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Novel Organophosphate Ligand O-(2-Fluoroethyl)-O-(*p*-Nitrophenyl)Methylphosphonate: Synthesis, Hydrolytic Stability and Analysis of the Inhibition and Reactivation of Cholinesterases

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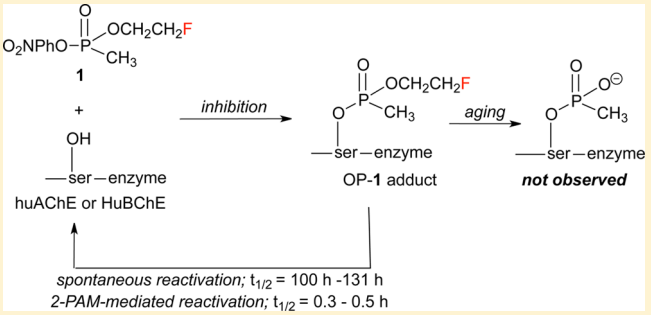
ABSTRACT: The organophosphate *O*-(2-fluoroethyl)-*O*-(*p*-nitrophenyl) methyphosphonate **1** is the first-in-class, fluorine-18 radiolabeled organophosphate inhibitor ([^{18}F]**1**) of acetylcholinesterase (AChE). In rats, [^{18}F]**1** localizes in AChE rich regions of the brain and other tissues where it likely exists as the $(CH_3)(^{18}FCH_2CH_2O)P(O)$-AChE adduct (ChE-**1**). Characterization of this adduct would define the inhibition mechanism and subsequent postinhibitory pathways and reactivation rates. To validate this adduct, the stability (hydrolysis) of **1** and ChE-**1** reactivation rates were determined. Base hydrolysis of **1** yields *p*-nitrophenol and (CH_3) $(FCH_2CH_2O)P(O)OH$ with pseudo first order rate constants (k_{obsd}) at pH 7.4 (PBS) of 3.25×10^{-4} min^{-1} ($t_{1/2}$ = 35.5 h) at 25 °C and 8.70×10^{-4} min^{-1} ($t_{1/2}$ = 13.3 h) at 37 °C. Compound **1** was a potent inhibitor of human acetylcholinesterase (HuAChE; $k_i = 7.5 \times 10^5$ M^{-1} min^{-1}), electric eel acetylcholinesterase (EEAChE) ($k_i = 3.0 \times 10^6$ M^{-1} min^{-1}), and human serum butyrylcholinesterase (HuBChE; 1.95×10^5 M^{-1} min^{-1}). Spontaneous and oxime-mediated reactivation rates for the (CH_3) $(FCH_2CH_2O)P(O)$-serine ChE adducts using 2-PAM (10 μM) were (a) HuAChE 8.8×10^{-5} min^{-1} ($t_{1/2}$ = 131.2 h) and 2.41×10^{-2} min^{-1} ($t_{1/2}$ = 0.48 h), (b) EEAChE 9.32×10^{-3} min^{-1} ($t_{1/2}$ = 1.24 h) and 3.33×10^{-2} min^{-1} ($t_{1/2}$ = 0.35 h), and (c) HuBChE 1.16×10^{-4} min^{-1} ($t_{1/2}$ = 99.6 h) and 4.19×10^{-2} min^{-1} ($t_{1/2}$ = 0.27 h). All ChE-**1** adducts undergo rapid and near complete restoration of enzyme activity following addition of 2-PAM (30 min), and no aging was observed for either reactivation process. The fast reactivation rates and absence of aging of ChE-**1** adducts are explained on the basis of the electron-withdrawing fluorine group that favors the nucleophilic reactivation processes but disfavors cation-based dealkylation aging mechanisms. Therefore, the likely fate of radiolabeled compound **1** *in vivo* is the formation of $(CH_3)(FCH_2CH_2O)P(O)$-serine adducts and monoacid $(CH_3)(FCH_2CH_2O)P(O)OH$ from hydrolysis and reactivation.



INTRODUCTION

Organophosphate (OP) compounds are primarily recognized as useful chemicals for crop protection. However, included in this class are structurally similar OP nerve agents that have been deployed as nondiscriminating chemical weapons. Both OP types are toxic to mammals and insects due to the initial inactivation of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) that is found within central and peripheral nervous tissues and blood.[1−7] The difference in toxic action is largely due to reactivity. Most OP insecticides are thionate (P═S) triesters that must first be converted into the more reactive oxon form (P═O) (Scheme 1; R = alkyl; Y = O-alkyl; leaving group = Z) to inactivate AChE. Conversely, OP nerve agents exist in the reactive oxon form and further differ from insecticide structures in that they are phosphonates that contain a direct carbon−phosphorus (C−P) bond (e.g., Y = Me, Scheme 1). Nerve agents typically contain highly labile leaving groups that lead to rapid inactivation of AChE (e.g., Z = F, thiocholine), as compared with insecticide structures that often contain *O*-aryl leaving groups with higher conjugate acid pK_a values.

Despite these differences, OP insecticide oxons and OP nerve agents inhibit AChE and other cholinesterases by the same mechanism, namely, covalent attachment of the OP to the AChE active site serine concomitant with the ejection of the leaving group to form an OP-AChE adduct (Scheme 1).[8−10] OP-AChE adducts are relatively stable, but many undergo secondary reactions by discrete mechanisms depending on the nature of the remaining groups attached to phosphorus. For example, OP-adducts containing small alkyl ester groups (R = Me, Et) are easily cleaved at the phospho-serine bond thereby reactivating AChE.[3,11] Conversely, OP-AChE adducts with highly branched alkyl ester groups (R = iPr, etc.) derived from OP nerve agents are less likely to be cleaved at the phospho-

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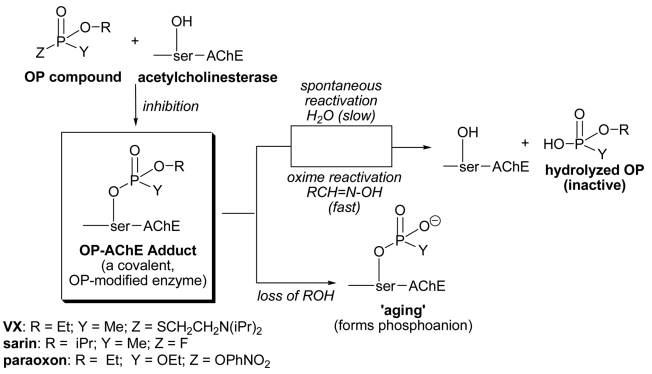
Scheme 1. Structure of OP Insecticides and Nerve Agents, Their Reaction with Acetylcholinesterase to Afford OP-AChE Adducts, and the Possible Adduct Fates



serine bond due to steric and electronic effects and are more susceptible to a dealkylation process known as "aging".[8,12,13] Therefore, the differences between the two OP-adduct mechanistic fates are manifested in distinct kinetic processes that are predicated to a large extent on the OP ester substituents of the OP-AChE adduct. Reactivation[14−16] may be further distinguished as a spontaneous process in which water acts as a nucleophile (hydrolysis) or more rapidly induced in an oxime-mediated nucleophilic process, both of which lead to scission of the AChE serine O−P bond.[8,11,13,17] Alternatively, aging is mechanistically aligned with a putative cationic process (protonation) leading to scission at an alkyl-oxygen bond affording a toxicologically deleterious AChE-phosphoanion (AChE-OP(O)(R)(O^-) species that is recalcitrant to reactivate even with oximes.[5,7,8,13,15,18] As a result, branched phosphoesters such as iPrO- and $OCH(CH_3)C(CH_3)_3$ that can better stabilize cations in the transition state favor aging and proceed via a path alternate to reactivation. Clearly, reactivation and aging mechanisms are competing postinhibitory processes whose rates and outcomes are influenced by substituents with opposing electronic properties.

We recently reported[19,20] the first-in-class fluorine-18 (^{18}F) labeled positron emission tomography (PET) imaging tracer [^{18}F]**1** where the ^{18}F-radionuclide is attached to the *beta*-position of an AChE-reactive phosphonate ethyl ester moiety (Figure 1). The tracer design was based on a hybridized

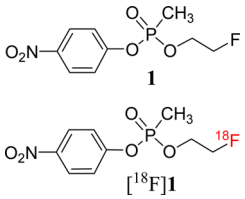


Figure 1. Structures of 2-fluoroethyl 4-nitrophenyl methylphosphonate **1** and PET imaging tracer [^{18}F]**1**.

scaffold of paraoxon, $(EtO)_2P(O)$-$OPhNO_2$, and the nerve agent VX, in which the resultant OP-cholinesterase adduct would closely resemble that formed from VX. The non-radioactive form of **1** possesses excellent activity as an AChE inhibitor (recombinant human AChE; $k_i \sim 10^6$ M^{-1} min^{-1}) by *in vitro* measures,[20] suggesting that the structure of the resultant OP-AChE adduct most likely results from ejection of the *p*-nitrophenoxy leaving group with retention of the *beta*-fluoroethoxy (Scheme 1; R = CH_2CH_2F). Similarly, tracer [^{18}F]**1** when evaluated *in vivo* within rats is found to localize within AChE rich central and peripheral nervous system tissues and in blood, where the majority of measured tissue radioactivity over the course of 1−2 h post-tracer administration is presumed to be the ^{18}F-OP-AChE adduct in the CNS and a mixture of ^{18}F-OP-BChE (butyrylcholinesterase) and ^{18}F-OP-AChE adducts in blood and elsewhere.

From these initial *in vitro* and tracer PET imaging studies, we sought to better understand the mechanism of action of the unique fluorine-containing OPs and the resultant cholinesterase adducts since the presence of a fluorine atom could influence the rate and preference of the reactivation or aging processes. We initially approached the adduct fate inquiry by employing *in vitro* kinetic measures using nonradioactive **1** and determining the rate constants of the OP-AChE and OP-BChE adduct reactivation processes relative to the competitive aging mechanism. Because the OP-ChE adducts formed from **1** differs from the OP-ChE adduct from the nerve agent VX only in the *beta*-fluorine atom, the role of this electronegative atom on the reactivation and aging reactions could be effectively appraised by considering similar *in vitro* measures previously conducted with VX.[21−23]

VX is a 10- to 100-fold more potent anticholinesterase inhibitor ($k_i \sim 10^7$ M^{-1} min^{-1}) than compound **1** due to the improved attraction for AChE and reactivity of the *beta*-diisopropylamino thiolester leaving group. VX yields an OP-AChE adduct in which the phosphylated serine bears an *O*-ethyl methylphosphonate.[18] The *O*-ethyl methylphosphonate OP-AChE adduct formed from VX undergoes spontaneous reactivation in humans (1% per hour over 70 h), and the ethyl ester moiety predictably undergoes aging very slowly relative to branched OP adducts derived from other nerve agents.[24,25] The rate constants of oxime-mediated reactivation (k_r) of rat brain homogenates inhibited by VX are 4.7×10^{-2} min^{-1} and 3.30×10^{-1} min^{-1} using at 10^{-1} to 10^{-8} M of the nucleophilic reactivation agents 2-pyridine aldoxime methiodide (2-PAM) and obidoxime, respectively.[22,26] Treatment of VX-inhibited blood using 2-PAM leads to 55−85% reactivation of AChE activity within 24 h further demonstrating a preference for reactivation over aging *in vivo* (Scheme 1).

On the basis of the structural similarity of **1** to VX, the ChE-**1** adduct would be expected to undergo similar spontaneous and oxime-mediated reactivation rates and show correspondingly slow aging rates. In part, a more detailed evaluation of the postinhibitory kinetic profile of ChE-**1** adducts for acetyl- and butyrylcholinesterase would support the proposed mechanism of the *in vivo* PET tracer in blood and AChE-rich tissues. Herein, we report the rates of spontaneous reactivation and oxime-mediated reactivation for the ChE-**1** adducts, namely, (CH_3) $(FCH_2CH_2O)P(O)$-ChE, resulting from inhibition of acetyl- and butyrylcholinesterase by compound **1**. Also included is an evaluation of the postinhibitory rates with electric eel AChE, which exhibits greatly extended solution stability and can be used to examine the progress of spontaneous reactivation over several days. To accomplish the kinetic evaluations, an improved synthesis and the *in vitro* hydrolytic stability of **1** are also reported since adequate quantities were needed, and the solution half-life is an important determinant in assessing the amount of intact inhibitor for *in vitro* and *in vivo* experiments.

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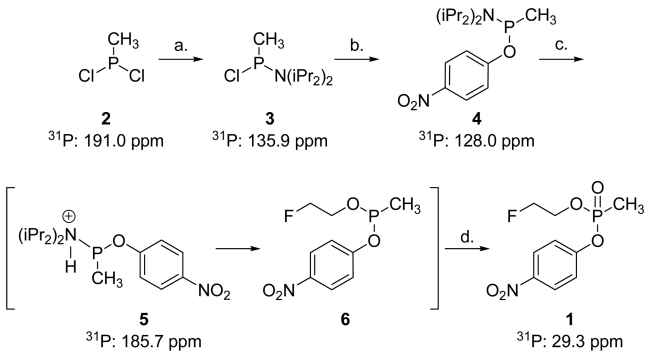
EXPERIMENTAL PROCEDURES

General.[19,20] Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Flash chromatography was conducted using silica gel (200−300 mesh). Thin-layer chromatography (TLC) was visualized by UV and/or staining by 2,6-dibromoquinone-4-chloroimide (DBQ) or iodine. The ^{1}H-, ^{19}F-, and ^{31}P NMR spectra were recorded on a Varian Avance 400-MHz spectrometer. High resolution mass spectrometry was performed with a Micromass LCT -Waters 2795 HPLC with a 2487 UV detector using caffeine as a molecular weight standard. Biochemical reagents were purchased from Fisher Scientific or Sigma-Aldrich (St. Louis, MO). Electric eel acetylcholinesterase (EEAChE), human recombinant acetylcholinesterase (HuAChE), human serum butyrylcholinesterase (HuBChE), acetylthiocholine iodide (ATChI), *S*-butyrylthiocholine iodide (BTChI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and asolectin were purchased from Sigma-Aldrich.

One-Pot Synthesis of 2-Fluoroethyl 4-Nitrophenyl Methylphosphonate 1.[19,20] A solution of diisopropylamine (0.13 mL, 0.98 mmol) and triethylamine (0.13 mL, 0.98 mmol) in dry THF (5 mL) was added dropwise to a solution of dichloromethylphosphine (0.095 mL, 1.1 mmol) at −10 °C with stirring (Scheme 2). The reaction was allowed to continue for 1 h at 25 °C whereupon a solution of *p*-nitrophenol (0.14 g, 0.98 mmol) and triethylamine (0.13 mL, 0.98 mmol) in dry THF (10 mL) were added at 25 °C with vigorous stirring over 3 h. Immediate formation of triethylamine hydrochloride occurred, and the formation of intermediate **4** (^{31}P NMR 128.0 ppm) was observed along with the protonated intermediate **5** (^{31}P NMR 185.71 ppm). To this reaction mixture was added 2-fluoroethanol (0.063 mL, 1.1 mmol) in dry THF (5 mL) at 25 °C. The reaction mixture was stirred for 14 h under argon to form methylphosphonite **6**. Subsequently, the flask was opened to an oxygen atmosphere to form **1**, stirred for 5 min, then the reaction was filtered and extracted with 5 mL $CHCl_3$, and the filtrate was concentrated *in vacuo*. Purification was conducted by flash chromatography using 4:6 ethyl acetate/hexanes to obtain pure **1** (148 mg, 52%): ^{1}H NMR (400.18 MHz, $CDCl_3$) δ/ppm 8.84 (d, J = 9.2 Hz, 2H), 7.41 (d, J = 9.2 Hz, 2H), 4.50−4.67 (m, 2H), 4.24−4.47 (m, 2H), 1.75 (d, J = 17.8 Hz, 3H). ^{13}C NMR (100.63 MHz, $CDCl_3$) δ/ppm 155.14, 144.75, 125.89, 121.11, 82.72, 81.35, 12.21 (d, J_{CP} = 144.15 Hz). ^{31}P NMR (162.0 MHz, $CDCl_3$) δ/ppm 29.33. ^{19}F NMR (376.55 MHz, $CDCl_3$) δ/ppm −224.47. ESI-MS 264 (M+1), HRMS: 264.0434 [(M+H)$^+$].

Scheme 2. Improved, One-Pot Synthesis of 2-Fluoroethyl 4-Nitrophenyl Methylphosphonate 1



a. $HN(iPr)_2$, Et_3N; b. $HOPhNO_2$, Et_3N; c. $HOCH_2CH_2F$; d. air

Hydrolysis of Compound 1. Compound **1** (5 mg; 0.019 mmol) was dissolved in acetonitrile (1 mL), and the solution was added to 0.1 M NaOH (99 mL) at 25 °C, where the hydrolysis was monitored for 30 min. The predominant hydrolytic product, *p*-nitrophenoxide (PNP), was spectrophotometrically quantified at 405 nm using a BioTek Synergy Mx microplate reader and covalidated versus the standard by visible detection and retention time by HPLC. For quantification, *p*-nitrophenol (PNP) was used as the reference standard in the same solution in which 1 mg/mL was equivalent to 1.89 mg/mL of compound **1**.

Evaluation of Stability of Compound 1. A stock solution of compound **1** in acetonitrile was diluted in acetonitrile/phosphate-buffered saline at pH 7.4 (1:9) to afford a concentration of 10.0 μg/mL. The absorbance at 405 nm was continuously monitored at 25 or 37 °C for 24 h using a BioTek Synergy Mx microplate reader or visible detection and retention time by HPLC. The observed rate constant (k_{obsd}) was calculated according to pseudo-first-order kinetics.

Inhibition of HuAChE, EEAChE, and HuBChE by Compound 1. The bimolecular inhibition constant (k_i) for compound **1** against recombinant human (HuAChE), human serum butyrylcholinesterase (HuBChE), and electric eel acetylcholinesterase (EEAChE) were determined using previously published methods[20] adapted from the original spectrophotometric assay by Ellman.[27]

Spontaneous Reactivation of HuAChE, HuBChE, and EEAChE Following Inhibition by 1. HuAChE (~0.01 mg; 0.16 nmol) or HuBChE (~0. One mg; 1.6 nmol) was diluted in phosphate-buffered saline (PBS, pH 7.4) containing 0.76% (w/v) asolectin (added to stabilize the protein over the course of the reactivation experiments) and a control rate of substrate hydrolysis (activity) recorded. The enzyme solution was then incubated with 10 μM of **1** at 25 or 37 °C to achieve >90% inhibition. Aliquots of each sample at various time points were placed in a 96-well microplate followed by mixing with the substrate (1.0 mM ATChI or BTChI) and Ellman's reagent (0.3 mM DTNB) in PBS. At the 25 or 37 °C temperature experiments, the absorbance at 412 nm was recorded at various time points correlating with the production of the thionitrobenzoic acid anion. The overall recovery and rate constant of reactivation (k_r) were determined for each enzyme according to the following equation:

$$\frac{(\text{enzyme activity})_t - (\text{enzyme activity})_0}{(\text{enzyme activity})_{max} - (\text{enzyme activity})_0} = 1 - e^{-k_r \cdot t}$$

An EEAChE solution in pH 7.4 PBS (50 mU/mL) was reacted with aliquots of a stock solution of **1** in acetonitrile/phosphate-buffered saline (1:9, 1 mg/mL) to achieve >90% inhibition. The inhibited EEAChE mixture was incubated at 25 °C and sampled for enzyme activity at 0, 0.25, 0.50, 1.0, 2.0, 4.0, 22, 94, 190, 358, 526, and 694 h, for which percent recovery and the rate constant of reactivation (k_r) were determined for as described above at 25 or 37 °C.

Oxime-Mediated Reactivation of HuAChE, HuBChE, and EEAChE. Compound **1** was prepared as 100.0, 200.0, 400.0, 800.0, or 1600 nM solutions in acetonitrile, and 20.0 μL aliquots of each concentration were added to 180.0 μL of enzyme solution in PBS (HuAChE and HuBChE contained 0.76% w/v asolectin). After vortexing, each mixture was incubated at 25 °C for 30 min to achieve >90% reduction in activity based on initial (untreated) velocity. A 2.50 μL aliquot from each inhibition solution was then added to a 250 μL solution of Ellman's reagent (1.0 mM ATChI or BTChI; 0.3 M DTNB) containing 2-pyridine-aldoxime methiodide (2-PAM) (10 μM) and the enzyme activity measured at 25 °C as the change in absorbance at 412 nm versus time. The rate constant of reactivation (k_r) was calculated as indicated above.

RESULTS AND DISCUSSION

Synthesis of 2-Fluoroethyl 4-Nitrophenyl Methylphosphonate 1. We previously reported the cold ligand synthesis of fluoroethoxy phosphonate **1** via carbodiimide coupling of fluoroethanol with the corresponding phosphonic acid.[20] For preparation of [^{18}F]**1**, *beta*-fluoroethyl tosylate was reacted with MeP(O) (OPNP)O$^-$Cs$^+$ using microwave-assisted accleration.[19] Since neither approach was found to be amenable to a larger scale, a new experimental protocol was examined[28−34] (Scheme 2). Commercially available dichloromethylphosphine **2** (^{31}P NMR 191.0 ppm) was reacted sequentially with diisopropylamine and *p*-nitrophenol to afford phosphonamidite **4**[31] (^{31}P NMR 128.0 ppm; 185.71 ppm in protonated form). When 2-fluoroethanol is added, the

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Table 1. Hydrolysis of *O*-(2-Fluoroethyl)-*O*-(*p*-nitrophenyl)methylphosphonate 1 (Pseudo-First-Order Kinetics)

structure	°C (°K)	k_{obsd} (min^{-1})[a]	$t_{1/2}$ (h)[b]	R^2
MeP(O)(OCH_2CH_2F)(OPNP)	25 (303)	3.25×10^{-4} (n = 4; ± 0.66)	35.6	0.962
	37 (310)	8.70×10^{-4} (n = 4; ± 1.08)	13.3	0.998
MeP(O)(OCH_2CH_3) (OPNP)[40]	37 (310)	4.03×10^{-5}	4.8	

[a] $\ln(C_t/C_0)$ vs $1/t$ where C_t = decrease in **1**. [b] $t_{1/2} = 0.693/k_{obsd}$.

Table 2. Rate Constants for the Inhibition and Reactivation of HuAChE, HuBChE, and EEAChE by Compound 1[a]

process	HuAChE	HuBChE	EEAChE
bimolecular inhibition constant (k_i; $M^{-1}min^{-1}$)[b]	7.49 (±1.37) × 10^5	1.95 (±0.48) × 10^5	5.90 (±0.15) × 10^6
spontaneous reactivation (k_r; min^{-1})	9.31 (±0.07) × 10^{-5} ($t_{1/2}$ = 124 h)	1.13 (±0.02) × 10^{-4} ($t_{1/2}$ = 102 h)	8.75 (±1.89) × 10^{-3} ($t_{1/2}$ = 1.3 h)
oxime-mediated reactivation[c] (k_{oxime}; min^{-1}) 30 min postinhibition	2.40 (±0.14) × 10^{-2} ($t_{1/2}$ = 0.48 h)	1.14 (±0.34) × 10^{-2} ($t_{1/2}$ = 1.0 h)	3.37 (±0.74) × 10^{-2} ($t_{1/2}$ = 0.34 h)
oxime-mediated reactivation[c] (k_{oxime}; min^{-1}) 18 h postinhibition	2.47 (±0.12) × 10^{-2} ($t_{1/2}$ = 0.47 h)	4.19 (±0.12) × 10^{-2} ($t_{1/2}$ = 0.28 h)	nd

[a] Mean ± SD ($n \geq 3$). [b] k_i for EEAChE from ref 20. [c] 2-Pyridine aldoxime methiodide (2-PAM). nd = not determined due to negligible inhibition.

intermediate trivalent phosphonate diester **6** is formed and upon exposure to air for 5 min is converted to the desired oxon (P═O) product **1**.

The one-pot, four-step process (Scheme 2) takes 18 h and affords a 52% yield of desired product, and **1** is characterized by a ^{31}P NMR shift of 29.3 ppm that is identical when compared to the authentic material.[19] The synthetic sequence relies upon *in situ* protonation of the phosphonamidite nitrogen to convert the diisopropylamine to a better leaving group and permit reaction with the weakly nucleophilic fluoroethanol. Reaction of alcohols with *N*,*N*-dialkylphosphonamidites is typically conducted in sequence with tetrazole and oxidizing agents.[28−30,35−39] However, in this reaction sequence triethylamine hydrochloride or *p*-nitrophenol serves as the proton donor, and the use of oxidizing agents such as *m*-CPBA and *t*-BuOOH to produce **1** from **6** was less effective than air exposure. Product **1** may also be accessed via purification of **4** by column chromatography followed by the addition of tetrazole[31] and fluoroethanol, and oxidation with *t*-BuOOH (~25% from **4**).

Hydrolytic Stability of 1. Hydrolysis rates were determined for compound **1** since any significant degradation over the time course of the reactivation or *in vivo* studies would influence the mechanistic interpretation. The *p*-nitrophenoxy (PNP) leaving group of **1** was specifically installed in the tracer design to minimize volatility, reduce toxicity relative to nerve agents, and slow the OP hydrolysis rate relative to a fluorine leaving group present on nerve agents (e.g., sarin, soman). However, the PNP group is labile (pK_a ~7.2), and an assessment of the hydrolysis of **1** under the assay conditions is warranted. Since the hydrolysis of compound **1** (λ_{max} = 281 nm) forms nitrophenol and (CH_3) (FCH_2CH_2O)P(O)OH, the nitrophenolate anion (λ_{max} = 405 nm) was used as an indicator. Aliquots (30 μg/mL) of a 10.8 mM stock solution of compound **1**, prepared from 2.86 mg/mL (1.51 mg/mL PNP) in CH_3CN, were diluted 100-fold with 0.1 M NaOH, and the phenolate absorbance was measured at 405 nm. Construction of a standard curve afforded a slope = 0.180 (R^2 = 0.99) that was used to determine an average maximal response of 10.2 ± 0.4 mM of **1**. Compound **1** was then formulated in phosphate buffered saline (PBS) at pH 7.4 and the stability of this solution determined at 25 and 37 °C (Table 1). The pseudo-first-order rate constants (k_{obsd}) at pH 7.4 (PBS) were 3.25×10^{-4} min^{-1} ($t_{1/2}$ = 35.6 h) at 25 °C and 8.70×10^{-4} min^{-1} ($t_{1/2}$ = 13.3 h) at 37 °C. The hydrolysis rate of **1** at 37 °C is expectedly slower than that found for the ethyl (nonfluorinated) compound (CH_3) (CH_3CH_2O)P(O)OPNP with (k_{hyd}) = 2.42×10^{-3} min^{-1} ($t_{1/2}$ = 4.8 h) conducted at pH 8.3.[40] The hydrolysis rates indicate that compound **1** is stable for the duration of the inhibition experiments but would hydrolyze to a large extent at the latter time points during reactivation measurements, which would suggest that reinhibition of reactivated enzyme would be unlikely. As noted, the rate constants for **1** are in general agreement with those found for triester OP compounds bearing a *p*-nitrophenoxy leaving group (e.g., paraoxon), although the reported hydrolysis conditions differed.[41] From the hydrolysis data, it is concluded that the fluorine atom does not cause enhanced hydrolytic degradation of compound **1** and is stable enough for accurate *in vitro* enzyme assays, in PBS formulations used *in vivo* for the tracer [^{18}F]**1**[19] and over the course of the tracer lifetime.

Inhibition of HuAChE, HuBChE, and EEAChE by 1. Compound **1** was previously shown to be a relatively potent inhibitor of HuAChE and EEAChE;[20] however, the inhibition of human butyrylcholinesterase (HuBChE) had not been evaluated. Compound **1** was found to be a relatively potent inhibitor of HuBChE with $k_i = 1.95 \times 10^5$ $M^{-1}min^{-1}$ (Table 2), which is only 7.4-fold weaker than paraoxon as the inhibitor ($k_i = 1.44 \times 10^6$ $M^{-1}min^{-1}$). This ratio correlates with data showing that compound **1** is an 8.4-fold weaker inhibitor ($k_i = 7.49 \times 10^5$ M^{-1} min^{-1}) than paraoxon (6.28×10^6 M^{-1} min^{-1}) toward HuAChE (Table 2).[20] The inhibition rate constants for **1** relative to paraoxon indicate that the anticholinesterase activity does not appear to be affected by the presence of the fluorine atom. In comparison, work by Chambers and coworkers demonstrated[23,42] that the *p*-nitrophenoxy analogue of VX, (Me)(EtO)P(O)(OPNP) and previously reported by Fukuto,[40] is a potent inhibitor ($k_i \sim 10^5$ $M^{-1}min^{-1}$) of various acetyl- and butyrylcholinesterases and simulates the mechanism of VX inhibition.[23,42]

Enzymatic Reactivation. HuAChE, EEAChE, and HuBChE were used to evaluate the rate and extent of spontaneous and oxime-mediated reactivation following inhibition by compound **1** to determine the influence and contribution of the fluorine atom. Human AChE and BChE were assessed to identify differences in the response of mammalian cholinesterases to **1**, which may advance our understanding of the previously evaluated tracer interaction

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with AChE- and BChE-rich CNS tissues and blood.[19] Electric eel acetylcholinesterase (EEAChE) was examined because it has excellent solution stability over an extended experimental time course that would permit multiday, extended monitoring of the reactivation experiments.

Assessment of Spontaneous Reactivation. Spontaneous reactivation occurs when the phosphoserine bond of an OP-cholinesterase adduct (Scheme 1) is cleaved without the aid of reactivating agents.[1,2,13,15,22,43] In principle, the resultant ChE, free of OP, may have part or all of the catalytic activity restored, although the fraction of ChE that is reactivated varies with the structure of the OP attached and the cholinesterase subtype and reactivator used. OP-ChE adducts with small phosphoester groups such as MeO- or EtO- undergo spontaneous reactivation more readily with half-lives ranging from a few minutes to hours.[5,13] Conversely, OP-ChE adducts containing branched esters such as iPrO- or $OCH(Me)C(Me)_3$ are slower to reactivate and prone to aging. Spontaneous reactivation occurs at rates far slower (>100×) than reactivation induced by oxime countermeasures such as 2-pyridine aldoxime methiodide (2-PAM). To evaluate spontaneous reactivation, a solution containing huAChE or huBChE was treated with compound **1** at various concentrations (10−160 nM) leading to greater than 95% inhibition at 30 min. The recovery of enzyme activity at each concentration was then monitored following a 40-fold dilution with PBS at pH 7.4 to minimize further inhibition.

The spontaneous reactivation rate constants k_r for HuAChE and HuBChE following inhibition by **1** were 9.31×10^{-5} min^{-1} ($t_{1/2}$ = 124 h) and 1.13×10^{-4} min^{-1} ($t_{1/2}$ = 102 h) (Table 2). The spontaneous reactivation rates did not show any evidence of dependence on the concentration of the inhibitor (10−160 nM). The mammalian reactivation data determined in this study were in general agreement with spontaneous reactivation rates found for VX and the *p*-nitrophenoxy VX surrogate.[22,25,42,44] However, the spontaneous rates for the mammalian enzymes were 75-fold slower than that observed for EEAChE (8.75×10^{-3} min^{-1}; $t_{1/2}$ = 1.32 h), which undergoes reactivations rapidly. The spontaneous reactivation of EEAChE inhibited by **1** is slightly faster than the $t_{1/2}$ = 2.6 h reported for sarin-inhibited EEAChE (Scheme 1; R = iPr, Z = F, Y = Me),[45] which is presumably due to the fact that sarin-modified EEAChE contains an isopropyl ester rather than the fluoroethoxy. Therefore, sarin-modified EEAChE would be expected to reactivate slower as a result of the bulkier substituent that impedes delivery of the hydroxyl/water to the phosphorus atom in the reactivation mechanism. Paraoxon and haloxon are related as ethyl and *beta*-chloro ethyl esters (Figure 2) that react with bovine red blood cell AChE to afford OP-AChE adducts bearing a diethoxyphosphyl and di-(chloroethoxy)phosphyl ester groups, respectively. In this comparison, the spontaneous reactivation rates for paraoxon $t_{1/2}$ = 58 h[46] and haloxon $t_{1/2}$ = 0.3 h[47] suggest that beta-halogen or related withdrawing substituents dramatically accelerate spontaneous reactivation rates.

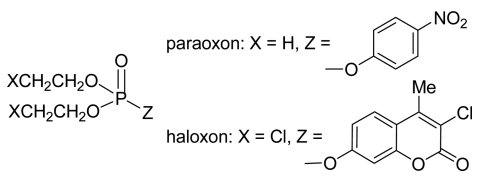

Figure 2. Structures of paraoxon and haloxon.

Because of the greater solution stability of EEAChE, the spontaneous reactivation was followed over multiple days. When EEAChE was inhibited >95% by 1−5 nM with compound **1**, complete restoration of the EEAChE activity was observed within a day, and inhibition using a higher concentration of **1** (20−100 nM) still led to >98% recovery of the original activity by 3 weeks. These data suggest that very little aging occurred, although aging cannot be completely ruled out for the mammalian enzymes.

Assessment of Oxime-Mediated Reactivation. Quaternary oximes such as 2-PAM and MMB-4 are well-recognized for their use as peripheral acting antidotes for organophosphate poisoning.[2,4,11,13,17,22,26,48−50] 2-PAM (pralidoxime) is the only FDA-approved countermeasure for OP chemical agent exposure for use in the US. The reaction of the ChE-1 inhibitor complex with 2-PAM was evaluated using the same concentrations of inhibitor **1** as that used for spontaneous reactivation. Following incubation of AChE or BChE (0.16 nM) with inhibitor **1** at 25 °C using a concentration range of 10−160 nM for 30 min, greater than 90% reduction in enzyme activity was observed based on initial (untreated) velocity. At 30 min and then at 18 h, the mixture was treated with 2-PAM (10 μM), and the rate of ATChI or BTChI hydrolysis activity was measured (Table 2). At both 30 min and 18 h postinhibition time points, dramatic accelerations of oxime-mediated reactivation rates were observed as compared to spontaneous reactivation. Reactivation rates for huAChE inhibited by **1** (10−160 nM) ranged from 1.762×10^{-2} min^{-1} to 2.410×10^{-2} min^{-1}, and huBChE inhibited by **1** varied from 1.080×10^{-2} min^{-1} to 4.051×10^{-2} min^{-1} indicating no dependence of reactivation rate on [1]. HuAChE inhibited by **1** undergoes oxime-mediated reactivation at statistically equivalent rates of 2.40×10^{-2} min^{-1} ($t_{1/2}$ = 0.48 h) and 2.47×10^{-2} min^{-1} ($t_{1/2}$ = 0.47 h) at 30 min and 18 h, respectively. The previous finding that no aging occurred by 4 h after the rat brain was inhibited by the *p*-nitrophenoxy VX surrogate supports reactivation as the major pathway; however, 12% aging was observed at 24 h suggesting partial loss of the ethoxy group.[23] Yet the presence of the fluorine atom in the AChE-1 adduct may favor greater oxime-mediated reactivation and less aging accounting for this small difference.

HuBChE inhibited by **1** undergoes oxime-mediated reactivation at a slightly slower rate at 30 min (1.14×10^{-2} min^{-1}; $t_{1/2}$ = 1.01 h) than at 18 h (4.19×10^{-2} min^{-1}; $t_{1/2}$ = 0.27 h), although both rates are comparable to that found for HuAChE and producing a similar dephosphylation mechanism. Because of the rapid spontaneous reactivation of the EEAChE-1 adduct (~20 min), an oxime-mediated rate at the 18 h time point was not possible. The oxime-mediated rate constant at 30 min correlates with paraoxon-inhibited EEAChE that is reactivated by 2-PAM (10 μM) with a rate constant k_r = 0.176 min^{-1} or $t_{1/2}$ = 0.065 h (4 min).[51] One explanation for the slightly slower oxime-mediated reactivation is that the AChE-inhibitor complex formed from 1 (ChE-1) contains a C−P bond rather than a C−O−P bond (paraoxon) that poses a greater steric barrier to nucleophilic attack by the oxime oxyanion.

The fluoroethoxyphosphonate compound **1** was previously reported as a potent acetylcholinesterase inhibitor *in vitro*, and the radiolabeled ^{18}F-form has recently been shown to be a useful PET imaging tracer in rodents.[19,20] In this article, we have improved the preparation of **1** by reducing the synthetic process to a one-pot sequence that affords **1** in a greater yield relative to that of the previous synthetic route reported. We

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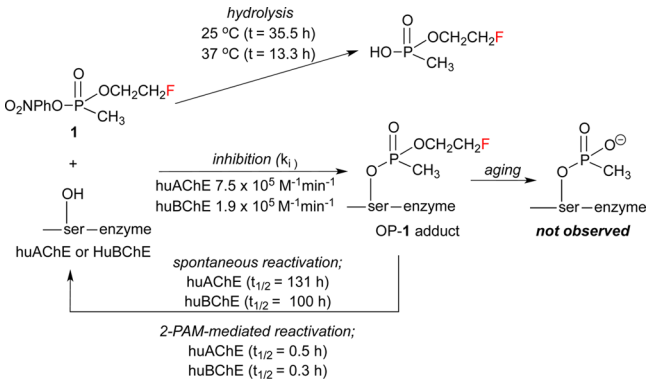
also demonstrated that **1** is hydrolytically stable *in vitro* at 25 °C ($t_{1/2}$ ~ 35.6 h) and at 37 °C ($t_{1/2}$ ~ 13.3 h) in pH 7.4 buffer. Compound **1** hydrolyzes with the loss of the *p*-nitrophenoxy group as the dominant degradation pathway (Scheme 3). The

Scheme 3. Compound 1 Hydrolysis, Inhibition, and Reactivation Rates



in vitro hydrolytic stability half-life suggests that a significant fraction of tracer **1** will survive *in vivo* dose formulation and administration, although hydrolytic breakdown *in vivo* by enzymes could also play a significant role. This notion is consistent with the demonstrated *in vivo* ability of tracer [^{18}F]**1** that remains intact within blood and penetrates into the brain. Conversely, if compound **1** had been found to undergo rapid *in vitro* hydrolytic breakdown to the corresponding phosphonic acid, the product phosphonic acid would ionize at physiologic pH and would be unlikely to penetrate and localize in the brain.

The reaction of **1** with ChE's affords *beta*-fluoroethoxy methylphosphonate adducts that undergo both spontaneous and oxime-mediated reactivation. Spontaneous and oxime-mediated reactivation rates were comparable for both HuAChE and HuBChE inhibited by **1** with the oxime-mediated rates 100- to 250-fold faster than spontaneous rates. Rate accelerations of 100-fold when using oximes for VX-inhibited cholinesterases have been reported.[21,22,26] There was little difference in the oxime-mediated reactivation rates found at 30 min and 18 h, and in general, mammalian enzyme inactivated at >95% of initial velocity fully recovered activity. EEAChE, used as a control enzyme for longer analysis time points, underwent spontaneous reactivation almost 100-times faster than HuAChE and recovered almost 50% of the original activity within an hour. No evidence of aging was observed as indicated by greater than 99% of the original activity being restored at all time points upon treatment with 2-PAM.

Although it is not surprising that the oxime-mediated reactivation rates occurred faster than spontaneous reactivation, most OP-AChE adducts formed from nerve agents (e.g., methylphosphonates) do not undergo complete reactivation due to competing aging processes. The nerve agent VX is an exception[25] that affords an ethoxy methylphosphonate AChE adduct that is largely reactivatable but undergoes some aging.[17,22−24] The OP-AChE adduct formed from **1** differs from the AChE-VX adduct in that the former possesses a *beta*-fluoro substituent where the rapid reactivation of the EEAChE-**1** adduct is explained on the basis of the electron-withdrawing fluorine atom favoring the nucleophilic process (water or an oxime). In this study, no evidence of aging of any of the ChE-**1** adducts was observed that is supported by the rapid reactivation rates and near complete restoration of activity using 2-PAM including the restoration of activity at 48 h. The absence of any significant aging for this adduct is likely due to the unbranched ethyl ester moiety in combination with the electron-withdrawing fluorine atom such that both contribute to the suppression of the putative cation-based aging mechanism. However, an aging pathway cannot be ruled out *in vivo* as was reported for the VX surrogate compound.[23]

Therefore, the presence of the beta-fluorine atom in the ChE-**1** adducts is thought to influence reactivation and aging rate processes by inductive effects. The reactivation rates are slightly faster than that observed for VX-modified AChE, and aging was not found. These *in vitro* findings with OP-**1** provide support for a similar mechanism of AChE inhibition with the radiotracer [^{18}F]**1** *in vivo*, in which the corresponding [^{18}F]**1**-AChE adduct most likely does not undergo aging over the PET imaging time course (e.g., 90−120 min) and tracer lifetime (fluorine-18 half-life, ~110 min). In that case, a significant portion of the measured tissue radioactivity by PET imaging over the course of 2 h post-tracer administration likely represents an [^{18}F]**1**−AChE adduct.

It is important to note that studies with recombinant huAChE, serum huBChE, and EEAChE provide relevant and useful mechanistic insights that may not be operative *in vivo*. In this study, the rate constants of inhibition and reactivation and proposed mechanisms do not take into account the asymmetry of **1** at phosphorus, which is known to affect inhibitory and postinhibitory rate and mechanistic processes.[52−56] Preparation or separation of the individual enantiomers of **1** and evaluation of their antiesterase properties are the subject of a future study.

On the basis of the studies described here, experiments in our laboratories are ongoing to quantitatively assess whether the *in vitro* spontaneous and/or oxime-induced reactivation rates are similar to the *in vivo* rates, where the fate of the [^{18}F]**1**−AChE adduct in live tissues prospectively can be observed and quantified by PET imaging. Results from the *in vivo* reactivation studies will be reported in due course.

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Notes

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ABBREVIATIONS

ACh, acetylcholine; AChE, acetylcholinesterase; huAChE, recombinant human acetylcholinesterase; huBChE, human butyrylcholinesterase; EEAChE, electric eel acetylcholinester-

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ase; PBS, phosphate-buffered saline; PNP, *para*-nitrophenoxy; OP, organophosphate

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