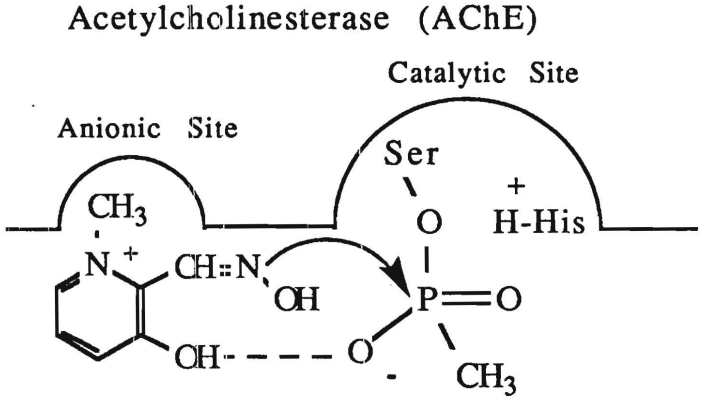
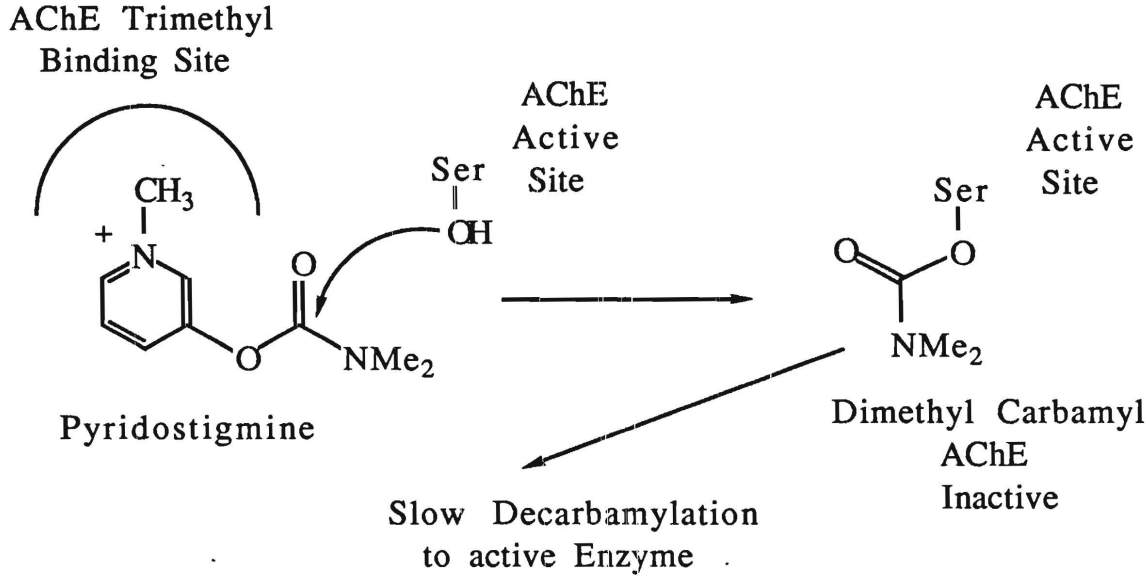


It should be pointed out that our primary design rationale has yet to be adequately tested. When this contract was funded, all the kinetic studies were removed and there are no current experiments which test whether any of the compounds are actually dephonylating aged inhibited acetylcholinesterase. We hope to perform such reactivation experiments in the future.

Later Approaches. An increasingly popular option to circumvent "aging" in nerve gas poisoning is through pretreatment with a reversible inhibitor of AChE. This can involve use of reversible (competitive) inhibitors of AChE which protect the active site until the nerve agent in cleared from the system or is hydrolyzed. One of our compounds (ICD 677, above figure, lower left) was shown to be active in the pretreatment assay. This was one of the first compounds which was tested in animal models. We speculated that the O-benzyl derivative could utilize additional hydrophobic binding sites in the active site of AChE and might be a very good competitive inhibitor of AChE. Thus, the initial positive test results stimulated the synthesis of several additional analogs. Unfortunately, retesting did not confirm the initial positive results. Indeed, this is not the only compound which gave widely differing test results in similar test situations at different times during this program.

Other widely studied pretreatment agents are the carbamates pyridostigmine (shown below) and physostigmine. This drugs are covalent inhibitors which transfer their carbamoyl group to the serine hydroxyl at the active site of AChE. This inhibits the

enzyme and also protects it from phosphonylation. The half-life for spontaneous hydrolysis of carbamylated enzyme is in the order of 30 min (Main, 1976) while that of phosphorylated or phosphonylated AChE (non-aged) is in the order of many hours to as long as 30 days. Thus the carbamylated enzyme will regenerate active enzyme in a few minutes giving the body a chance to clear or hydrolyze the nerve agent. Since we prepared a number of hydroxy pyridine derivatives, the corresponding carbamates were also synthesized for testing.

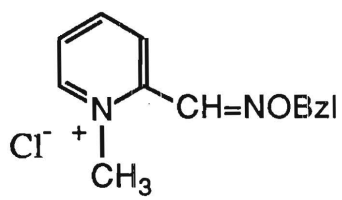


RESULTS AND DISCUSSION

Biological Testing. This program has suffered from a lack of timely testing data. A large number of compounds still remain to be tested. Much testing data was received as the program was ending. Thus, for most of the three year contract, it was not possible to direct our synthetic efforts based on testing of structures already submitted. As mentioned above, we still have no kinetic evidence to test our initial dephosphonylation hypothesis.

Enzymatic Activity-Pyridine Oximes. In order to compensate for the lack of timely testing and prepare for publication, Mr. Steve Thornton has been performing kinetic studies with most of the compounds which have been synthesized. He has tested every compound as a reversible inhibitor of AChE from electric eel and some with the human erythrocyte enzyme. The carbamate have been tested as irreversible inhibitors with the electric eel enzyme and will be tested in the future with the human erythrocyte enzyme. This work was not supported by the army, but is included for completeness.

With the exception of pyridine derivatives, none of the synthesized compounds were competitive inhibitors of AChE. The results obtained with pyridine oxime derivatives are shown in Table I. The best inhibitor of the human erythrocyte enzymes was ICD891. This compound showed no activity either in the pretreatment or adjunct efficacy assays. Interestingly, addition of a 3-OH group or substituents on the benzyl group resulted in poorer inhibitors. The methyl oxime was inactive which shows that the benzyl group is probably interacting with a aromatic binding site on the surface of AChE.

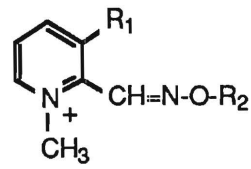


ICD 891

The 3-OH derivative, ICD677, was the compound that lead to the synthesis of most of those analogs. Interestingly, it binds less tightly to the enzyme. Although, ICD 677 was initially shown to have good activity in the pretreatment assay, it lost all of its activity upon retesting.

Enzymatic Activity-Pyridine Oxime Carbamates. The corresponding carbamates were also prepared and irreversible inhibition kinetic determined with electric eel AChE (Table I), results with the human enzyme have not yet been obtained. The $k_{obs}/[I]$ values are second order inhibition rate constants. The benzyl oxime carbamate ICD 1099 (shown below) was a good

Table I. Inhibition of Electric Eel and Human Erythrocyte Acetylcholinesterase by Pyridine Oxime Derivatives.



Competitive Inhibition.

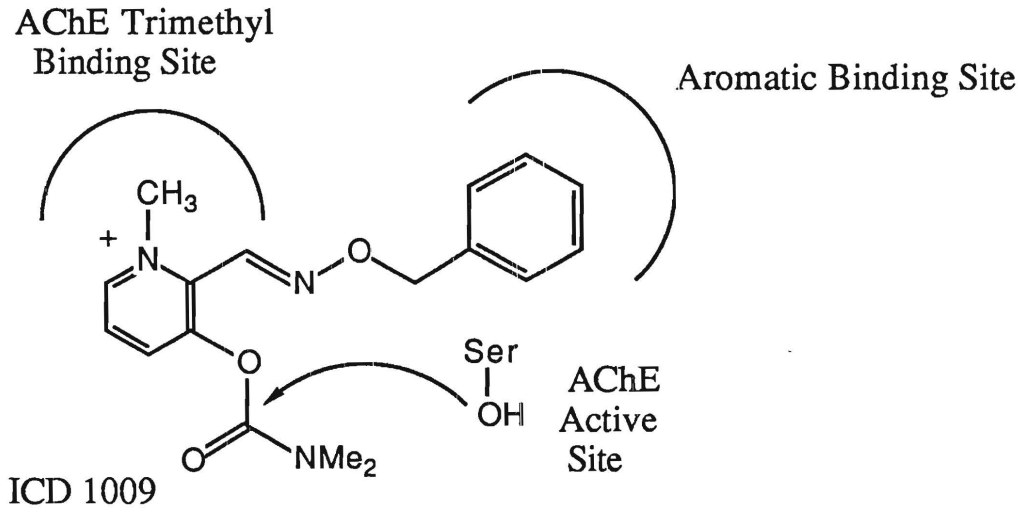
R_1	R_2	Electric Eel K_I	H. Erythrocyte I_{50}
H	benzyl	40 uM	160 uM
OH	benzyl	---	500 uM
OH	methyl	---	No Inhib.
OH	*p*-Cl-Benzyl.	---	400 uM
OH	*p*-OMe-Benzyl.	---	400 uM

Irreversible Inhibition.

R_1	R_2 $k_{obs}/[I]$	Electric Eel $k_{obs}/[I]$	H. Erythrocyte
$OCON(Me)_2$	benzyl	1650 $M^{-1}s^{-1}$	
$OCON(Me)_2$	$CH\text{-}(Ph)_2$	870	
$OCON(Me)_2$	p-OMe-Bz.	5000	

The enzymes used were electric eel acetylcholinesterase (type VI-S) and human erythrocyte (membrane bound, type XIII) acetylcholinesterase.

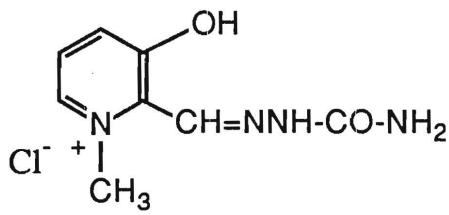
inhibitor. Obviously, the benzyl group is interacting with an aromatic binding site as shown.



The diphenylmethyl oxime (ICD 1038) was a 2 fold poorer inhibitor, while the p-methoxybenzyl oxime derivative (ICD???, BL52696) was over 2 fold better as an inhibitor.

In the pretreatment assay, the benzyl oxime was effective at a dose of 0.003 mg/Kg at both 15 min. (90% survivors) and 60 min. (80% survivors). The diphenylmethyloxime was also effective, but a higher dose was required. With 0.14 mg/Kg, there were 90% survivors at 15 min. pretreatment and 60% at 60 min. This compound was also less toxic (LD50 = 9.2 mg/Kg vs 0.18 mg/Kg for ICD1009). The p-methoxybenzyl derivative which would be predicted to be the most effective, has not yet been tested in the pretreatment assay.

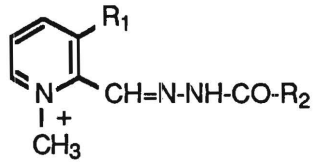
Enzymatic Activity-Pyridine Semicarbazones. A number of pyridine semicarbazone were synthesized and tested as reversible inhibitors for acetylcholinesterase. The results are shown in Table II. These compounds were based on ICD 722 which was also an early compound tested and shown to be active as a reactivator.



ICD722

This semicarbazone was a moderate inhibitor of human erythrocyte AChE. Addition of a phenyl to the terminal nitrogen resulted in a slight improvement, although the kinetics were

Table II. Inhibition of Electric Eel and Human Erythrocyte Acetylcholinesterase by Pyridine Semicarbazone Derivatives.



Competitive Inhibition.

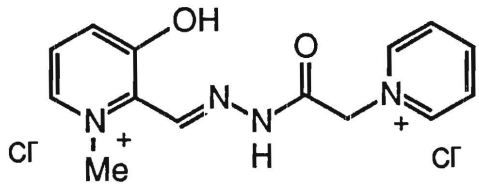
R_1	R_2	Electric Eel K_I	H. Erythrocyte K_I	I_{50}
OH	NH_2	-----	-----	300 uM
OH	NH-C_6H_5	*	*	240 uM
$OCON(Me)_2$	NH-C_6H_5	85 uM		
OH	CH_2-$NC_5H_5^+$	10 uM	20 uM	

The enzymes used were electric eel acetylcholinesterase (type VI-S) and human erythrocyte (membrane bound, type XIII) acetylcholinesterase.

*The two K_I values were 30 uM and 340 uM (EE), multiple binding with both enzymes.

All semi-carbazones show mixed-type inhibition (linear non-competative).

complex and gave two K_I values. The corresponding carbamate was also a good inhibitor. But the best inhibitor was the bis-quarternary derivative (ICD???, BL54770) shown below. This showed a KI value of 10 and 20 μM respectively with electric eel and human erythrocyte AChE (Table II). The corresponding carbamate would be an excellant compound to test also.

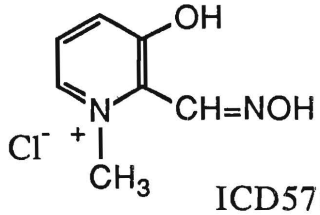


BL54770

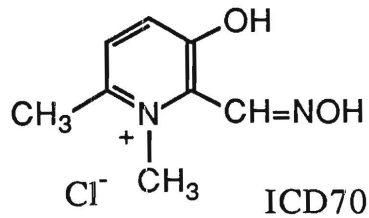
The parent semicarbazone ICD722) was active as reactivator at a high dose of 100 mg/Kg (70% survivors). It was also active in the adjunct efficacy assay (90% survivors at 100 mg/Kg). Interestingly, the corresponding N-phenyl derivative (ICD1041) was not active in a pretreatment assay. The corresponding carbamate and BL54770 have yet to be tested.

Biological Testing-3-Hydroxypyridine Derivatives. Biological testing data with all the 3-hydroxypyridine derivatives is shown in Table III. Unfortunately, there are still major gaps in the testing data. In addition, the data should be treated with some skeptism. Two compounds exhibited a complete reversal upon retesting. As described above, ICD 677 went from active to inactive, while ICD 721 (Table IV) when from inactive to active.

The PIs find it hard to believe that several simple derivatives of PAM were found to be inactive. Both the 3-hydroxy derivatives ICD 576 and ICD 701 showed no activity. It cann't be steric since much larger derivatives of ICD 576 did show activity.



ICD576



ICD701

Of all the 3-hydroxypyridine derivatives tested, the only ones that are active are those previously discussed in the kinetics section (ICD 722, ICD 1009, ICD 1038, and ICD 1039 with moderate activity). Future testing and kinetic studies may shed more light on the validity of the current results and allow the development of additional structure activity relationships.

Table III. 3-Hydroxypyridine Derivatives

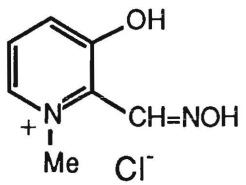
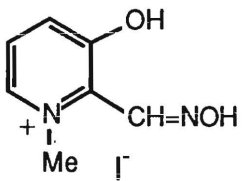
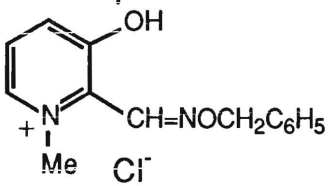
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL23999	3g	9/86				
	BL20229 ICD 576	2g	5/86	>110	reactivator	110	0
	BL23980 ICD 677	3g	9/86	>245	reactivator (diazepam)	1.6	20
					pretreatment 15 min	1.6	20
						12.5	80
						100	80
					pretreatment 60 min	1.6	30
						12.5	100
						100	100
					pretreatment 15 min	6.25	0
						25	0
					pretreatment 60 min	6.25	10
						25	10
	BL50012	9g	11/87				

Table III. (Continued) 3-Hydroxypyridine Derivatives

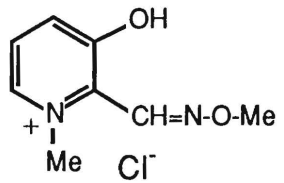
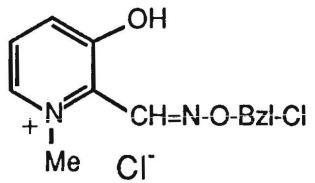
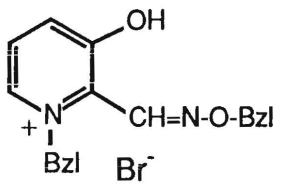
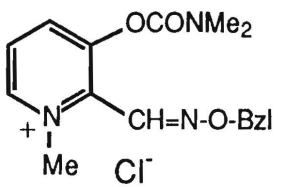
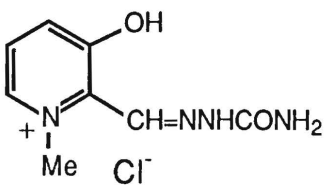
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL50478 ICD 944	3g	12/87	>400	reactivator	100	0
	BL51546	3g	2/88				
	BL51528 ICD 1008	3g	2/88	>93	pretreatment	46.5	0
	BL51537 ICD 1009	3g	2/88	0.18	pretreatment 15 min.	.003 .01 .05	90 80 90
					pretreatment 60 min.	.003 .01 .05	80 80 90
	BL30985 ICD722	3g	12/86	>255 >255	reactivator (0.1 M NaOH) adjunct efficacy	100 6.25 25 100	70 50 70 90
	BL55777	6.9	10/88		behavioral deficit free dose	>63.8 mg/Kg	

Table III. (Continued) 3-Hydroxypyridine Derivatives

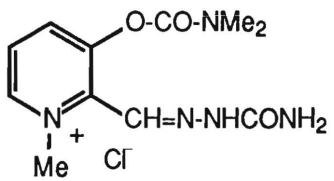
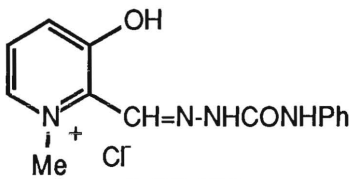
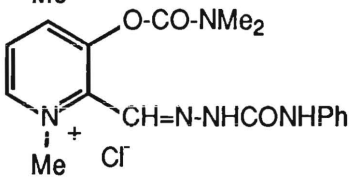
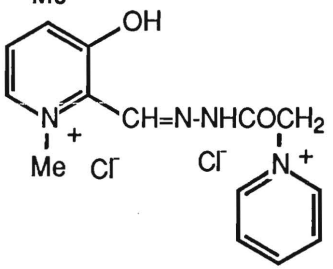
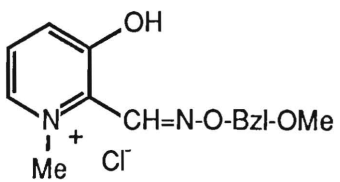
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL55786	2.3g	10/88				
	BL52507 ICD 1041	3g	4/88	>13	pretreatment 15 min. pretreatment 60 min.	6.5 6.5	0 10
	BL52516	3g	4/88				
	BL54770	2.7g	8/88				
	BL52525 ICD 1039	3g	4/88	>235	pretreatment 15 min. pretreatment 60 min.	100 100	40 0

Table III. (Continued) 3-Hydroxypyridine Derivatives

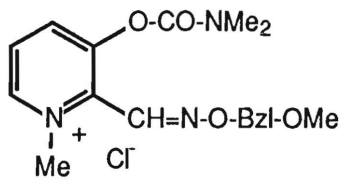
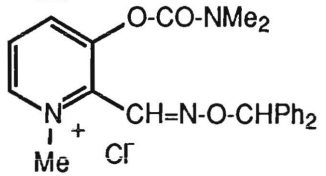
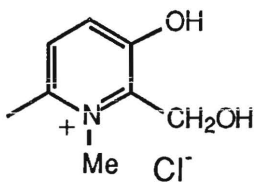
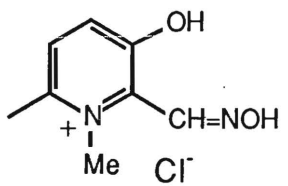
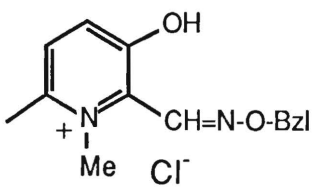
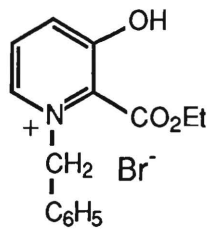
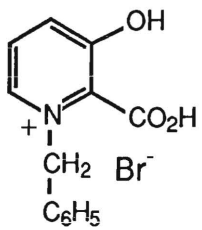
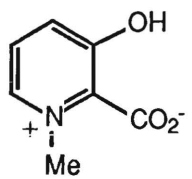
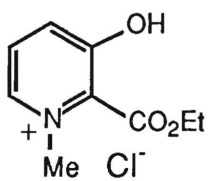
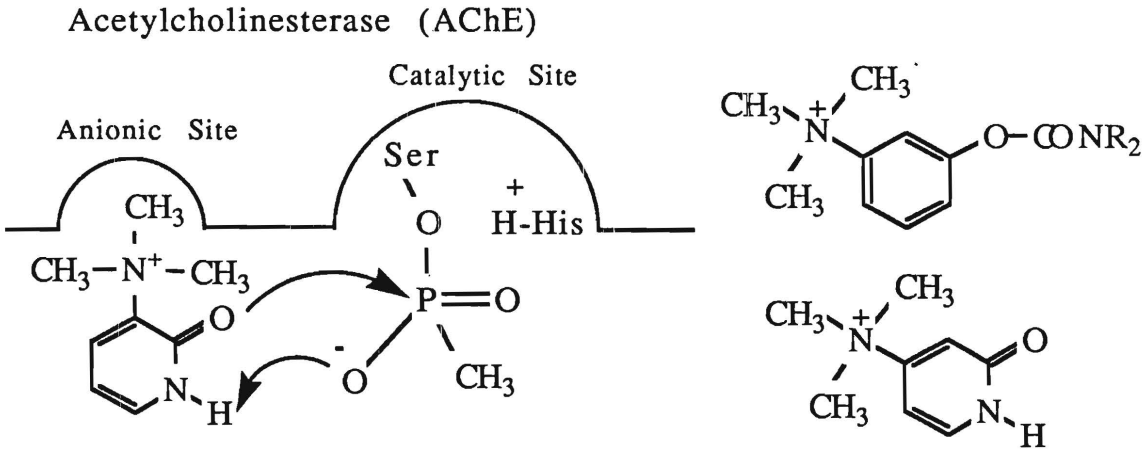
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL52696	3g	4/88				
	BL52534 ICD 1038	3g	4/88	9.2	pretreatment 15 min. pretreatment 60 min.	0.14 0.60 2.3 0.14 0.60 2.3	90 60 30 60 70 60
	BL28350	3g	11/86				
	BL28092 ICD 701	3g	11/86	>392	reactivator pretreatment 15 min. pretreatment 60 min.	100 100 100	0 20 0
	BL49457 ICD 895	3g	9/87	>275	pretreatment 15 min pretreatment 60 min	100 100	10 0

Table III. (Continued) 3-Hydroxypyridine Derivatives

Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	Bl39817 ICD 786	3g	4/87	>400	reactivator (water)	6.25	10
	BL39808 ICD785	3g	4/87	>20	adjunct efficacy reactivator	0.625 2.5 10 10	70 50 40 0
	BL40696 ICD 816	3g	5/87	>94	pretreatment 15 min. pretreatment 60 min	47 47	0 10
	BL40687	3g	5/87				

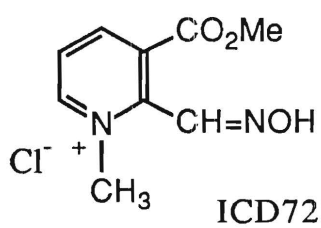
Biological Testing-Other Pyridines and Pyridones. Testing data with other pyridines and pyridones are shown in Table IV. Some of the pyridines are simply analogs of 3-hydroxypyridines (Table III) which lack the hydroxyl group. Several of these compounds were synthesized when enzyme inhibition studies showed that compounds without a 3-OH group bound better to AChE.

Another approach to AChE reactivators involved the synthesis of 2-pyridones and pyridone-like structures containing dimethylamino or trimethylammonium functional groups. One representative compound is shown below (left). This structure is based on the classic observation of Swain that 2-pyridones are excellent bifunctional or acid-base catalysts, and on the observation that carbamate esters containing quaternary methyl ammonium functional groups (below, top right) are capable of binding to the active site of AChE and carbamylating the active site serine residue (Cannon, 1981). In the reactivation mechanism, we expected the oxyanion of the aged phosphonyl derivative of AChE to assist in its own hydrolysis. Since dephosphonylation is quite analogous to the deacylation step in the normal catalytic cycle of AChE, we expected that the protonated histidine in the active site of the enzyme would act as the proton donor during dephosphonylation (Rosenberry, 1975).



Thus far, none of the 2-pyridones have shown any significant activity as reactivators.

The only active compound (upon retesting) in Table IV was ICD 721.



ICD721

Table IV. Other Pyridines and Pyridones

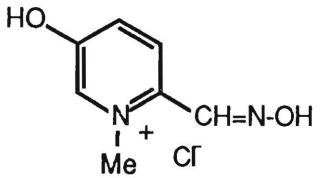
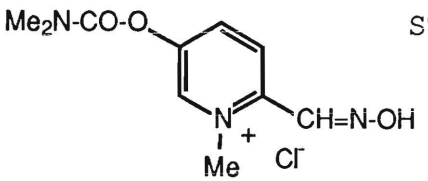
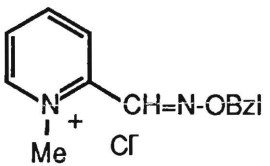
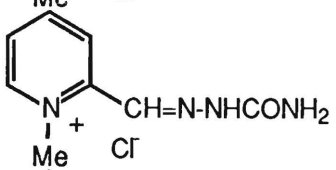
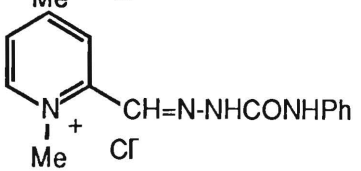
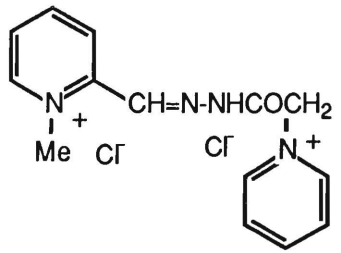
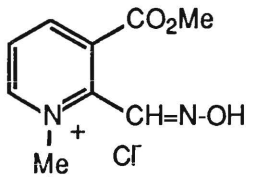
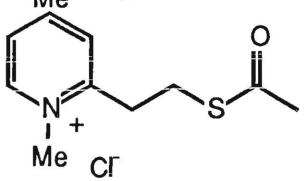
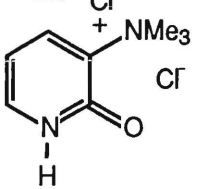
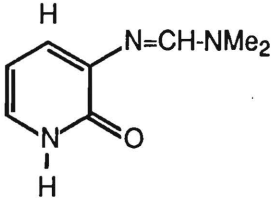
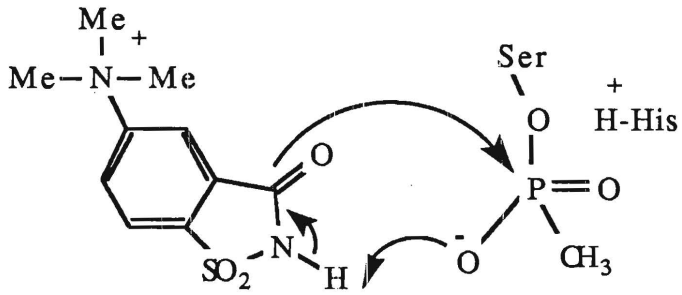
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL28109 ICD 702	3g	11/86	>97	pretreatment 15 min pretreatment 60 min reactivator	48.5 48.5 48.5	0 20 0
	ST-41B	0.78g	12/88				
	BL49260 ICD 891	3g	9/87	47.4	pretreatment 15 min pretreatment 60 min adjunct efficacy	11.9 11.9 11.9	20 10 10
	BL53497	3.1g	6/88				
	BL53504	3.1g	6/88				

Table IV. (Continued) Other Pyridines and Pyridones

Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL56238	2.8g	11/88				
	BL30976 ICD 721	3g	12/86	154	reactivator (water) reactivator (retest)	38.5 38.5	10 70
	BL22456	3.1g	8/86				
	BL31964 ICD 747	2.5g	1/87	113	reactivator (water) pretreatment	28 15 60	10 0 0
	BL40703 ICD 817	3g	5/87	>200	adjunct efficacy	87.5	60

Biological Testing-Aromatic Compounds and Saccharins. Testing results with aromatic and saccharin reactivators are shown in Table V. The design rationale for the aromatic reactivators are shown before.

The rationale for the saccharins is shown below. With saccharin derivatives, this process may be especially favored due to the relatively high acidity of the proton on a nitrogen alpha to both a carbonyl and a sulfonyl moiety. The most promising saccharin has not yet been tested.



The rationale for the aromatic reactivators is shown below for one of the compounds. This compound has moderate activity in the adjunct efficacy assay, but has not been tested as a reactivator. Unfortunately, we didn't have the time or manpower to adequatedly pursue either the aromatic or saccharins potential reactivators.

Table V. Aromatic Compounds and Saccharins

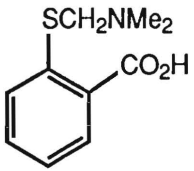
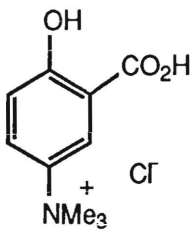
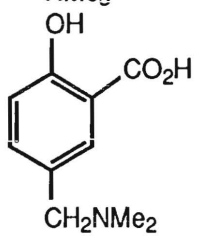
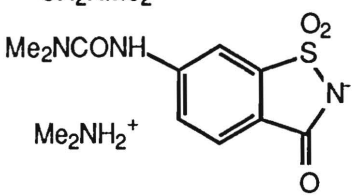
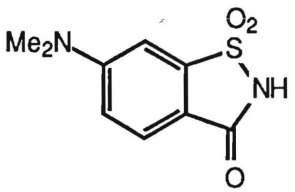
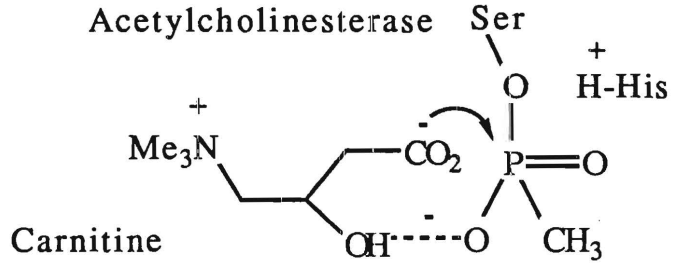
Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL40516	3g	4/87	>220	reactivator (diazepem)	25	10
	BL40525 ICD811	3g	4/87	>230	reactivator (water)	100	10
	BL39791 ICD 784	3g	4/87	>69	adjunct efficacy	2.09 8.39 33.5	70 20 50
	BL40605 ICD 818	3g	5/87	400 1000	pretreatment (15 min water) pretreatment (60 min) oral pretreatment (15 min) oral pretreatment (60 min.)	100 100 250 250	20 10 30 0

Table V. (Continued) Aromatic Compounds and Saccharins

Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL49251	3g	9/87				

Biological Testing-Amino Acid Derivatives and Miscellaneous Compounds. The results are shown in Table VI for a series of amino acid derivatives and miscellaneous compounds. None have yet shown any activity. Carnitine was proposed to reactivate Soman inhibited AChE by the following mechanism, but was inactive in the pretreatment assay and has not been tested in the reactivator assay.



Appropriate amino acid derivatives were proposed to reactivate soman inhibited AChE by the following mechanism. Several derivatives were submitted, but none have yet been shown to have any activity. We were not able to submit enough compounds to fully test this hypothesis.

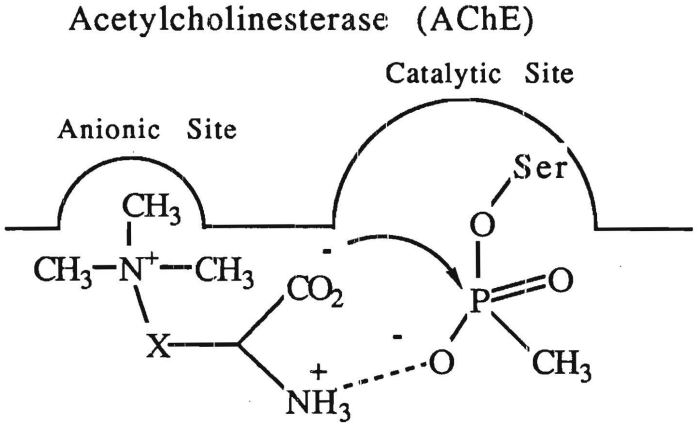
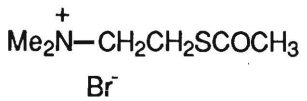
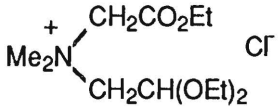
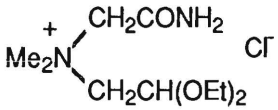
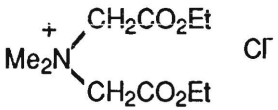
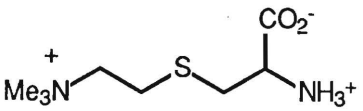
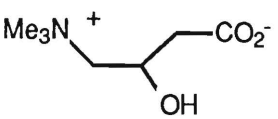
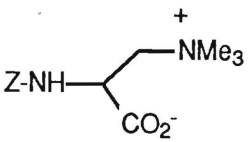
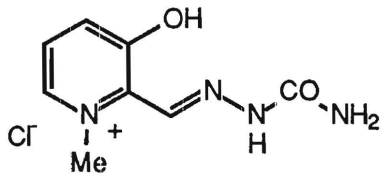


Table VI. Miscellaneous Compounds

Compound	Compound Number	Sample Size	Date Submit	LD_{50} (mg/Kg)	Assay	Dose (mg/Kg)	Survival (%)
	BL21173 ICD 598	4g	7/86	18	pretreatment (15 min, water)	4.5	20
	BL21235 ICD 617	3.1g	8/86	190	reactivator (water) reactivator	47 2.96 47	0 20 0
	BL21244 ICD 618	2.9g	8/86	297	reactivator (water)	74 18.6	0 30
	BL21253 ICD 619	3.7g	8/86	205	reactivator (water)	51 3.2	0 10
	BL21968 ICD 650	2.7g	7/86	>400	reactivator (water) adjunct efficacy	100 100	0 0
	BL31811 ICD 737	5g	1/87	>400	pretreatment (60 min, water)	1.5	30
	BL34287 ICD 762	2.5g	2/87	>400	reactivator	100	10

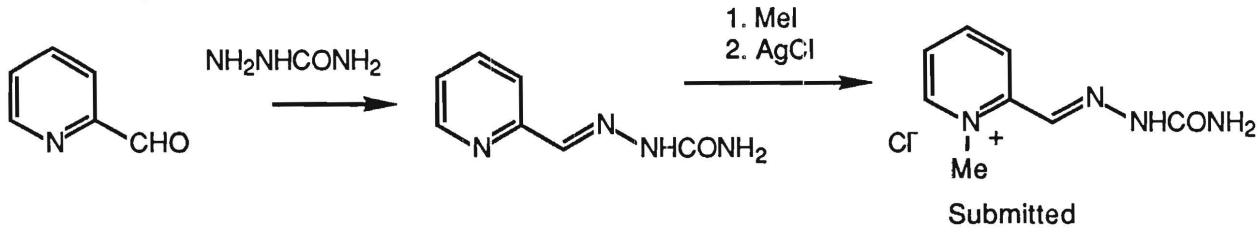
SYNTHESIS

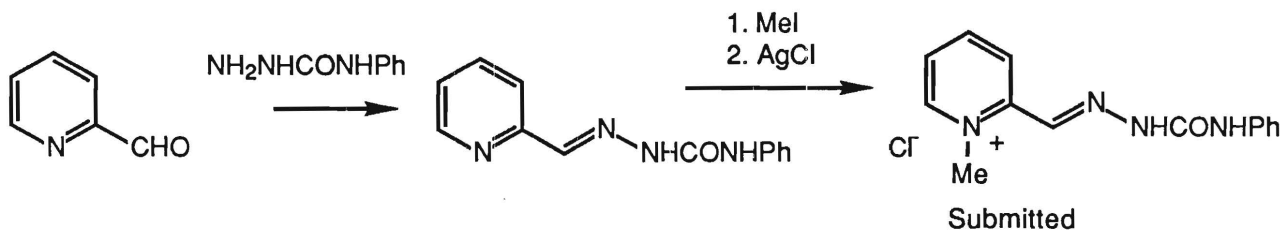
Pyridinium Compounds. Much of our recent synthetic work has revolved arount ICD 722, a pyridinium compound which showed good activity in the adjunct assay (3-hydroxy-1-methylpyridinium-2-aldehyde semicarbazone, BL-30985, ICD 722, WR 256438).



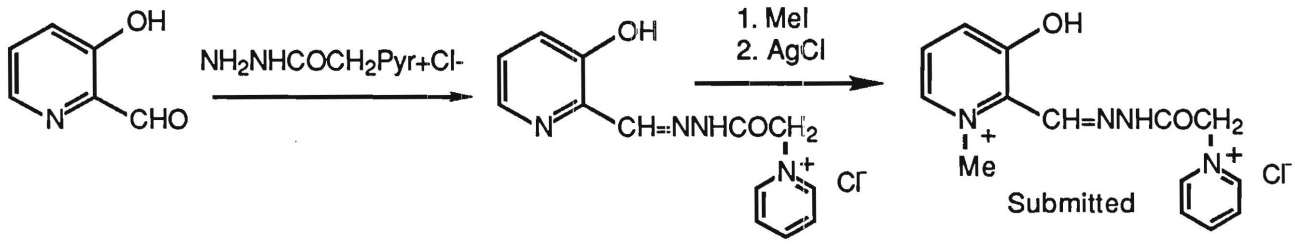
722

Kinetic studies carried out by Steve Thornton suggested that the 3-OH group might actually decrease binding affinity for the active site of AChE. Therefore, we prepared and submitted the following two compounds which lack the 3-OH.

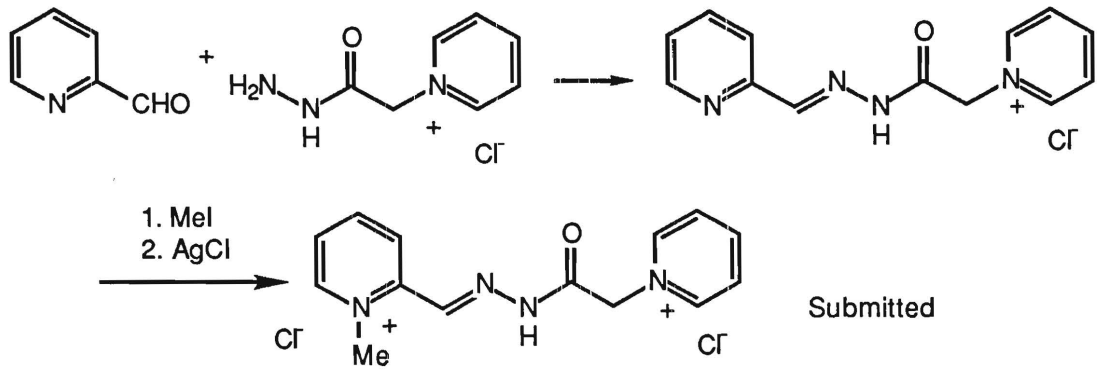




The latter derivative could take advantage of the hydrophobic binding site in AChE. Another related derivative is shown below. This derivative contains an additional pyridinium moiety on the semicarbazone side chain. By analogy with the well-known bis-pyridinium PAM reactivators, we reasoned that this additional pyridinium moiety may aid binding, thus making the compound a good competitive inhibitor and therefore a good prophylactic agent against Soman.



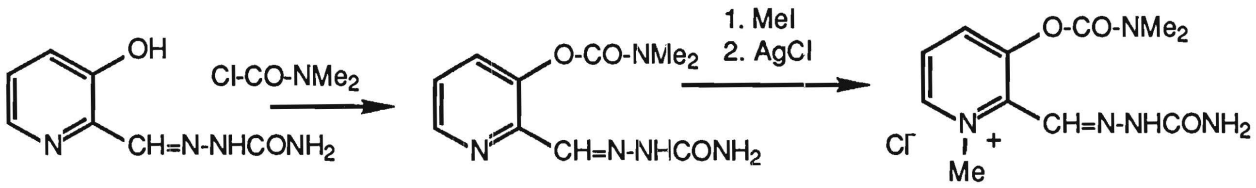
We also synthesized the analog without the 3-hydoxyl group by the route shown below.



It will be quite interesting to compare testing results for these two compounds. The *in-vivo* results might simply parallel the enzyme inhibition results. Alternatively, other factors such as bioavailability, permeability, pharmacodynamics, etc. may be overriding factors in the relative efficacy of these pyridinium compounds.

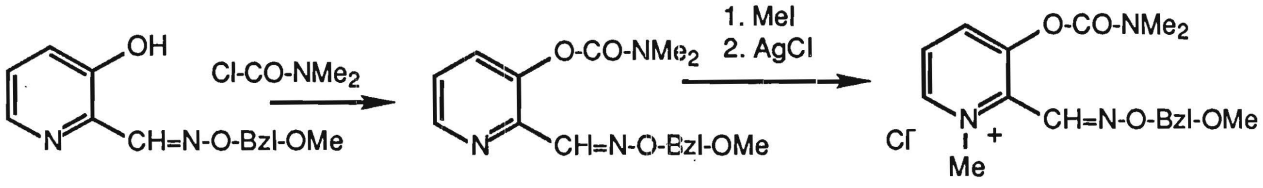
Carbamates. The dimethylamino carbamates are another family of compounds related to our 3-hydroxy pyridinium derivatives. These compounds were designed based on the good prophylactic activity of well known anticholinesterases such as pyridostigmine and physostigmine. Depending on the lability of the dimethylaminocarbamoyl group towards hydrolysis <u>in vivo</u>, these compounds may serve as prodrug forms of their parent compounds (free 3-hydroxyl group) or as reversible inhibitors of AChE which transfer a carbamoyl group to the serine hydroxyl in the active site.

Synthesis of the carbamate analog of ICD 722 was completed as shown below. The incorporation of the carbamoyl group was done after functionalization of the 2-aldehyde moiety.



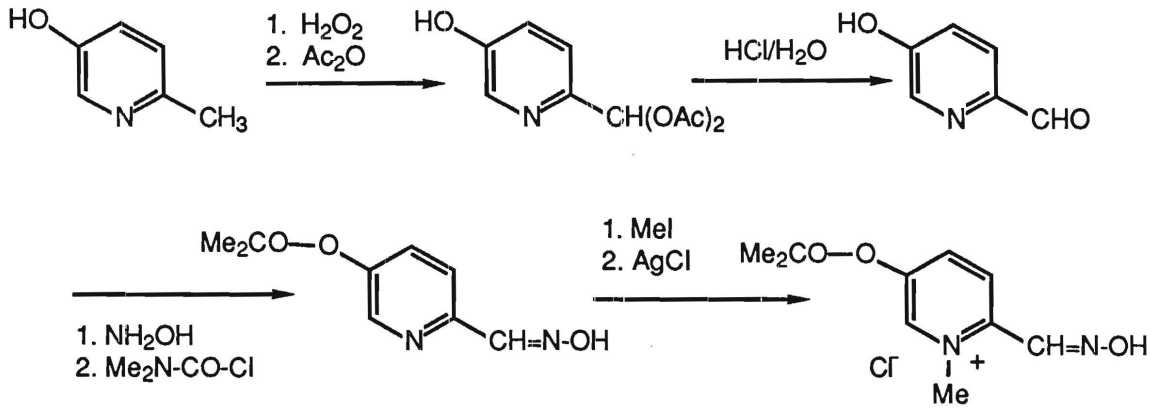
Submitted

A related oxime carbamate was synthesized as shown below.



Submitted

An isomeric carbamate has also been synthesized by the route shown below.



Submitted

These compounds round out a logical series of carbamates for in-vivo evaluation.

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Synthesis of 1-Methyl-2-Aldehyde Semicarbazone Pyridinium Chloride

1-Methyl-2-Aldehyde Semicarbazone. Sodium acetate (4.92 g, 0.06 mol) was dissolved in 60 mL of water and to this solution were added semicarbazide hydrochloride (6.69 g, 0.06 mol) and pyridine-2-aldehyde (5.7 mL, 0.06 mol). The reaction mixture was stirred at room temperature for 14h. The solid in the reaction mixture was collected by filtration and washed with water. After drying, 6.8 g (69%) of product is obtained as a beige solid which was used in the next step without further purification; m.p. 197-198°C.

1-Methyl-2-Aldehyde Semicarbazone Pyridinium Chloride. Pyridine-2-aldehyde semicarbazone (4.14 g, 0.025 mol) was divided into two pressure glass vessels and each portion was dissolved in 20 mL of acetonitrile. Methyl iodide was added to the solutions (3.5 mL each, 0.056 mol) and the resulting mixtures were heated at 65°C for 3 days. The reaction mixture was cooled to 10°C and filtered to obtain an orange yellow solid which was washed with acetone and ether to yield 7.1 g (93%) of the iodide salt m.p. 241-2°C (dec.). Freshly made AgCl (from 7.88 g $AgNO_3$ and excess conc. HCl) was washed well with water and added to a solution of the iodide salt (7.1 g, 0.023 mol) in 400 mol acetonitrile/water, 1:1. The resulting suspension was stirred at room temperature for 2h. The silver iodide was filtered through celite and the filtrate was concentrated to dryness in vacuo to yield a solid which is washed with acetone and ether. After drying, the beige product was obtained as the pure chloride salt (5.53 g, quantitative), mp 227-228°C (dec.). ^{1}HNMR (d_6-DMSO) δ: 11.37 (s, 1H); 8.89 (d of d, 2H); 8.45 (t, 1H); 8.28 (s, 1H); 7.93 (t, 1H); 7.05 (br.s., 2H); 4.31 (s, 3H). Anal. Calcd. for $C_8H_{11}ClN_4O \cdot 2 H_2O$: C, 38.33; H, 6.03; N, 22.35; Cl, 14.14. Found: C, 38.38; H, 6.05; N, 22.31; Cl, 14.19.

Synthesis of 2-Aldehyde-(4'-Phenyl) Semicarbazone-1-Methyl Pyridinium Chloride.

Pyridine-2-Aldehyde-(4'-Phenyl) Semicarbazone. Pyridine-2-Aldehyde (5.7 mL, 0.06 mol) was added to a suspension of sodium acetate (4.92 g, 0.06 mol) and 4-phenyl semicarbazide (9.07 g, 0.06 mol) in 60 mL of water. The resulting mixture was stirred at room temperature for 14h and the solid in suspension was filtered and washed thoroughly with water. After drying, the semicarbazone was obtained as a dark beige solid (12.54 g, 87%) and was used in the next step without further purification; m.p. 181-182°C.

2-Aldehyde-(4'Phenyl) Semicarbazone-1-Methyl Pyridinium Chloride. Pyridine-2-aldehyde-(4'-phenyl semicarbazone (4.47 g, 0.02 mol) was divided into two pressure glass tubes and dissolved in acetonitrile (20 mL each). Methyl iodide was then added (2.6 mL each, 0.04 mol), the tubes were tightly closed and heated at 65°C for 3 days. The reaction flasks were cooled to 10-15°C and the solid was filtered. The yellow solid was washed with acetone and ether and dried to yield 6.66 g (92%) of the iodide salt; m.p. 192-193°C (dec.). It was dissolved in 400 mL of acetonitrile/water 1:1 and exchanged with freshly made AgCl in the following manner: 5.92 g of $AgNO_3$ (0.035 mol) were dissolved in 20 mL of water and excess conc. HCl was then added. The precipitated silver chloride was then filtered and washed well with water. It was added to the solution of the iodide salt and the resulting suspension was stirred at room temperature for 2h. The silver iodide was filtered off and the filtrate was concentrated to dryness in vacuo to yield a yellow solid that was triturated with acetone and ether. It was filtered and dried to yield 4.86 (96%) of the pure chloride salt as a yellow solid; m.p. 187-188°C (dec.). ^{1}HNMR (d_6-DMSO) δ: 11.87 (s, 1H); 9.45 (s, 1H); 8.98 (d of d, 2H); 8.56 (t, 1H); 8.43 (s, 1H); 8.01 (t, 1H); 7.62 (d, 2H); 7.34 (t, 2H); 7.08 (t, 1H); 4.36 (s, 3H). Anal. Calcd. for $C_{14}H_{15}ClN_4O \cdot 0.48\ H_2O$: C, 56.15; H, 5.37; N, 18.71; Cl, 11.84. Found: C, 56.15; H, 5.38; N, 18.69; Cl, 11.88.

Synthesis of 2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone]-3-Hydroxy-1-Methyl Pyridinium Chloride

2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone-3-Hydroxy Pyridine. 1-(carboxymethyl) pyridinium chloride hydrazide (2.63 g, 0.014 mol) in 10 mL of methanol was added to a solution of 2-aldehyde-3-hydroxy pyridine (1.72 g, 0.014 mol) in 20 mL of CH_3CN. The resulting dark yellow solution is stirred at room temperature for 2h and at the end of this period a beige solid filled the reaction mixture. Ethyl ether was added (10 mL) and the reaction mixture was cooled to 5-10°C before filtering the beige precipitate. The solid was washed with ethyl ether and dried to yield 3.72 g (91%) of product; m.p. 229-30°C (dec.). ^{1}HNMR (d_6-DMSO) δ: 9.05 (t, 2H); 8.69 (m, 1H); 8.45 (s, 1H); 8.25-8.17 (m, 3H); 7.38-7.34 (m, 2H); 6.02 (s, 1H, exchanges with D_2O); 5.66 (s, 1H, exchanges with D_2O).

2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone]-3-Hydroxy-1-Methyl Pyridinium Chloride. 2-[1'-(carboxymethyl) pyridinium chloride hydrazone]-3-hydroxy pyridine (3.5 g, 0.012 mol) was divided into three pressure glass vessels and each portion was dissolved in 20 mL of acetonitrile. Methyl iodide was added to these solutions (1.2 mL each, 0.02 mol) and the resulting mixtures were heated at 70°C for 3 days. The reaction mixtures were concentrated in vacuo to a dark residue which was triturated with acetone/ethanol 5:1. The resulting yellow solid was washed with acetone/ethanol 5:1 and dried to obtain 4.39 g (84%) of the iodide salt, m.p. 193-94°C (dec.). Freshly made AgCl (from 3.43 g of $AgNO_3$ and excess conc. HCl) was washed well with water and added to a solution of the iodide salt (4.39 g, 0.01 mol) in 300 mL acetonitrile/water 1:1. The resulting suspension was stirred at room temperature for 2h. The silver iodide was filtered through celite and the

filtrate was concentrated to dryness *in vacuo* to yield a solid that was triturated with acetone. The dark beige solid was filtered, washed with acetone and dried to yield 2.89 g (84%) of pure product as the chloride salt; m.p. 230-31°C (dec.). ^{1}HNMR (d_6-DMSO) δ: 9.1 (d of d, 2H); 8.82-8.64 (m, 2H); 8.54 (s, 1H); 8.32-8.22 (m, 3H); 7.93 (d of d, 1H); 6.08 (s, 2H); 4.49 (s, 3H). Anal. Calcd. for $C_{14}H_{16}Cl_2N_4O_2 \cdot 0.96\ H_2O$: C, 46.65; H, 5.01; N, 15.55; Cl, 19.67. Found: C, 46.65; H, 4.95; N, 16.33; Cl, 20.66

Synthesis of 2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone] -1- Methyl Pyridinium Chloride

2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone] Pyridine. 1-(Carboxymethyl) pyridinium chloride hydrazide (5.25g, 28mmol) and pyridine -2- carboxaldehyde (2.7mL, 28mmol) were dissolved in 25mL acetonitrile: methanol (4:1). The resulting mixture was stirred at room temperature for 2h, with the product beginning to come out of solution after 20 min of reaction. After 2h the white solid in the reaction mixture was filtered and washed with acetonitrile and ether. After drying, the product was obtained as a white solid (5.27g, 68%) and was used in the next step without further purification; m.p. 240-241°C(dec.). ^{1}HNMR (d_6-DMSO) δ: 12.43 (br.s., 1H); 9.07 (d, 2H); 8.70 (t, 1H); 8.66 (d, 1H); 8.24 (d of d, 2H); 8.19 (s, 1H); 8.01-7.90 (m, 2H); 7.48-7.44 (m, 1H); 6.07 (s, 2H).

2-[1'-(Carboxymethyl) Pyridinium Chloride Hydrazone]-1- Methyl Pyridinium Chloride. 2-[1'-Carboxymethyl) pyridinium chloride hydrazone] pyridine (2.88g, 10mmol) was divided into two pressure vessels and each portion was dissolved in 25mL of DMF. Methyl iodide was added to these solutions (1.62mL each, 25mmol) and the resulting mixtures were heated at 70°C for 3 days. The solvent was removed in vacuo to yield a dark residue which was triturated with acetone. The resulting solid was filtered, washed with acetone and ether and dried to yield 4.25g (quantitative) of the methiodide as a yellow solid. Freshly made AgCl (from 3.77g $AgNO_3$ and excess conc. HCl) was washed well with water and added to a solution of the iodide salt (4.18g, 10mmol) in 200mL acetonitrile/water 1:1. The resulting suspension was stirred at room temperature for 1.5h. The silver iodide was filtered through Celite and the filtrate was concentrated to dryness in vacuo to yield 3.63 g of a yellow solid. Recrystallization from ethanol/acetone affords the pure chloride as a beige solid (3.0 g, 92%); m.p. 209-210°C(dec). ^{1}HNMR (d_6-DMSO) δ : 9.13 (d, 2H); 9.08 (d, 1H); 8.75-8.59 (m, 4H); 8.26 (t, 2H); 8.10 (m, 1H); 6.19 (br.s., 2H); 4.42 (s, 3H). Anal. Calcd. for $C_{14}H_{16}Cl_2N_4O \cdot 0.91H_2O$: C, 48.94; H, 5.23; N, 16.31; Cl, 20.64. Found: C, 48.94; H, 5.25; N, 16.31; Cl, 20.43.

Synthesis of 3-(N,N-Dimethylcarbamoyl)hydroxy-1-Methylpyridinium-2-Carboxaldehyde Semicarbazone Chloride

3-Hydroxy-2-pyridine Carboxaldehyde Semicarbazone. Sodium acetate (2.21g, 16 mmol) was dissolved in 25 ml of water and then semicarbazide hydrochloride was added (1.81 g, 16 mmol), followed by 3-hydroxy-2-pyridine aldehyde (2.0 g, 16 mmol). The resulting mixture was stirred at room temperature for 2 h. The solid in the reaction mixture was then filtered, washed with water and dried to yield 2.71 g (93%) of the semicarbazone. It was used in the next step without further purification; m.p.: 215-217°C(dec.)

3-(N,N-Dimethylcarbamoyl)hydroxy-2-Pyridine Carboxaldehyde Semicarbazone. 3-Hydroxy-2-pyridine carboxaldehyde semicarbazone (2.71 g, 15 mmol) was suspended in 20 mL of pyridine and then N,N-dimethylcarbamyl chloride was added (4.2 mL, 45 mmol). The reaction flask was sealed with a septum and the mixture was stirred at room temperature for 20h. The reaction mixture becomes a clear solution after one hour and the product begins to come out of solution after 2h. When the reaction is complete, the mixture is cooled to 5-10°C in ice bath and filtered. The beige solid that results is washed with acetone - ether 1:1 and dried to yield 3.63 g (96%) of the carbamate which is used in the next step without further purification; m.p. 182-3°C(dec.). ^{1}HNMR (d_6-DMSO) δ : 10.58 (s, 1H), 8.48 (d, 1H), 7.98 (s,1H), 7.63 (d, 1H), 7.42 (d of d, 1H), 6.30 (br.s., 2H), 3.07 (s, 3H), 2.90 (s, 3H).

3-(N,N-Dimethylcarbamoyl)hydroxy-1-Methylpyridinium-2-Carboxaldehyde Semicarbazone Chloride. The carbamate obtained in the previous step (3.63 g, 14 mmol) was dissolved in 50mL hot (90°C) DMF. When a clear solution was obtained, the reaction flask was cooled under running water until the inner

temperature reached 26°C. Methyl iodide (6.3 mL, 100 mmol) was then added and the reaction mixture was left at 65°C in a Parr shaker for 3 days. The solvent was removed in vacuo to yield a dark viscous residue which was triturated with acetone. The yellow solid that resulted was washed with acetone/ether 1:1, then with ether and dried to yield 5.15 g (94%) if the methiodide. Freshly made AgCl (from 4.45 g $AgNO_3$ and excess conc. HCl) was washed well with water and added to a solution of the methiodide (5.15g, 13 mmol) in 200 mL acetonitrile/water 1:1. The resulting suspension was stirred at room temperature for 1.5h. The silver iodide was filtered through Celite and the filtrate was concentrated to dryness in vacuo to yield a residue which is triturated with acetone. The resulting solid is filtered and recrystallized from CH_3CN/EtOH and acetone to yield 2.44 g (62%) of the pure chloride salt; m.p. 194-195°C (dec.). ^{1}HNMR (d_6-DMSO): 11.10 (br.s., 1H), 8.97 (d, 1H, $J_{6,5}$=6.1 Hz), 8.51 (d, 1H, $J_{4,5}$=8.6 Hz), 8.17 (s, 1H), 8.05 (d of d, 1H, $J_{5,4}$=8.6 Hz, $J_{5,6}$=6.1 Hz), 6.68 (br.s., 2H), 4.44 (s, 3H), 3.08 (s, 3H), 2.94 (s, 3H). Anal. Calcd. for $C_{11}H_{16}ClN_5O_3 \cdot 0.96\ H_2O$: C, 41.42; H, 5.66; N, 21.96; Cl, 11.12. Found : C, 41.42; H, 5.72; N, 21.81; Cl, 11.20.

Synthesis of 3-(N,N-Dimethylcarbamoyl) hydroxy-2-[O-(p-methoxy)benzyl] aldoxime-1-methyl Pyridinium Chloride.

3-(N,N-Dimethylcarbamoyl)hydroxy-2-[O(p-methoxy) benzyl] aldoxime Pyridine. Dimethylcarbamyl chloride (10 ml, 0.11 mol) was added to a solution of 3-hydroxy-2-[O-(p-methoxy) benzyl] aldoxime pyridine (3.3 g, 12.8 mmol) in 30 ml of pyridine. The resulting mixture was stirred at room temperature for 14 h and then poured over 400 ml of crushed ice. Since it was not possible to induce precipitation of the product, the aqueous mixture was extracted with ether (4 x 100 ml) and the organic extract was concentrated to dryness in vacuo. The oily residue was coevaporated with water to remove traces of pyridine and then with acetone. The 300 MHz ^1H NMR of the resulting yellow oil shows no pyridine or water and was used in the next step without further purification. ^1H NMR ($CDCl_3$) δ: 8.50 (d of d, 1H); 8.32 (s, 1H); 7.50 (d of d, 1H); 7.32 (d of d, 2H); 7.28 (t, 1H); 6.88 (d of d, 2H); 5.20 (s, 2H); 3.77 (s, 3H); 3.05 (s, 3H); 2.96 (s, 3H).

3-(N,N-Dimethylcarbamoyl)hydroxy-2-[O-[p-methoxy)benzyl]aldoxime-1-methyl Pyridinium Chloride. Methyl iodide (2.9 ml, 46.4 mmol) was added to each of two pressure glass tubes containing a solution of 3-(N,N-dimethyl carbamoyl) hydroxy-2-[O-(p-methoxy)benzyl]aldoxime pyridine (3.82 g, 11.6 mmol, divided into two) in 40 ml of acetonitrile. The sealed reaction vessels were heated at 70°C for 3 days and then the solvent was removed in vacuo. The resulting oil crystallizes upon standing at 0-5°C for 14 h in a 2:1 mixture of acetone/ether (75 ml). The yellow solid was filtered, washed with ether and dried to yield 4.94 g (90%) of crude methiodide. It was dissolved in 1:1 mixture of H_2O/acetonitrile (200 ml) and then freshly made AgCl (21 mmol, washed well with water) was added. The resulting suspension was stirred at

room temperature for 2 h and filtered through celite. The filtrate was concentrated to dryness in vacuo and the residue was triturated with 1:1 acetone/ether. The white solid that resulted was filtered, washed with ether and dried to yield 3.72 g (84.4%) of pure chloride, m.p. 145-6°C (dec.). ^1H NMR (d_6-DMSO) δ: 9.04 (d, 1H, J=6.0Hz); 8.67 (s, 1H); 8.58 (d, 1H, J=8.0Hz); 8.17 (d of d, 1H, J=6.0 Hz, J=8.0 Hz); 7.35 (d, 2H, J=8.7 Hz); 6.95 (d, 2H, J=8.7 Hz); 5.23 (s, 2H); 4.36 (s, 3H); 3.75 (s, 3H); 3.01 (s, 3H); 2.93 (s, 3H). Anal. Calcd. for $C_{18}H_{22}ClN_3O_4 \cdot 0.5\ H_2O$: C,55.59; H, 5.96; N, 10.81; Cl, 9.12. Found: C, 55.59; H, 6.03; N, 10.76; Cl, 9.17.

Synthesis of 5-(N,N-Dimethylcarbamoyl)hydroxy-2-aldoxime-1-methyl Pyridinium Chloride Monohydrate.

2-Formyl-5-hydroxypyridine. To a stirred solution of 21.8 g of 5-hydroxy-2-picoline (0.2 mole) in 200 ml of glacial acetic acid was added 18 ml of 30% hydrogen peroxide (0.159 mole) in one portion. The mixture was heated in an oil bath at 80-85 C with stirring for 3 hrs. Then another 18 ml of hydrogen peroxide was added and the mixture was stirred for 3 hrs at the same temperature. Excess solvent was removed in vacuuo followed by the addition of acetone, which caused the pyridine N-oxide to crystallize. Without further purification, 200 ml of acetic anhydride was added to the solid and the mixture was heated at 120 C with stirring in an oil bath for 2 hrs. After cooling to room temperature, excess acetic anhydride was removed by high vacuum distillation. The oily material was again oxidized with 30% hydrogen peroxide twice and rearranged with acetic anhydride following the same procedure and amounts depicted above. The black oily material obtained was hydrolyzed with 200 ml of 1N HCl (0.2 mole) at room temperature for 3 weeks (hydrolysis at higher temperature may be harmful to the pyridine nucleus). The mixture was neutralized with anhydrous sodium carbonate, indicated by litmus paper and then extracted three times with 300 ml of diethylether. The combined ether extracts were dried (anh. $MgSO_4$), filtered and evaporated to leave a solid material in a small amount of oil. The pure product (7.8 g, 32%) was obtained by filtering the solid, followed by washing it with a small amount of ether. It is yellow in color and shows a sharp melting point at 186-187 C. H^1 NMR (d_6-DMSO): 11.10 (br. s, 1H, OH); 9.87 (s, 1H, CHO); 8.35 (d, J= 2Hz, 1H); 7.58 (d, J = 9 Hz, 1H); 7.35 (d of d, J = 2Hz, 9Hz, 1H).

5-Hydroxy-2-pyridinealdoxime. To 200 ml of 2.5% (w/w) sodium hydroxide solution was dissolved 5.5 g of 5-hydroxy-2-formylpyridine (45 mmol). Subsequently 12.5 g of hydroxylamine HCl (180 mmole) was added to the solution in one portion. The solution turned cloudy after being stirred for 10 minutes. After stirring at room temperature for 3 hrs, the precipitate was

filtered under vacuum and dried in the air for several days to yield 4.8 g of 5-hydroxy-2-pyridinealdoxime (78%). The solid is off white and decomposes at 195 C. H^1 NMR (d_6-DMSO): 11.27 (br. s, 1H); 10.83 (br. s, 1H); 8.18 (d, J = 2 Hz, 1H); 8.05 (s, 1H, CH=N); 7.72 (d, J = 9Hz, 1H); 7.25 (d of d, J = 2Hz, 9Hz, 1H).

5-(N,N-Dimethylcarbamoyl)hydroxy-2-aldoxime Pyridine. Dimethylcarbamoyl chloride (5.2 ml, 57 mmol) was added to a solution of 5-hydroxy-2-pyridinealdoxime (6.53 g, 47 mmol) in 40 ml of pyridine. It was stirred at room temperature overnight and then the solvent was removed in vacuuo, leaving a light green paste. The paste was dissolved in a minimum quantity of acetone and then triturated with ethyl acetate and placed in the freezer overnight. Glassy needles formed and were filtered and washed with ethyl acetate to yield 3.0 g of pure product (30%), mp = 134-135 C (sharp).

5-(N,N-Dimethylcarbamoyl)hydroxy-2-aldoxime-1-methyl Pyridinium Chloride Monohydrate. Methyl iodide (5.3 ml, 85 mmol) was added to a solution of 5-(N,N-Dimethylcarbamoyl) hydroxy-2-aldoxime pyridine (2.8 g, 13.4 mmol) in 25 ml of acetonitrile. The resulting mixture was put into a glass pressure vessel and heated at 80 C for 3 days. The solvent was removed in vacuuo and the residue was dissolved in a minimum quantity of acetone, triturated with ether and placed in the freezer overnight. A dark yellow solid resulted and was filtered under vacuum and washed with 1:1 ether/acetone to yield 3.25 g of the iodide salt. Freshly made AgCl (2 eq, washed well with water) was added to the iodide salt in acetonitrile/water (60 ml/100 ml) and stirred for several hours. After filtration, the residue was crystallized from acetone/ether as above and dried in the air for several days to yield 2.5 g of the chloride salt (monohydrate, 72% yield). The mp was sharp, 159-160 C. ^1H NMR (d_6-DMSO): 9.18 (s, 1H); 8.65 (s, 1H); 8.40 (d of d, 2H, J = 2 Hz, 9 Hz); 4.36 (s, 3H); 3.3 (s, H_2O); 3.1 (s, 3H); 2.95 (s, 3H). Anal. Calcd. for $C_{10}H_{14}N_3O_3Cl \cdot H_2O$ (259.70 g/mol): C, 43.25; H, 5.81; N, 15.13; Cl, 12.76. Found: C, 43.42; H, 5.81; N, 15.09; Cl, 12.87.

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