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Synthesis and anti-acetylcholinesterase properties of novel β- and γ-substituted alkoxy organophosphonates

S. Kaleem Ahmed, Yamina Belabassi, Lakshmi Sankaranarayanan, Chih-Kai Chao, John M. Gerdes, Charles M. Thompson *

Department of Biomedical and Pharmaceutical Sciences, The University of Montana, Missoula, MT 59812, USA

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ABSTRACT

Activated organophosphate (OP) insecticides and chemical agents inhibit acetylcholinesterase (AChE) to form OP-AChE adducts. Whereas the structure of the OP correlates with the rate of inhibition, the structure of the OP-AChE adduct influences the rate at which post-inhibitory reactivation or aging phenomena occurs. In this report, we prepared a panel of β-substituted ethoxy and γ-substituted propoxy phosphonoesters of the type *p*-NO_2PhO-P(X)(R)[$(O(CH_2)_nZ$] (R = Me, Et; X = O, S; n = 2, 3; Z = halogen, OTs) and examined the inhibition of three AChEs by select structures in the panel. The β-fluoroethoxy methylphosphonate analog (R = Me, Z = F, n = 2) was the most potent anti-AChE compound comparable (k_i $\sim 6 \times 10^6$ M^{-1} min^{-1}) to paraoxon against EEAChE. Analogs with Z = Br, I, or OTs were weak inhibitors of the AChEs, and methyl phosphonates (R = Me) were more potent than the corresponding ethyl phosphonates (R = Et). As expected, analogs with a thionate linkage (P═S) were poor inhibitors of the AChEs.

Organophosphate (OP) compounds (e.g., diazinon, parathion; Fig. 1; P═S) have been widely employed as insecticides in which their mode of toxic action first requires conversion to the more reactive P═O (oxon) form.[1,2] Conversely, OP compounds that contain the oxon functional group and possess a leaving group that is readily stabilized upon release (e.g., F^-, CN^-) are readily reactive and define the properties present in nerve agents like VX, sarin (GB) and soman (GD) (Fig. 1).[3,4]

The mechanism of toxicity of most highly reactive OPs results from the inhibition of acetylcholinesterase (AChE), specifically phosphorylating an active site serine to produce an OP-AChE adduct.[1,2,4] Formation of OP-AChE adducts diminish or halt the hydrolysis of the neurotransmitter, acetylcholine (ACh) causing a surplus of ACh in the neuronal synapse that triggers overexcitation of nicotinic and muscarinic receptors leading to neurotoxic events. Once the OP-AChE adduct is formed it can undergo reactivation (restoration of activity) or aging, which is defined as loss of a phosphoester group leading to an oxyanion that is intractable to reactivation (Scheme 1).[5–7]

The reactivation of OP-AChE adducts occurs by nucleophilic displacement at phosphorus (by water, oximes), whereas aging is influenced by branching and/or inductive properties that favor cationic (proton-assisted) mechanisms. For example, dimethoxyphosphoryl-AChE (R = CH_3, Y = OCH_3) undergoes reactivation within 1–2 h (or minutes using oxime antidotes) and ages with a $t_{½}$ ~2–10 h. Conversely, OP-AChE adducts containing branched alkoxy groups like sarin (R = $CH(CH_3)_2$) or soman (R = $CH(CH_3)(C(CH_3)_3$) undergo aging to the methyl phosphonate anion (Y = CH_3) in 3–6 h and 2–5 min, respectively, affording little chance for the OP-adducts to reactivate.[8]

When the OP-ester component attached to AChE is a small, unbranched alkoxy group (R = CH_2CH_3, $CH_2CH_2CH_3$), these adducts are known to reactivate slowly and do not undergo appreciable aging ($t_{½}$ ~36–48 h) suggesting that these OP-AChE adducts are relatively stable.[9] Herein, we report the synthesis and anti-AChE activity of OP compounds designed to form analogs of these stable OP-AChE adducts, for example, (ZCH_2CH_2O)(Y)P(O)-AChE and ($ZCH_2CH_2CH_2O$)(Y)P(O)-AChE (Y = CH_3, CH_2CH_3) in which Z is a halogen or tosylate substituent. It was thought that these customized inhibitors would form OP-AChE adducts in which the Z-substituent is positioned to influence the adduct stability and post-inhibitory reaction pathways. Since there are no systematic reports that prepare AChE-reactive, organophosphate compounds containing β- or γ-substituent phosphoester groups, this study was aimed to identify useful synthetic strategies to these structure supported by preliminary enzymatic evidence demonstrating their potential as enzyme inhibitors.

The OP targets (4 and 5) were designed and synthesized (Scheme 2) to include a number of key features. To produce OPs with useful inhibitory properties, a *p*-nitrophenol ester was installed as a leaving group. To examine the role, if any, of the alkyl group on the inhibition mechanism, both methyl and ethyl phosphonates were considered as targets. Both phosphonothionate

* Corresponding author. Tel.: +1 406 243 4643; fax: +1 406 243 5227.
E-mail address: charles.thompson@umontana.edu (C.M. Thompson).

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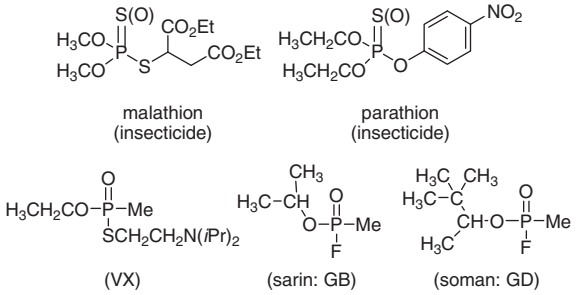
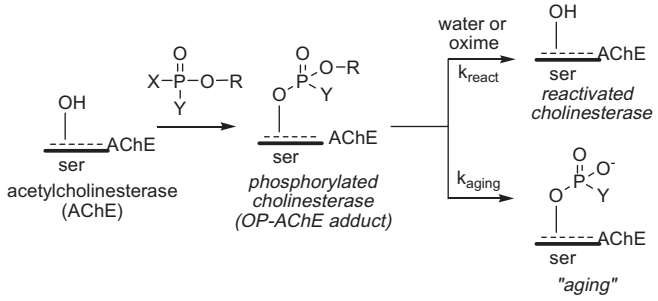


Figure 1. Representative OP insecticides and nerve agents.



Scheme 1. OP-inhibition of AChE and subsequent reactivation and aging reactions.

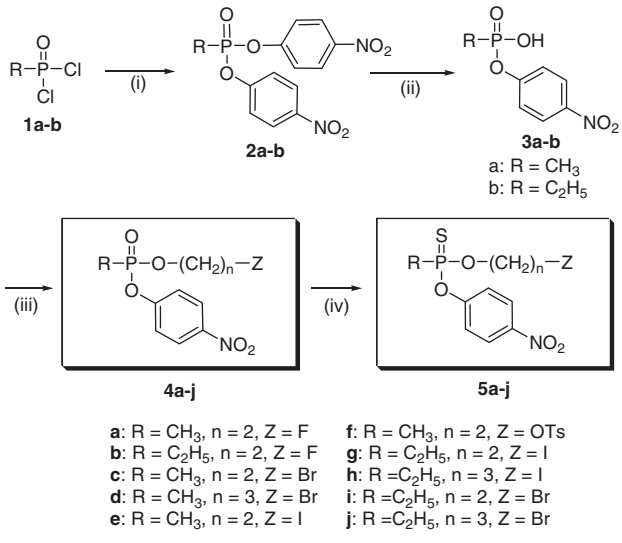
(P═S) and phosphonate oxon (P═O) analogs were prepared. Phosphonothionates are expected to be less reactive toward nucleophilic displacement than oxons, and therefore likely to be poor inhibitors of AChE.[10] However, the thionate P═S bond reduces reactivity at the phosphorus center allowing functional group transformations to more readily proceed elsewhere on the molecule. Substituted, ethoxy and *n*-propoxy phosphoester groups were considered to evaluate the ease of substituent installation and potentially examine the contribution of inductive effects on inhibition. Additionally, if certain alkoxy substituents (Z) are leaving groups, the alkylation of AChE residues residing in proximity to the active site serine will be examined in a future study. To this end, twenty novel OP structures were produced with the most notable variation occurring in the alkoxy β, γ-substituents as F, Br, I and OTs.

Compounds **4a–j** and **5a–j** were first synthesized (Scheme 2) starting from the corresponding phosphonyl chlorides **1a** (R = CH_3) and **1b** (R = CH_3CH_2). Reaction with *p*-nitrophenol (PNP) in base affords the bis(4-nitrophenoxy) alkylphosphonates (**2a–b**) in good yield 87–91%.[11] To convert **2a–b** to the 4-nitrophenyl, hydrogen alkylphosphonic acids, LiOH, NaOH, KOH, $Ba(OH)_2$, $Ca(OH)_2$ were examined. Most conditions formed the hydrolyzed diacids or mixtures of hydrolysis products[11] except LiOH (0.5 M aq) with CH_3CN that led to monoesters **3a–b** in moderate yield 51–54%.[12]

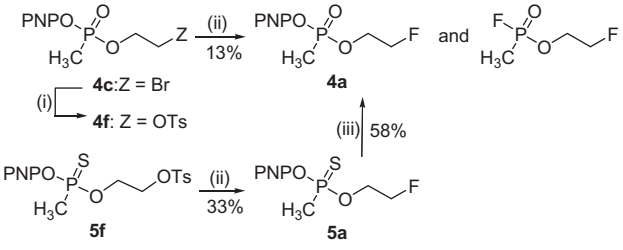
Monoesters **3a–b** were converted to the target compounds **4a–j** by reaction of the corresponding haloalcohol or tosyl alcohol in the presence of a coupling reagent, dicyclohexylcarbodiimide (DCC) or benzotriazol-1-yl-oxy-*tris*-(dimethylamino)-phosphonium hexafluorophosphate (BOP·PF_6). Both coupling reactions were successful; however, the use of DCC afforded higher yields.[13] Coupling of monoesters **3a–b** to the alcohols was also attempted using Mitsunobu conditions or water soluble carbodiimides, however, neither worked.[14,15] Oxons **4a–j** were treated with Lawesson's reagent to form the corresponding phosphonothionates **5a–j** in 60–65% yield.[16]

A more straightforward route to the target OPs **4a–j** and **5a–j** was undertaken by attempting to directly introduce the Z substituent by nucleophilic displacement of a leaving group on the ester side chain (Scheme 3). For example, the bromoethyl ester **4c** was treated with silver tosylate to form **4f** in 48% yield. We next attempted to prepare the 2-fluoro derivative **4a** using tetrabutyl ammonium fluoride (TBAF; 1.0 M aq THF; 10 min) and the yield was low (13%). We attributed this low product yield to competing nucleophilic substitution at phosphorus and formation of a P–F bond as confirmed by the appearance of a d_{F-P} in the ^{31}P (J = 1074 Hz). To circumvent the P–F bond formation problem, which results in a toxic product, we examined the TBAF reaction using the thionate (P═S) **5f** as starting material, which owing to stronger pπ–dπ overlap, would be less reactive toward nucleophiles at phosphorus. When **5f** was treated with TBAF, fluoride ion preferably displaced the OTs leaving group rather than the *p*-nitrophenol ester moiety in 10 min to afford **5a** in 33% yield. This yield was acceptable because there was no evidence of P(S)–F bond formation. The β-fluoroethoxy thionate **5a** was treated with *m*-CPBA to obtain **4a** in 58% yield.[17–19] In sum, nucleophilic displacement of a side chain leaving group (Z) works with phosphonothionates as substrates but poorly with phosphonates leading to the reaction occurring preferentially at phosphorus producing hazardous phosphonofluoridates. All compounds were characterized by ^{1}H, ^{13}C, ^{31}P, ^{19}F NMRs and HRMS data.[23–26]

Many of the new compounds were screened for their inhibitory potency against EEAChE (Table 1), and five were assessed in detail for their anti-cholinesterase activity. Owing to variations in the



Scheme 2. (i) *p*-NO_2PhOH, Et_3N, CH_2Cl_2, 0 °C to rt, 4 h; (ii) 0.5 M LiOH, CH_3CN, rt, 1 h; (iii) $Z(CH_2)_nOH$, DCC or BOP·PF_6, CH_2Cl_2, rt, 24 h; (iv) Lawesson's reagent, toluene, 80 °C, 3 h.



Scheme 3. Alternate route to target organophosphates: (i) $TsO^{-}Ag^{+}$, CH_3CN, 65 °C, 4 h; (ii) 1.0 M TBAF in THF, rt, 10 min; (iii) *m*-CPBA, CH_2Cl_2, rt, 30 min.

Table 1
Inhibition rate constants (k_i M^{-1} min^{-1}) for **4a**, **4b**, **4c**, **4f** and **5c** against EEAChE, rHAChE and RBAChE

Compd	Method	EEAChE	rHAChE	RBAChE
4a	CD	$5.90 \pm 0.15 \times 10^6$	$7.51 \pm 0.21 \times 10^6$	$6.11 \pm 0.25 \times 10^6$
	TD	$2.50 \pm 0.50 \times 10^6$	$1.40 \pm 0.59 \times 10^7$	$6.16 \pm 0.28 \times 10^6$
4b	CD	$5.52 \pm 0.09 \times 10^4$	$6.16 \pm 0.16 \times 10^4$	$1.16 \pm 0.06 \times 10^6$
	TD	$8.64 \pm 2.20 \times 10^4$	$1.02 \pm 0.05 \times 10^5$	$3.77 \pm 0.48 \times 10^6$
4c	CD	$8.11 \pm 0.29 \times 10^4$	nd	nd
4f	TD	$1.14 \pm 0.03 \times 10^5$	nd	nd
5c	TD	$1.17 \pm 0.14 \times 10^4$	nd	nd
Paraoxon	CD	$1.96 \pm 0.05 \times 10^6$	nd	$1.58 \pm 0.22 \times 10^8$
	TD			$3.39 \pm 0.14 \times 10^7$

EEAChE: electric eel acetylcholinesterase; rHAChE: recombinant human acetylcholinesterase; RBAChE: rat brain acetylcholinesterase. All k_i values (mean ± SEM) were determined from 2 to 4 different experiments at 25 °C. nd: Not determined; CD: concentration-dependent; TD: time-dependent. All rate constants (k_i) were determined by Ellman assay.[21]

inhibitor solubility and reactivity, the bimolecular inhibition constants (k_i) for select compounds were determined using two kinetic methods: (a) a set inhibitor concentration [I] incubated with AChE for various time points (time-dependent) and (b) variable [I] incubated with AChE and measured at a set time point (concentration-dependent[20]) kinetics (Table 1). Compounds **4a** and **4b** differ only in the alkyl-P bond as methyl- and ethyl phosphonates, respectively, and were examined by concentration- and time-dependent methods. Compounds **4a**, **4c** and **4f** are all methyl phosphonates that vary in the β-ethyl substituent Z and were examined by concentration or time-dependent kinetics. Paraoxon was also examined as a control.

First, the difference between methyl- and ethyl phosphonates was examined. The fluoroethoxy methyl phosphonate **4a** was found to be a 30- to 100-fold better inhibitor of EEAChE than the corresponding ethyl phosphonate **4b** as determined by both time- and concentration-dependent kinetic methods. The inhibitory potency of **4a** was $k_i \sim 5 \times 10^6$ M^{-1} min^{-1} toward EEAChE, which is comparable to the highly potent inhibitor, paraoxon.

Changing from 2-fluoroethoxy **4a** to the 2-bromoethoxy methylphosphonate **4c** led to a 70-fold decrease in inhibitory potency. This large difference in inhibition is somewhat surprising in light of the relatively small inductive and steric differences between F and Br that would be expected. However, additional or unforeseen steric considerations that affect the inhibitory potency other than those indirectly influencing the phosphorus atom cannot be excluded.

Replacing halogen substituents with a tosylate **4f** afforded a 50-fold weaker inhibitor of EEAChE than the corresponding fluoro analog **4a** but comparable to the bromo analog **4c**. This result suggests that the larger tosylate group did not adversely affect the inhibition of EEAChE, whereas altering the phosphonate from methyl to ethyl played as significant role in reducing the reactivity at phosphorus.

None of the phosphonothionates (**5**: P═S) were inhibitors of EEAChE ($k_i < 10^2$ M^{-1} min^{-1}) except **5c** that blocked enzyme activity at only sevenfold less than that of the corresponding oxon (P═O) (**5**: P═S). Typically, oxons are 100-fold more potent anticholinesterases than the corresponding thionates.[10]

Since the methyl β-fluoroethoxy phosphonate **4a** results in an OP-AChE adduct that closely resembles inhibition by the nerve agent VX, the concentration- and time-dependent inhibition of two mammalian acetylcholinesterases, recombinant human and rat brain, was also examined. For comparison, the ethylphosphonate analog **4b** and paraoxon were also studied. In general, the methyl β-fluoroethoxyphosphonate **4a** was a marginally more potent an inhibitor of the mammalian enzymes than that of EEAChE. As found for EEAChE, compound **4a** was a 100-fold more potent inhibitor of rHAChE than the ethyl analog **4b**. To our surprise, the methylphosphonate **4a** and ethyl phosphonate analog **4b** showed comparable inhibition values ($\sim 10^6$ M^{-1} min^{-1}) against RBAChE suggesting that this enzyme is less sensitive to the added occlusion and inductively electron-donating properties of the ethyl group at phosphorus. Compounds **4a** and **4b** were 25- to 100-fold less potent inhibitors of RBAChE than paraoxon by their respective concentration-dependent analyses.

As noted, the $(CH_3)(CH_3CH_2O)P(O)$–serine adduct neither reactivates nor ages rapidly, which may be due to a combination of inductive and steric effects that reduce reactivity at the phosphorus atom and also reduce the propensity to form cation-like intermediates thought to underscore the aging mechanism. Since the phosphylation of AChE inhibition by compounds **4a**, **4b**, **4c**, **4f**, and **5c** occur with concomitant loss of the *p*-nitrophenoxy group, the resulting OP-AChE adducts closely resemble that formed from VX, namely, the $(CH_3)(CH_3CH_2O)P(O)$–serine adduct, which differs only in the retained β-substituent.[22] For example, reaction of AChE with **4a** results in phosphylation at ser-200 to produce a $(Me)(FCH_2CH_2O)P(O)$–serine adduct.

In summary, a series of β- and γ-substituted alkoxy methylphosphonates were successfully prepared. The synthetic pathway is highlighted by a new approach that allows selective nucleophilic displacement at a phosphoester side chain rather than at a reactive phosphorus center. The enzymatic analyses demonstrated that the β-fluoroethoxy analog **4a** was the best inhibitor of acetylcholinesterases of those tested, and comparable in strength to paraoxon. The kinetic analyses also revealed two noteworthy results. First, the size of the alkoxy group substituent does not adversely affect the inhibitor strength. Second, the phosphonothionate analog **5c** was shown to possess anti-cholinesterase activity whereas P═S structures are normally inactive. The data produced in this study now makes possible advanced studies to further elucidate the mechanism of reactivation and aging by alkylphosphonates bearing substituted esters.

Acknowledgments

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23. *General procedure for synthesis of bis(4-nitrophenyl) alkylphosphonate (**2a–b**)*: To the alkyl phosphonyl dichloride (11.3 mmol) was added 4-nitrophenol (22.5 mmol) in CH_2Cl_2 (20 mL). The mixture was cooled in an ice-water bath and triethylamine (TEA; 45.1 mmol) in dry CH_2Cl_2 (5 mL) was added drop wise with stirring. TEA–HCl formed as the mixture stirred for 4 h at rt whereupon the reaction mixture was poured into 100 mL of ice cold water and extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the solvent removed to yield crude bis(4-nitrophenyl) alkylphosphonate that was purified on a short silica column using 3:7, ethyl acetate/hexanes. Bis(4-nitrophenyl)methylphosphonate (**2a**) isolated as a pale yellow solid (3.49 g, 91%); ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm 8.22 (d, J = 9.29 Hz, 4H), 7.37 (d, J = 9.29 Hz, 4H), 1.95 (d, J = 17.71 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ/ppm 154.53, 145.08, 125.88, 121.11, 12.62 (d, J_{CP} = 144.25 Hz); ^{31}P NMR (500 MHz, $CDCl_3$) δ/ppm 25.34; HRMS Calcd for $C_{13}H_{11}N_2O_7P$ 338.0304. Found: 339.0307 [(M+H)$^+$]. See: Ghanem, E.; Li, Y.; Xu, C.; Raushel, F. M. *Biochemistry* **2007**, *46*, 9032.
24. *General procedure for the synthesis of 4-nitrophenyl hydrogen alkylphosphonates (**3a–b**)*: To the bis(4-nitrophenyl) alkylphosphonate (7.4 mmol) in CH_3CN (28.8 mL) was added 0.5 M LiOH (28.8 mL) drop wise using a pressure equalizing funnel over 20 min and stirred at rt for 1 h. The CH_3CN was removed under reduced pressure, and the aqueous solution extracted with CH_2Cl_2 (3 × 250 mL) to remove *p*-nitrophenol. The aqueous phase was then acidified with 3 N HCl to pH 0.5 and extracted with CH_2Cl_2 (3 × 250 mL). The organic phases were combined, and concentrated to give a crude semisolid that was re-crystallized using 10% EtOAc, 90% *n*-pentane. 4-Nitrophenyl hydrogen methylphosphonate (**3a**) was isolated as a crystalline light brown solid (0.86 g, 54%); ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm 8.20 (d, J = 9.13 Hz, 2H), 7.32 (d, J = 9.16 Hz, 2H), 1.66 (d, J = 16.69 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ/ppm 154.76, 144.81, 125.70, 121.26, 12.53 (d, J_{CP} = 139.25 Hz); ^{31}P NMR (500 MHz, $CDCl_3$) δ/ppm 31.14; HRMS Calcd for chemical formula $C_7H_8NO_5P$ 217.0140. Found: 218.0188 [(M+H)$^+$]: Ghanem, E.; Li, Y.; Xu, C.; Raushel, F. M. *Biochemistry* **2007**, *46*, 9032.
25. *General procedure for the synthesis of 2/3-haloalkylnitrophenyl alkylphosphonates and 2-(methyl (4-nitrophenoxy)phosphoryloxy)ethyl-4-ethylbenzene sulfonate (**4a–j**)*: To 4-nitrophenyl hydrogen alkylphosphonate (0.5 mmol) in dry CH_2Cl_2 (5 mL) was added the 2/3-haloalcohol or 2-hydroxyethyl 4-methylbenzenesulfonate (0.5 mmol) and DCC (0.9 mmol) at rt with stirring for 24 h. The reaction mixture was filtered through filter paper to remove DCU, the filtrate diluted with CH_2Cl_2 (50 mL), washed with DI water (3 × 50 mL), and the CH_2Cl_2 layer dried (Na_2SO_4). Filtration of Na_2SO_4 and removal of the solvent yielded the crude product that was purified over silica using 6:4 EtOAc/hex to afford the product. *2-Fluoroethyl 4-nitrophenyl methylphosphonate* (**4a**; a colorless sticky mass) (76.3 mg; 58%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.85 (d, J = 9.27 Hz, 2H), 7.40 (d, J = 9.27 Hz, 2H), 4.50–4.67 (m, 2H), 4.24–4.47 (m, 2H), 1.75 (d, J = 17.85 Hz, 3H); ^{13}C NMR (500 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 (500 MHz, $CDCl_3$) δ/ppm 29.33; ^{19}F NMR (500 MHz, $CDCl_3$) δ/ppm −224.47; HRMS Calcd for chemical formula $C_9H_{11}NFO_5P$ 263.0359. Found: 264.0434 [(M+H)$^+$].
26. *General procedure for synthesis of O-2/3-haloalkyl O-4-nitrophenyl alkylphosphonothioates and 2-(methyl(4-nitrophenoxy)phosphorothioyloxy)-ethyl-4-methylbenzene sulfonate (**5a–j**)*: Compound **4a–j** (0.2 mmol) taken up in 3 mL of dry toluene was added Lawesson's reagent (0.1 mmol) and the reaction brought to reflux for 3 h after which the reaction mass was filtered, washed with 2 mL $CHCl_3$, and the filtrate directly loaded on preparative TLC plate using 1:3, ethyl acetate/hexanes to obtain the pure product. *O-2-Fluoroethyl-O-p-nitrophenyl methyphosphonothioate* (**5a**; isolated as a semisolid) (35.7 mg; 64%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, J = 9.27 Hz, 2H), 7.34 (d, J = 9.27 Hz, 2H), 4.49–4.66 (m, 2H), 4.24–4.48 (m, 2H), 2.11 (d, J = 15 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 154.99, 145.01, 125.43, 122.41, 82.70, 81.33, 66.35, 22.67 (d, J_{CP} = 460 Hz); ^{31}P NMR (500 MHz, $CDCl_3$) δ 96.15; ^{19}F NMR 500 MHz, $CDCl_3$) δ −224.29; HRMS Cacld for $C_9H_{11}FNO_4PS$ 279.0130. Found: 280.0128 [(M+H)$^+$].