

# Kinetic Assessment of *N*-Methyl-2-methoxypyridinium Species as Phosphonate Anion Methylating Agents

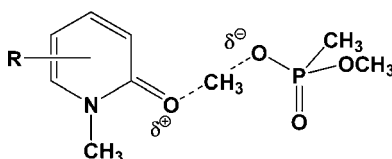
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## ABSTRACT



Organophosphate nerve agents and pesticides are potent inhibitors of acetylcholinesterase (AChE). Although oxime nucleophiles can reactivate the AChE-phosphyl adduct, the adduct undergoes a reaction called aging. No compounds have been described that reactivate the aged-AChE adduct. A family of 2-methoxypyridinium species which reverse aging in a model system is presented. A kinetic study of this system, which includes an SAR analysis, demonstrates that the reaction is highly tunable based on the ring substituents.

Organophosphorus (OP) agents have been used as both pesticides and chemical warfare agents for most of the past century.<sup>1,2</sup> Pesticides based on phosphate or thiophosphate agents (e.g., parathion, diazinon, or malathion) are or have been used in domestic and commercial agriculture to protect crops from a variety of destructive species. Despite numerous efforts to ban or limit the use of OP pesticides, their continued use results in over 200,000 fatalities and over 1 million instances of morbidity annually due to OP exposure. Phosphonate nerve agents, including sarin, soman, VX, and tabun, are extremely toxic and are considered a serious threat to national security due to their potential use in terrorist actions.<sup>1</sup>

These nerve agents elicit their acute toxicity by inhibiting the cholinergic system which results in an overstimulation of muscles due to the buildup of acetylcholine in the neuromuscular junction. The ultimate result is muscle fasciculation and subsequent cardiopulmonary arrest.<sup>1,2</sup> Specifically, OPs inhibit acetylcholinesterase (AChE) by covalently bonding to the serine residue in the catalytic triad of the active site (Figure 1). The well documented and

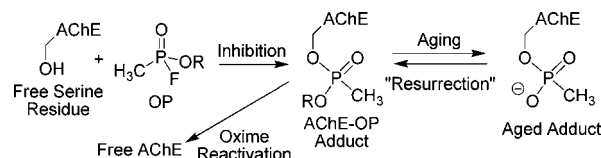


Figure 1. AChE inhibition by organophosphonates.

tremendous affinity of OP agents for the active site of AChE is a result of an OP's structural similarity to the tetrahedral transition state of the cognate hydrolysis reaction of acetylcholine.<sup>3</sup>

There are several potential treatments for both acute and chronic organophosphate poisoning. Most include a nucleophilic oxime to reactivate the enzyme along with atropine as an acetylcholine receptor agonist and diazepam to treat seizures that arise from inhibition of AChE in the CNS.<sup>1,2</sup> If administered shortly after exposure, the oxime (i.e., 2-PAM) is able to displace the bound OP and liberate the serine residue (Figure 1).<sup>4</sup> If administration of

(1) Mercey, G.; Verdelet, T.; Renou, J.; Kliachyna, M.; Baati, R.; Nachon, F.; Jean, L.; Renard, P.-Y. *Acc. Chem. Res.* **2012**, 45, 756.

(2) Jokanovic, M.; Prostran, M. *Curr. Med. Chem.* **2009**, 16, 2177.

(3) Quinn, D. M. *Chem. Rev.* **1987**, 87, 955.

the oxime is delayed a process called aging occurs where the enzyme bound phosphonate experiences a solvolytic loss of an alkyl group (Figure 1: R = isopropyl for sarin, pinacolyl for soman).<sup>1,5</sup> The aged adduct is a phosphonate ester monoanion, which is intrinsically less reactive as an electrophile than the neutral phosphonate ester of the initial phosphonyl-AChE adduct. The aged adduct is further stabilized by several key interactions with the AChE active site and is refractory to oxime reactivation.<sup>5</sup> No compounds have yet been discovered that are able to recover AChE activity once aging has occurred, and therefore there are no antidotes against aged AChE-OP adducts.<sup>1,4</sup>

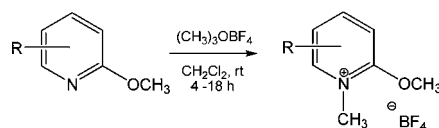
We hypothesize that realkylation of the aged adduct could lead to a reactivatable AChE adduct and have initiated studies to demonstrate this. Because the aged complex has long been thought of as a dead enzyme, we term this concept “resurrection” of the aged adduct (Figure 1). Presented here is the description of a family of *N*-methyl-2-methoxypyridinium species which are effective at “resurrecting” a model phosphonate system. An analysis of the kinetics of these reactions demonstrates the highly tunable reactivity of *N*-methyl-2-methoxypyridinium species as alkylating agents for the methyl methane-phosphonate monoanion.

Our studies began with a literature search of alkylating agents that are known to react with phosphate or phosphonate anions. Although several alkylating agents or reactive intermediates are capable of phosphonate anion alkylation, few provide practical solutions to the aging problem due to their aqueous instability, poor selectivity, or toxicity (i.e., MeOTf is not suitable).<sup>6</sup> This led to the consideration of less traditional methyl transfer reagents. Particularly appealing were classic reports that describe the equilibration of 2-methoxypyridine to *N*-methylpyridone

that utilized *N*-methyl-2-methoxypyridinium as a catalyst.<sup>7</sup> The methyl transfer reactivity of these species and their structural similarity to the oxime AChE reactivator 2-PAM lead to the consideration of them as aged AChE-OP resurrecting agents.

The synthesis of *N*-methyl-2-methoxypyridinium species was conducted by exposure of various commercial pyridines to trimethoxonium tetrafluoroborate (Scheme 1). This resulted in a family of nine pyridinium tetrafluoroborates with varied substituents. Other work demonstrated the utility of <sup>1</sup>H NMR in measuring constants associated with this family of compounds.<sup>8</sup> Previous reports have evaluated the <sup>1</sup>H NMR chemical shift of the pyridinium *N*-methyl as a measure of electron density.<sup>8a</sup> In an expansion of this, Figure 2 shows a multiple linear correlation of the methoxy methyl shift against the Swain–Lupton field and resonance parameters<sup>9,10</sup> of the ring substituents. The correlation shows that the chemical shift of the methoxy moiety, and hence its electron density, is markedly dependent on both field and resonance effects of substituents. Further correlations with reactivity are discussed below.

**Scheme 1.** Synthesis



The effectiveness of these compounds as methyl transfer agents was determined by using sodium methyl methane-phosphonate as an analogue of the aged AChE-OP adduct. Model reactions were conducted by mixing equimolar concentrations of phosphonate salt with the pyridinium species (27 mM in *d*<sub>6</sub>-DMSO), and the reaction was monitored by <sup>1</sup>H NMR spectroscopy (see header of Table 1).

All reactants and products in the header of Table 1 could be simultaneously monitored. Moreover, a broad resonance in the <sup>1</sup>H NMR spectra centered at 3.4 ppm was interpreted as low levels of water, which gives rise to a competing hydrolysis reaction of the *N*-methyl-2-methoxypyridinium reactants to generate methanol. Indeed, methanol was detected in product spectra through its methyl resonance at 3.2 ppm. Consequently, the overall kinetics of the reaction system can be described by the

(4) The synthesis of improved oximes is an ongoing field of research: (a) Kalisiak, J.; Ralph, E. C.; Zhang, J.; Cashman, J. R. *J. Med. Chem.* **2011**, *54*, 3319. (b) Kalisiak, J.; Ralph, E. C.; Cashman, J. R. *J. Med. Chem.* **2012**, *55*, 465. (c) Mercey, G.; Verdelt, T.; Saint-Andre, G.; Gillon, E.; Wagner, A.; Baati, R.; Jean, L.; Nachon, F.; Renard, P.-Y. *Chem. Commun.* **2011**, *47*, 5295. (d) Sit, R. K.; Radic, Z.; Gerardi, V.; Zhang, L.; Garcia, E.; Katalinic, M.; Amitai, G.; Kovarik, Z.; Fokin, V. V.; Sharpless, K. B.; Taylor, P. *J. Biol. Chem.* **2011**, *286*, 19422.

(5) Characterization of aged OP-AChE adducts: (a) Millard, C. B.; Kryger, G.; Ordentlich, A.; Greenblatt, H. M.; Harel, M.; Ravess, M. L.; Segall, Y.; Barak, D.; Shafferman, A.; Silman, I.; Sussman, J. L. *Biochemistry* **1999**, *38*, 7032. (b) Sanson, B.; Nachon, F.; Colletier, J.; Froment, M.; Tokar, L.; Greenblatt, H. M.; Sussman, J. L.; Ashani, Y.; Masson, P.; Silman, I.; Weik, M. *J. Med. Chem.* **2009**, *52*, 7593. (c) Carletti, E.; Colletier, J.; Dupeux, F.; Trovaslet, M.; Masson, P.; Nachon, F. *J. Med. Chem.* **2010**, *53*, 4002. (d) Masson, P.; Nachon, F.; Lockridge, O. *Chem.-Biol. Interact.* **2010**, *187*, 157.

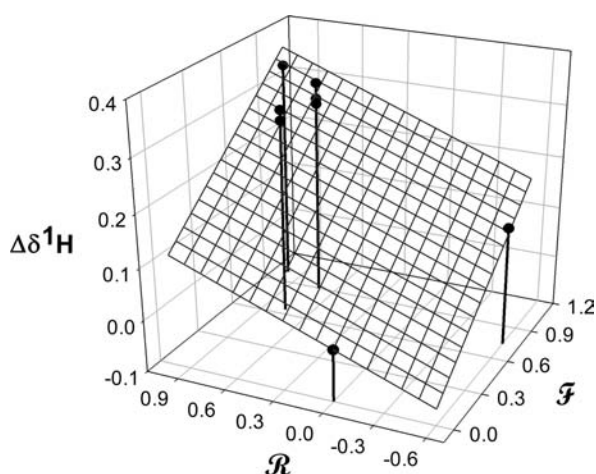
(6) Phosphonate or phosphate anion alkylation with bioincompatible reagents: (a) Moriarty, R. M.; Condeiu, C.; Tao, A.; Prakash, O. *Tetrahedron Lett.* **1997**, *38*, 2401. (b) Di Raddo, P.; Chan, T.-H. *J. Chem. Soc., Chem. Commun.* **1983**, *16*. (c) Schole, J.; Schole, C.; Eikemeyer, J. *Tetrahedron* **1994**, *50*, 1125. (d) Andre, V.; Lahrache, H.; Robin, S.; Rousseau, G. *Tetrahedron* **2007**, *63*, 10066. (e) Pitsch, S.; Pombo-Villar, E.; Eschenmoser, A. *Helv. Chim. Acta* **1994**, *77*, 2251. (f) Jankowski, S.; Kovacs, J.; Huben, K.; Blaszczyk, M.; Glowka, M.; Keglevich, G. *Heteroat. Chem.* **2006**, *17*, 369. (g) Ayukawa, H.; Ohuchi, S.; Ishikawa, M.; Hata, T. *Chem. Lett.* **1995**, *81*. (h) Chavez, M. R.; Zhao, P.; Kovacs, Z.; Sherry, A. D. *Lett. Org. Chem.* **2004**, *1*, 194.

(7) (a) Beak, P.; Bonham, J.; Lee, J. T., Jr. *J. Am. Chem. Soc.* **1968**, *90*, 1569. (b) Beak, P.; Bonham, J. *Chem. Commun.* **1966**, 631. (c) Beak, P.; Lee, J. T., Jr. *J. Org. Chem.* **1969**, *34*, 2125.

(8) (a) Huang, S.; Wong, J. C. S.; Leung, A. K. C.; Chan, Y. M.; Wong, L.; Fernandez, M. R.; Miller, A. K.; Wu, W. *Tetrahedron Lett.* **2009**, *50*, 5018. (b) Wong, F. M.; Capule, C. C.; Chen, D. X.; Gronert, S.; Wu, W. *Org. Lett.* **2008**, *10*, 2757. (c) Titsky, G. D.; Mitchenko, E. S.; Dereza, L. I. *Ukrainskii Khimicheskii Zhurnal* **1993**, *59*, 2077. (d) Tummalam, J.; Albiniak, P. A.; Dudley, B. D. *J. Org. Chem.* **2007**, *72*, 8962.

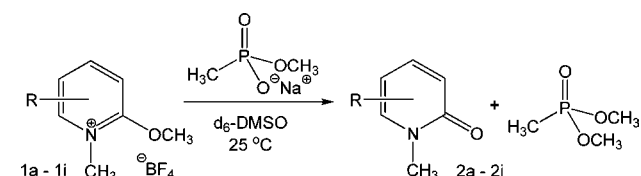
(9) (a) Swain, C. G.; Lupton, E. C. *J. Am. Chem. Soc.* **1968**, *90*, 4328. (b) Swain, C. G.; Unger, S. H.; Rosenquist, N. R.; Swain, M. S. *J. Am. Chem. Soc.* **1983**, *105*, 492. (c) Ewing, D. F. Correlation of NMR chemical shifts with Hammett sigma values and analogous parameters. In *Correlation Analysis in Chemistry-Recent Advances*; Chapman, N. B., Shorter, J., Eds.; Plenum Press: New York, 1978; pp 357–392. (d) Hamer, G. K.; Peat, I. R.; Reynolds, W. F. *Can. J. Chem.* **1973**, *51*, 897.

(10) Carpenter, B. K. *Determination of Organic Reaction Mechanisms*; John Wiley & Sons: New York, 1984; pp 76–82.



**Figure 2.** Multiple linear correlation of OCH<sub>3</sub> chemical shift. Parameters of the fit are field =  $0.25 \pm 0.02$ , res =  $0.11 \pm 0.03$ ,  $r^2 = 0.93$ ,  $n = 8$ . See text for elaboration.

**Table 1.** Methylation of Model System<sup>a</sup>



entry	R =	$k_{Me}$	$k_{hyd}$	$k_{Me}/k_{Me}^H$
<b>1a</b>	H	$(4.6 \pm 0.1) \times 10^{-5}$	$(2.8 \pm 0.2) \times 10^{-6}$	1
<b>1b</b>	3-F	$(3.1 \pm 0.1) \times 10^{-2}$	$(1.1 \pm 0.1) \times 10^{-3}$	670
<b>1c</b>	5-F	$(1.6 \pm 0.2) \times 10^{-4}$	ND <sup>b</sup>	3.5
<b>1d</b>	5-CF <sub>3</sub>	$(2.83 \pm 0.03) \times 10^{-3}$	$(6.0 \pm 0.1) \times 10^{-4}$	60
<b>1e</b>	6-CF <sub>3</sub>	$(4.63 \pm 0.08) \times 10^{-3}$	$(8.7 \pm 0.2) \times 10^{-4}$	100
<b>1f</b>	5-NO <sub>2</sub>	$(5.6 \pm 0.1) \times 10^{-3}$	$(2.2 \pm 0.1) \times 10^{-2}$	120
<b>1g</b>	4-CN	$(2.47 \pm 0.06) \times 10^{-3}$	$(3.3 \pm 0.2) \times 10^{-4}$	50
<b>1h</b>	5-CN	$(5.13 \pm 0.07) \times 10^{-3}$	$(3.9 \pm 0.1) \times 10^{-3}$	110
<b>1i</b>	6-CN	$(5.8 \pm 0.2) \times 10^{-3}$	$(2.1 \pm 0.1) \times 10^{-3}$	125

<sup>a</sup>Reactions were conducted in *d*<sub>6</sub>-DMSO at  $25.0 \pm 0.5$  °C and were monitored by <sup>1</sup>H NMR spectroscopy at 500 MHz. <sup>b</sup>ND = not determined.

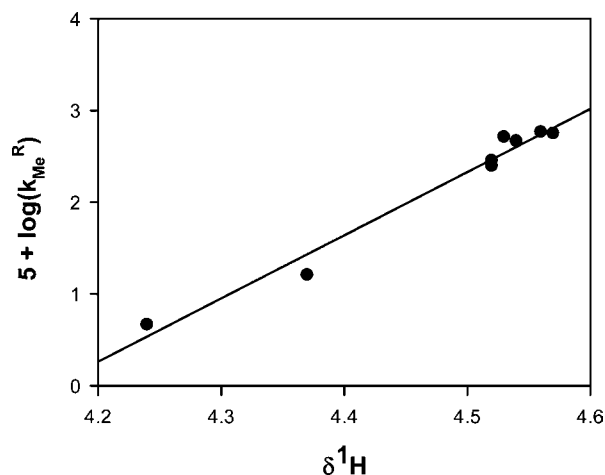
following component differential equations:

$$-d[B]/dt = d[Q]/dt = k_{Me}[A][B] \quad (1)$$

$$-d[A]/dt = d[P]/dt = k_{Me}[A][B] + k_{hyd}[H_2O][A] \quad (2)$$

In these equations, A and B are the methoxypyridinium and phosphonate monoanion reactants, respectively; P and Q are the pyridone and neutral phosphonate products, respectively; and  $k_{Me}$  and  $k_{hyd}$  are the respective second-order rate constants for the methyl transfer and hydrolysis reactions. Since time-dependent changes in the concentrations of all four species A, B, P, and Q can be measured, one can write from eqs 1 and 2 four independent differential equations, one each for  $-d[A]/dt$ ,  $d[P]/dt$ ,  $-d[B]/dt$ , and

$d[Q]/dt$ . For each of the nine methyl transfer reactions, these four differential equations were numerically integrated by fourth-order Runge–Kutta integration<sup>10</sup> (see Supporting Information (SI) for details), which provided least-squares estimates of the rate constants  $k_{Me}$  for all nine *N*-methyl-2-methoxypyridinium reactants and  $k_{hyd}$  values for all but the compound for which R = 5-F (see Table 1 for rate constants). Nearly 3 orders of magnitude distinguish the values of the fastest and slowest rate constants of methyl transfer among the nine compounds evaluated. Interestingly, an excellent linear free energy correlation, plotted in Figure 3, is noted between methyl transfer rate constants and the methoxy methyl chemical shifts that are plotted in the multiple linear correlation of Figure 2. This correlation and that in Figure 2 suggest in turn that there will be a good correlation between  $\log(k_{Me}^R)$  and the Swain–Lupton parameters, and this is indeed the case (see SI for plot); the corresponding parameters are field =  $1.4 \pm 0.2$ , res =  $1.0 \pm 0.2$ ,  $r^2 = 0.93$ ,  $n = 8$ . These data indicate that  $k_{Me}$  is markedly sensitive to both the field and resonance effects of substituents, as one would expect if the transition state for methyl transfer from *N*-methyl-2-methoxypyridinium species is relatively late and the leaving group strongly resembles the *N*-methylpyridone product. Moreover, the correlation between  $\log(k_{Me})$  and the methoxy methyl chemical shift indicates that additional methyl donor substrates can be readily screened by simple <sup>1</sup>H NMR spectroscopy.



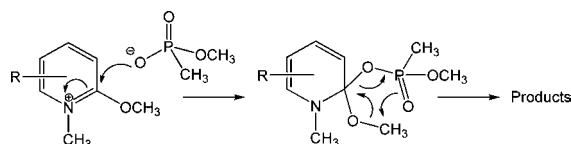
**Figure 3.** Linear free-energy correlation between methyl transfer rate constants and methoxy methyl chemical shifts. Parameters of the fit are slope =  $6.9 \pm 0.4$ , intercept =  $-29 \pm 2$ ,  $r^2 = 0.98$ ,  $n = 8$ .

In a practical sense, the fastest compound (R = 3-F) was capable of 40% methyl transfer to the methyl methanephosphonate monoanion in less than 10 min. This is within the expected time frame for the treatment of OP exposure. The marked sensitivity of the methyl transfer rate constant to the substituent provides a temporal tuneability that will likely be important in the eventual design of resurrection agents for aged OP-AChE adducts.

The hydrolytic stability of the *N*-methyl-2-methoxypyridinium species is an issue of considerable significance, since antidotes against aged OP-AChE adducts must survive the aqueous media of the bloodstream to reach their target. Consequently, initial rates of hydrolysis were obtained by observing pseudo-first-order hydrolysis in D<sub>2</sub>O by <sup>1</sup>H NMR spectroscopy (Table 2). Since the initial rate is  $V_i = k_{\text{hyd}}[A]$ , the corresponding first-order rate constant is calculated as  $k_{\text{hyd}} = V_i/[A]$ . No hydrolysis was observed for compounds R = H, 5-F, and 6-CF<sub>3</sub> over one week in D<sub>2</sub>O. The rate constants of hydrolysis for all compounds were significantly slower than those of methyl transfer in DMSO, typically by 2 orders of magnitude. The 5-NO<sub>2</sub> compound showed the lowest selectivity for methyl transfer over hydrolysis. Another and perhaps more germane measure of hydrolytic stability versus methyl transfer activity was gauged by measuring rate constants of hydrolysis in phosphate buffer. Table 2 shows that the rate constants of hydrolysis in buffer at near-physiological pH are significantly faster than those in pure D<sub>2</sub>O but are still markedly slower than methyl transfer in DMSO. The only compound that showed methyl transfer to the phosphate buffer was that with R = 3-F, which showed slower kinetics of methyl transfer in aqueous media than hydrolysis. The kinetic selectivity of methyl transfer from pyridinium compounds in DMSO, as a model of the AChE active site, versus aqueous hydrolysis suggests that blood serum lifetimes of select pyridinium compounds will be sufficient to reach the aged OP-AChE targets at neuromuscular junctions. On the other hand, the kinetic selectivity of methyl transfer in DMSO versus that to phosphate buffer components in aqueous media suggests that unselective alkylation of other biological phosphorus molecules (e.g., DNA, RNA, or ATP) will be minimal.

In conclusion, a family of *N*-methyl-2-methoxypyridinium species has been shown to be capable of methylating

(11) An S<sub>N</sub>2 reaction is depicted in the abstract. The absence of observable intermediates in these reactions would support this. However, a reviewer suggested an alternative mechanism, which is shown below.



**Table 2.** Rate Constants of Hydrolysis<sup>a</sup>

entry	R =	$k_{\text{hyd}}$ in D <sub>2</sub> O	$k_{\text{hyd}}$ in buffer <sup>b</sup>
<b>1a</b>	H	ND <sup>c</sup>	ND <sup>c</sup>
<b>1b</b>	3-F	$(2.3 \pm 0.2) \times 10^{-5}$	$(7.1 \pm 0.3) \times 10^{-5}$
<b>1c</b>	5-F	ND <sup>c</sup>	ND <sup>c</sup>
<b>1d</b>	5-CF <sub>3</sub>	$(1.10 \pm 0.05) \times 10^{-6}$	$(7.0 \pm 0.5) \times 10^{-5}$
<b>1e</b>	6-CF <sub>3</sub>	ND <sup>c</sup>	$(1.20 \pm 0.06) \times 10^{-4}$
<b>1f</b>	5-NO <sub>2</sub>	$(6.4 \pm 0.4) \times 10^{-5}$	$(2.40 \pm 0.07) \times 10^{-3}$
<b>1g</b>	4-CN	$(1.40 \pm 0.03) \times 10^{-6}$	$(1.60 \pm 0.04) \times 10^{-4}$
<b>1h</b>	5-CN	$(1.40 \pm 0.05) \times 10^{-5}$	$(8.3 \pm 0.2) \times 10^{-4}$
<b>1i</b>	6-CN	$(4.1 \pm 0.3) \times 10^{-6}$	$(4.10 \pm 0.04) \times 10^{-4}$

<sup>a</sup> Rate constants given in units of min<sup>-1</sup> were determined by <sup>1</sup>H NMR spectroscopy. <sup>b</sup> 50 mM phosphate buffer pH = 7.2. <sup>c</sup> Not determined, the reaction was too slow to observe hydrolysis.

a weakly basic phosphonate anion, which is a model for an aged AChE-OP adduct. This reaction is highly tunable based on substituent dependent electronic factors, and gratifyingly high rates of methyl transfer have been observed.<sup>11</sup> On the other hand, these compounds are relatively stable in aqueous solution. The resurrection activity of these compounds versus aged AChE-OP adducts is being explored and will be reported in due course.

**Acknowledgment.** Mr. Alexander Lodge (U-Iowa) is acknowledged for the preparation of sodium methyl methanephosphonate. Supported by the CounterACT Program, National Institutes of Health Office of the Director, and the National Institute of Neurological Disorders and Stroke, Grant No. 5 R21 NS076430.

**Supporting Information Available.** Experimental preparation, <sup>1</sup>H and <sup>13</sup>C NMR for all compounds, and expanded kinetic analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.