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Journal

Journal of Labelled Compounds and Radiopharmaceuticals, 60(7)

ISSN

0362-4803

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Publication Date

2017-06-15

DOI

10.1002/jlcr.3511

Peer reviewed

Published in final edited form as:

J Labelled Comp Radiopharm. 2017 June 15; 60(7): 337–342. doi:10.1002/jlcr.3511.

An improved radiosynthesis of O-(2-[^{18}F]fluoroethyl)-O-(*p*-nitrophenyl)methylphosphonate: A first-in-class cholinesterase PET tracer

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Abstract

O-(2-Fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate **1** is an organophosphate cholinesterase inhibitor that creates a phosphonyl-serine covalent adduct at the enzyme active site blocking cholinesterase activity *in vivo*. The corresponding radiolabeled O-(2-[^{18}F]fluoroethyl)-O-(p-nitrophenyl) methylphosphonate, **[^{18}F]1**, has been previously prepared and found to be an excellent positron emission tomography imaging tracer for assessment of cholinesterases in live brain, peripheral tissues, and blood. However, the previously reported **[^{18}F]1** tracer synthesis was slow even with microwave acceleration, required high-performance liquid chromatography separation of the tracer from impurities, and gave less optimal radiochemical yields. In this paper, we report a new synthetic approach to circumvent these shortcomings that is reliant on the facile reactivity of bis-(O,Op-nitrophenyl) methylphosphonate, **2**, with 2-fluoroethanol in the presence of DBU. The cold synthesis was successfully translated to provide a more robust radiosynthesis. Using this new strategy, the desired tracer, **[^{18}F]1**, was obtained in a non-decay–corrected radiochemical yield of 8 ± 2% (n = 7) in >99% radiochemical and >95% chemical purity with a specific activity of 3174 ± 345 Ci/mmol (EOS). This new facile radiosynthesis routinely affords highly pure quantities of **[^{18}F]1**, which will further enable tracer development of OP cholinesterase inhibitors and their evaluation *in vivo*.

Keywords

[^{18}F]-fluoroethanol; acetylcholinesterase; bis-(p-nitrophenyl) methylphosphonate; fluorine-18; positron emission tomography (PET); β-fluoroethoxyphosphonate

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

1 | INTRODUCTION

Organophosphorus esters (OP) are a class of compounds that include insecticides (parathion, malathion, etc) and chemical nerve agents (sarin, soman, VX, etc).[1,2] The OP nerve agents (Figure 1) are particularly lethal because of their reactive P=O bond and leaving group Z that combine to rapidly react with the neurotransmitter-hydrolyzing enzyme acetylcholinesterase (AChE). OP insecticides first require a conversion to form the reactive oxon form that reacts with AChE.[3] The OP nerve agents and insecticide oxons are structurally similar sharing a phosphoester group (R = alkyl) and leaving group Z but differ in that nerve agents possess a P—CH_3 bond whereas insecticide oxons are typically diesters.

Despite this minor difference, nerve agents and insecticide oxons are covalent inhibitors of AChE. Given these common structural components, we developed a hybrid OP structure based upon the nerve agent VX and paraoxon (insecticide oxon)[4] modified to incorporate an [^{18}F]fluorine atom to produce an OP-based PET imaging tracer[5] (Figure 2). The resultant fluoro-VX surrogate, O-(2-fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate, uses a *p*-nitrophenoxy leaving group and acts similarly *in vitro* and *in vivo* to VX without handling concerns.[6,7] The fluorine tracer atom is strategically positioned 4 bonds from the reactive phosphorus atom to minimize steric or electronic perturbation.[8] Injection of high specific activity O-(2-[^{18}F] fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate, **[^{18}F]1**, in rats shows localization of radioactivity in AChE-rich regions of brain and other tissues[5] where it likely exists as (CH_3)($^{18}FCH_2CH_2O$)P(O)-AChE and other esterase adducts.[8]

Although *in vivo* studies with O-(2-[^{18}F]fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate were highly successful, the synthesis was slow (approximately 150 min), requiring microwave acceleration and yields were low because of low-purity acquisition of the precursor [^{18}F]-2-fluoroethyltosylate.[5] To further advance tracer **[^{18}F]1** studies, our goal was to develop a faster, more reliable, and higher yielding synthetic approach.

2 | EXPERIMENTAL

2.1 | Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, Missouri) and used without purification, unless otherwise noted. Solvents were purchased in their anhydrous forms and used without any additional purification. For cold chemistry, flash chromatography was conducted using silica gel (200–300 mesh) and thin-layer chromatography (TLC) was visualized by UV and/or staining by 2,6-dibromoquinone-4-chloroimide (DBQ) or iodine. The nonradioactive molecular characterizations used ^{1}H-, ^{19}F-, and ^{31}P-NMR that were recorded in $CDCl_3$ on a Varian Avance 400-MHz spectrometer; chemical shifts (δ) are reported in ppm (relative to 7.26 ppm) and coupling constants (J) in Hertz (Hz). For radiochemical transformations, analytical high-performance liquid chromatography (HPLC) was performed using a Waters 590 LC pump (Milford, Massachusetts), connected in series to a Shimadzu SPD-UV-visible detector (Columbia, Maryland) (λ = 254 nm) and a gamma counting in-line radiation flow detector (Model 105 s, CRA; Berkeley, California). Semipreparative HPLC was performed with a Waters 600 LC pump (Milford, Massachusetts) connected in series to a Shimadzu SPD-UV-visible detector (Columbia,

Maryland) (λ = 254 nm) and a gamma counting in-line radiation flow detector (Model 105 s, CRA; Berkeley, California). The HPLC chromatograms were acquired using SRI PeakSimple software (version 304—Torrence, California). The QMA cartridges for concentrating [^{18}F]fluoride from cyclotron production were purchased from Myja Scientific (O'Neill, Nebraska).

2.2 | Synthesis of O-(2-fluoroethyl)-O-(4-nitrophenyl) methylphosphonate (1)

Solid *bis*-(O,O-*p*-nitrophenyl) methylphosphonate (0.068 g; 0.200 mmol)[9] was added to a Wheaton vial (0.5-mL total volume) containing 0.2 mL of anhydrous dichloromethane. The flask was fitted with a septum, a triangular stir vane added, and the atmosphere exchanged for nitrogen (balloon needle inlet) at 25 °C. To this solution was added 1,8-diazabicycloundec-7-ene (DBU; 0.016 mL; 0.100 mmol), whereupon the solution turned a light yellow (due to formation of some *p*-nitrophenoxy oxyanion). The solution was stirred at 25°C for 15 minutes (TLC shows no decomposition of the starting material), and 2-fluoroethanol (6.6 mg; 6.5 μL; 0.1 mmol) was added via a 10-μL Hamilton syringe. Within a few seconds, the yellow solution turns to darker yellow or burnt orange. Thin-layer chromatography shows formation of the product (R_f is approximately 0.25–0.30, EtOAc:hex, 7:3) that was confirmed by ^{31}P-NMR (29.3 ppm) at 3-minute post-addition of 2-fluoroethanol. After an additional 2 minutes, the reaction indicates complete conversion to O-(2-fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate **1** as determined by ratio of product to starting material by ^{31}P-NMR along with co-identification of the starting material (R_f is approximately 0.40; twofold excess in this experiment) and *p*-nitrophenol (Rf is approximately 0.55). ^{19}F NMR indicates >95% consumption of fluoroethanol and formation of O-(2-fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate (−224.5 ppm). Flash chromatography with EtOAc:hex (7:3) affords 24 mg (90% based on fluoroethanol) that was >98% pure as determined by HPLC using a Sonoma C_{18}(2) 5 μm 100Å 15 cm × 4.6 mm (ES Industries; SMA-C18) column at 275 nm with 35% CH_3CN/0.01% H_3PO_4 in H_2O at 1.50 mL/min. Spectral data:[10] ^{1}H NMR (400.18 MHz, $CDCl_3$) δ 8.84 (d, J = 9.2 Hz, 2H), 7.41 (d, J = 9.2 Hz, 2H), 4.50 to 4.67 (m, 2H), 4.24 to 4.47 (m, 2H), 1.75 (d, J = 17.8 Hz, 3H). ^{13}C NMR (100.63 MHz, $CDCl_3$) δ 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 29.3. ^{19}F NMR (376.55 MHz, $CDCl_3$) δ −224.47.

2.3 | Radiosynthesis of O-(2-[^{18}F]fluoroethyl)-O-(*p*-nitrophenyl) methylphosphonate; [^{18}F]1

A Kryptofix K2.2.2/$KHCO_3$ solution was prepared from K2.2.2 (12 mg) in 0.8 mL CH_3CN followed by the addition of 0.1 mL of a 10 mg/mL $KHCO_3$ solution in water. [^{18}F] Fluoride, obtained by the nuclear reaction of ^{18}O(p,n)^{18}F from a GE PETtrace cyclotron, was eluted from a miniature QMA cartridge (Myja Scientific) using the Kryptofix/$KHCO_3$ solution, and the solvent was azeotropically distilled at 115°C under a stream of nitrogen and reduced pressure. Once the vial was dry, an additional 0.8 mL of anhydrous CH_3CN was added to the reaction vial and the solvent was azeotropically distilled again affording apparent dry reaction vial contents. The reaction vial was cooled to 25°C, and a solution of ethylene sulfite (20 μL in 300 μL of anhydrous CH_3CN) was added. The vial was sealed, then heated to 85°C for 20 minutes on an aluminum block heater. The reaction vial was allowed to cool for 3 minutes whereupon *bis*-(O, O-*p*-nitrophenyl) methylphosphonate (5.0 mg dissolved in

400 μL of anhydrous CH_3CN and 2 μL DBU) was added. The vial contents were manually mixed for 5 seconds and allowed to stand at 25°C for 10 minutes. The resultant reaction mixture was passed through a C18 light cartridge (Waters) into a clean reaction vial, and the solution was evaporated at 40°C under a stream of nitrogen gas and reduced pressure. The resultant residue was dissolved in 300 μL of acetonitrile and injected onto a semipreparative HPLC column (Luna $C_{18}(2)$ column, 250 × 10 mm, 5 μm) using a gradient mobile phase (1:9 CH_3CN:H_2O containing 0.1% ascorbic acid for 4 min then linear increase to 8:2 over 30 min, R_t = 16 min, see Supporting Information) at a flow rate of 3 mL/min and detection at 254 nm. The isolated product was diluted with 20 mL of water, supplemented with 0.1% *w/v* L-ascorbic acid. The solution was passed through a C18 light cartridge at a rate of 4 mL/min. The cartridge is then washed with 5 mL of deionized water, and the final product is eluted from the cartridge with 200 μL of ethanol and collected in a glass vial. The ethanol is then removed under vacuum for 20 seconds. The desired tracer product identity, **[^{18}F]1**, was confirmed using the identical HPLC conditions and coelution with cold standard **1**. Specific activity was determined by integrating area against a standard curve. Non-decay–corrected radiochemical yields (EOB) of **[^{18}F]1** were 8 ± 2% (n = 7). Chemical and radiochemical purities were >95% and >99%, respectively. Specific activity was calculated to 3174 ± 345 Ci/mmol at the end of the synthesis (n = 7). Overall synthesis time, including HPLC and product reformulation, from EOB was 65 minutes. Starting from 150 to 300 mCi of [^{18}F]fluoride, the range of final isolated yields of **[^{18}F]1** were 10 to 50 mCi.

3 | RESULTS AND DISCUSSION

Phosphorylation of AChE by **1** requires nucleophilic attack by the primary alcohol of an active serine residue, which liberates the *p*-nitrophenoxy (Z) moiety. Therefore, the [^{18}F]-label in the VX surrogate structure (Figure 2) must be introduced in either the methyl or ethoxy groups to remain attached to AChE after inhibition. Incorporation of fluorine at the methyl or alpha carbon of the ethoxy substituent would increase hydrolytic reactivity at phosphorus atom and ester group, respectively, and were dismissed as possible fluorine atom introduction sites leaving the beta ethyl position as the sole option for positron atom labeling. The suitable viability of this beta ethyl position strategy has recently been further confirmed by cold ligand *in vitro* cholinesterase studies.[8]

In our prior tracer preparation report, we demonstrated the microwave-assisted S_N2 reaction of 2-fluoroethyl tosylate ([^{18}F]FCH_2CH_2OTs) with the cesium salt of methyl (*p*-nitrophenoxy) phosphonic acid, leading to **[^{18}F]1**.[5] Although successful, a consistent radiosynthesis (ndc yields approximaltely 1%–2.5% for 150-min preparation) was found routinely limited as a result of the sensitive coupling between the phosphonic acid salt and 2-fluoroethyl tosylate, and the inconsistent technical limitations using a microwave apparatus. Thus, a higher yielding and technically streamlined synthetic methodology was sought. Herein, we report a higher yielding and effectively reactive approach to synthesize **[^{18}F] 1** quickly and in efficient conversion directly from *bis*-(O,O-*p*-nitrophenyl) methylphosphonate (**2**) (prepared from methyl phosphonic dichloride).[9] This approach may be generally amenable to prepare other radiolabeled OP agents shown in Figure 1.

Previous investigators showed that **2** (^{31}P-NMR δ 25.2) reacts effectively with a variety of alcohols in the presence of 1,8-diazabicycloundec-7-ene (DBU) to afford methyl *p*-nitrophenyl phosphonates.[9] We successfully extended this reaction using 2-fluoroethanol as the coupling alcohol to prepare compound **1** in near quantitative yield. Reaction monitoring by chromatographic and spectroscopic (^{1}H- and ^{31}P-NMR) methods indicated immediate formation of compound **1** with minimal side product formation, namely, the undesired formation of *p*-nitrophenol. Aqueous reaction workup using 1% HCl followed by 0.1 N NaOH effectively removed DBU and *p*-nitrophenol, respectively. Evaporation of the solvent after workup indicated a sole peak in the ^{31}P-NMR at δ 29.3 ppm and in the ^{19}F NMR at δ −224.5 ppm. The relatively rapid reaction along with the high conversion was somewhat unexpected as the fluorine group was anticipated to reduce the nucleophilicity of the alcohol group. It should be noted that substituting DBU for other tertiary amine bases (TEA, Hunig base, lutidine, etc) led to dramatically lower yields and increased number of side products.

The previous report using **2** also indicated using a fivefold to tenfold excess of the alcohol.[9] This stoichiometry would not be possible with standard radiolabeling methods in which [^{18}F]-containing alcohols (eg, 2-[^{18}F]fluoroethanol) would require a high surplus of **2** for the transformation to tracer **[^{18}F]1**. We were surprised to find that a tenfold or greater excess of **2** could be used with 2-fluoroethanol as the limiting reagent to form **1** quantitatively, based upon the conversion of 2-fluoroethanol to product **1** followed by ^{19}F NMR. Thus, reversing the stoichiometric ratio of reactants did not appear to adversely influence the rate of the transesterification reaction to produce **1**. Hence, the synthetic strategy showed promise for radiochemical translation.

Prior reports have shown that [^{18}F]-2-fluoroethanol could be prepared by reaction of *n*-$Bu_4N^+[^{18}F]F^-$ with ethylene carbonate,[11] tosylate displacement with $Cs^{18}F$, reduction of the fluoroacetic acid with an aluminum hydride[12] or $Cs^{18}F$ ring opening of ethylene sulfite.[12,13] The latter process was chosen for tracer synthesis owing to minimal steps and ease of workup. With 2-[^{18}F]fluoroethanol in hand, we envisioned a mild and facile transesterification with **2**, as depicted in Scheme 1.

A previous report[12] described nucleophilic attack of [^{18}F] fluoride ion on ethylene sulfite, which in the presence of trace amounts of water, yielded 2-[^{18}F]fluoroethanol. The S_N2-like reaction was conducted at 85°C for 20 minutes, which routinely afforded 2-[^{18}F]fluoroethanol in yields of 50% to 60%, as determined by radioTLC. While the previous report[12] also described intermediate purification of 2-[^{18}F] fluoroethanol by distillation, we did not anticipate the subsequent transesterification reaction would be affected by the presence of noncompeting reactants from the labeling reaction. Indeed, upon cooling the reaction vial to room temperature after completion of the 2-[^{18}F]fluoroethanol reaction, the direct addition of **2** (dissolved in acetonitrile), in the presence of DBU, resulted in formation of **[^{18}F]1** within 10 minutes at room temperature in 30% yield, as determined by analytical HPLC.

As the transesterification of **2** to produce **[^{18}F]1** is ultimately limited by the amount of 2-[^{18}F]fluoroethanol present, we sought to increase the radiochemical yields of 2-[^{18}F] fluoroethanol. Neither an increase in temperature nor reaction time increased the yields in

any appreciable amount. Owing to the apparent lack of sensitivity of this reaction to polar protic solvent, we postulated the presence of bulky, aliphatic alcohols would increase the yields of 2-[^{18}F] fluoroethanol as has been previously described[14] for [^{18}F] fluorination of aliphatic electrophiles. Indeed, as evidenced by radioTLC, yields of 2-[^{18}F]fluoroethanol increased to 75% to 85%; however, this yield increase, which was the direct result of *t*-amyl alcohol in the reaction mixture, did not result in a subsequent increase in yields of **[^{18}F]1**. This outcome is thought presumably due to competitive transesterification reaction of the excess *t*-amyl alcohol at the phosphorus atom.

The previous radiochemical synthesis reported[5] for **[^{18}F] 1** was also complicated by the need of a normal phase chromatographic separation using ethyl acetate and hexanes, which required substantial reformulation for mammalian use; namely, the final product required a 10% (*v*/v) supplement of acetonitrile in PBS to ensure tracer stability prior to injection. We attempted to streamline the isolation of pure **[^{18}F]1** by simple dilution of the acetonitrile reaction mixture with water for HPLC purification. While the radiochemical separation was simple, a significant amount of chemical impurity (as detected at 254 nm) coeluted with **[^{18}F]1**. Initially, we attributed the production of this impurity to a lack of appropriate buffer under acidic (pH 4–5) conditions. However, supplementation of the HPLC mobile phase with various acidic buffers, including acetic acid, phosphoric acid, and citric acid, did not reverse the formation of the chemical impurity. Supplementing the mobile phase with 0.1% L-ascorbic acid, with direct injection of the crude reaction mixture in 300 μL of acetonitrile, completely attenuated the formation of the coeluting impurity. As a result, **[^{18}F]1** could be purified by reverse-phase HPLC and reformulated in 1% ethanol/saline and 0.1% L-ascorbic acid to provide **[^{18}F]1** in 8 ± 2% (n = 7) non-decay–corrected radiochemical yield with >99% radiochemical purity and >95% chemical purity in a total preparation time of 65 minutes. In the presence of aqueous *L*-ascorbic acid, **[^{18}F]1** showed no evidence of degradation over 4 hours in solution (as evidenced by HPLC).

The method for the radiochemical preparation of **[^{18}F]1** reported herein is superior to our prior effort.[5] The improved methodology gives **[^{18}F]1** in 8 ± 2% non-decay–corrected radiochemical yield with respect to starting [^{18}F]fluoride, while the prior method gave a 1% to 2% non-decay–corrected yield with respect to starting [^{18}F]fluoride. The optimized method reported herein is a single-pot, two-step, 65-minute synthesis and offers a significant technical and practical advantage for the radiochemical syntheses. The lower yields for the initial preparation may reflect the excess handling required including the need for microwave irradiation to perform the phosphonate alkylation with 2-[^{18}F]fluoroethyl tosylate leading to a 150-minute total radiochemical synthesis time. The increase in radiochemical yields of **[^{18}F]1** herein may also be attributed to a modification of synthetic strategy with the phosphorus behaving as an electrophile on incident nucleophilic [^{18}F]fluoroethanol as opposed to phosphorus oxyanion behaving as a nucleophile on incident [^{18}F] fluorethyltosylate[5]. Additionally, the new methodology also provides nearly a 50% increase in specific activity as compared to the previous method, which among other advantages, will minimize the potential side effects of mass OPs in *in vivo* studies.

4 | CONCLUSIONS

Although a number of radiotracers and imaging approaches to investigate AChE have been reported,[15–20] there are very few reports of OP PET imaging radiotracers that are based on the covalent modification of this target. Tracer **[^{18}F]1** is the first-in-class example of an OP agent that simulates the mechanism of inhibition of OP insecticides and chemical warfare agents, specifically, phosphonylation of the active site serine.[8] For this reason, radioligand **[^{18}F]1** is of strategic importance to evaluate *in vivo* tissue exposures to OP compounds and to interrogate new and existing countermeasures to OP toxicity. In this paper, we reported a new nonradioactive (cold) synthesis to circumvent these shortcomings that is reliant on the facile reactivity of *bis*-(O,O-*p*-nitrophenyl) methylphosphonate, **2**, with 2-fluoroethanol in the presence of DBU. This approach was successfully translated to provide a routine and more optimal radiosynthesis that is mild, rapid, and high yielding, which is considered generally applicable for the radiolabeling of many other important OP analogs. This new facile radiosynthesis routinely affords highly pure **[^{18}F]1** in sufficient quantities that will further enable the tracer development as a novel radiolabeled OP cholinesterase inhibitor for assessing OP modes of action with PET imaging *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the CounterACT Program, National Institutes of Health Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke (NINDS), grant award number U01NS092495. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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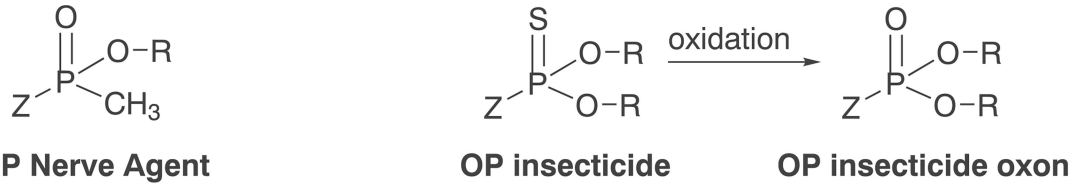


OP Nerve Agent **OP insecticide** **OP insecticide oxon**

VX: R = Et ; Z = $SCH_2CH_2N(iPr)_2$
sarin: R = iPr; Z = F
soman: R = $CH(CH_3)C(CH_3)_3$, ; Z = F
cyclosarin: R = cyclohexyl ; Z = F

malathion: R = Me, Z = $SCH(CO_2Et)CH_2CO_2Et$
parathion: R = Et, Z = O-Ph-$_p$$NO_2$
chlorpyriphos: R = Et, Z = O-[2,3,5-trichloropyridinol]
diazinon: R = Et, Z = O-[6-Me,4-iPr-pyrimidinol]

FIGURE 1.
Examples of organophosphate chemical nerve agents and insecticides

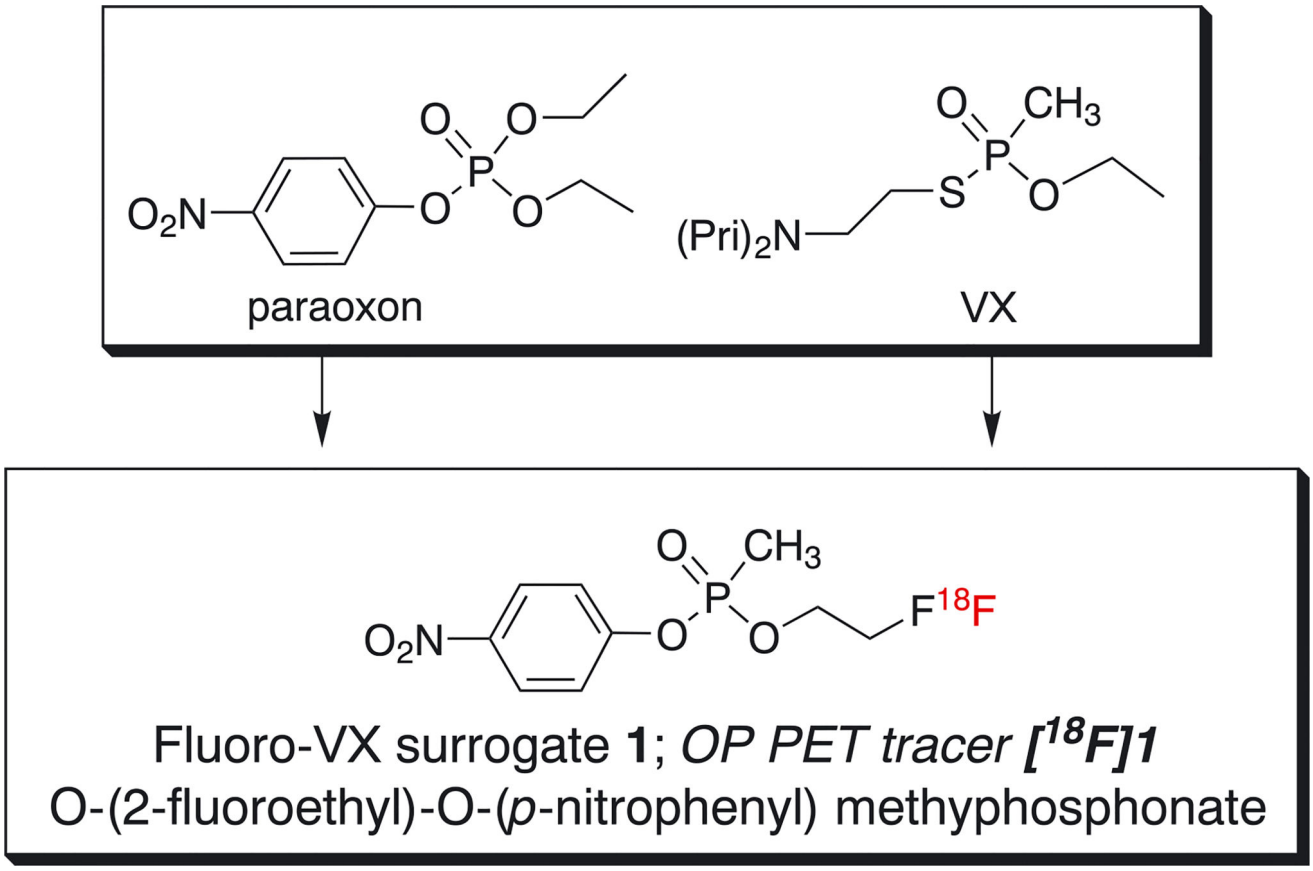
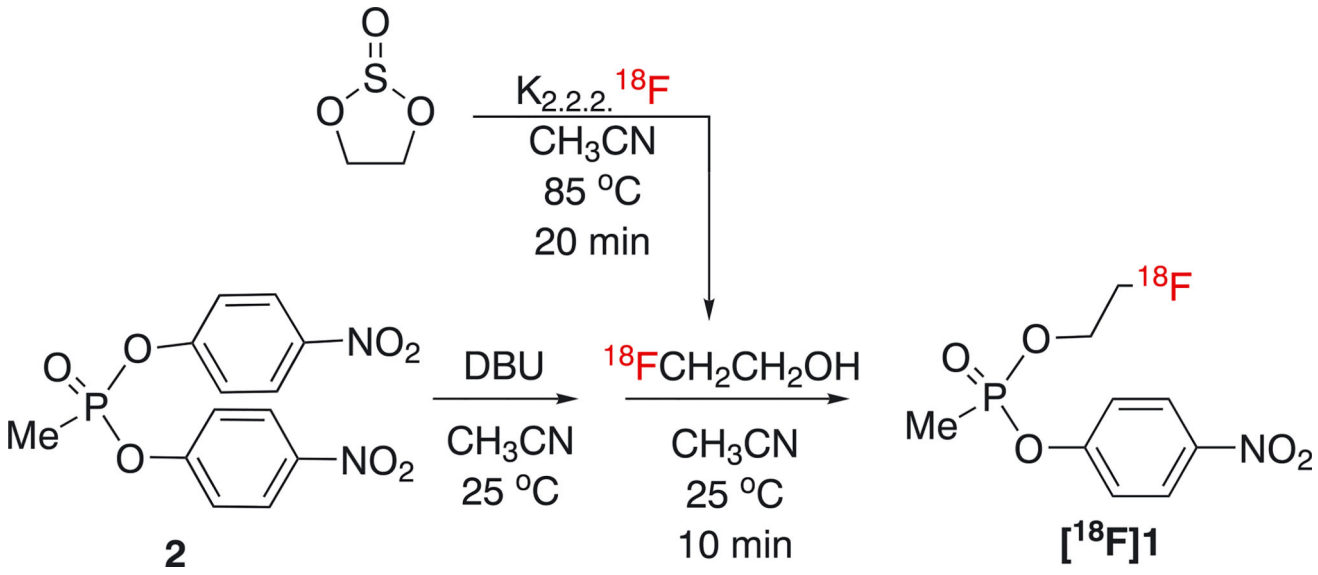


FIGURE 2.
Fluoro-VX surrogate based on VX and paraoxon structures



SCHEME 1.
Radiochemical synthetic scheme of **[^{18}F]1**