Supplementary Material

Characterization of a Phosphotriesterase Capable of Hydrolyzing EA 2192, the Most Toxic Degradation Product of the Nerve Agent VX

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The structures for the compounds synthesized below are presented in **Scheme 3**.

General: ^{1}H NMR spectra were collected using Inova-300 NMR spectrometer with residual $CHCl_3$ in $CDCl_3$ as internal standard. ^{31}P NMR were collected with Inova-300 NMR or Inova-400 broad band spectrometer with 95% aqueous phosphoric acid as external standard (reference as 0 ppm). Mass Spectra were measured with ESI method by laboratory for Biological Mass Spectrometry in the Department of Chemistry, Texas A&M University at College Station, Texas.

Compounds **2**, **3**, **4**, **5, 7, 8** were prepared according to the general method described below:

The corresponding alkylphosphonic dichloride (~ 10 mmol, methyl, ethyl or phenylphosphonic dichloride for **3**, **4**, and **8**) or alkyl phosphorodichloridate (methyl, ethyl or phenyl phosphorodichloridate for **2**, **5** and **7**) was reacted with 4-nitrophenol (2 eq.) in anhydrous ethyl ether (around 200 mL) in the presence of triethylamine (2.1 eq.) to yield the bi(*p*-nitrophenyl phosphonate or phosphate) which was then hydrolyzed by sodium hydroxide (1 eq.) in acetone to yield the desired phosphonate or phosphate. For example, into a solution of phenyl phosphorodichlodridate (9.8 mmol) and *p*-nitrophenol (2 eq) in ethyl ether, stirred in an ice-water bath, was dripped triethylamine (TEA) (2.1 eq) slowly over 30 minutes. The resulting suspension was then stirred at room temperature for another 2 hours. After removal of the solvent under reduced pressure, the residue was suspended in water (800 mL) and filtered. The solid was washed with water, then ethyl ether (3 x 30 mL) and dried at reduced pressure. The crude product was then recrystallized from benzene to give bis(*p*-nitrophenyl) phenyl phosphate in a yield of 80%. Into a solution of bis(*p*-nitrophenyl) phenyl phosphate (3.7 mmol) in acetone (40 mL) was added NaOH (2.0 eq, 10 M in water). The reaction was stirred vigorously at room temperature for 12 hours. After removal of the precipitate by filtration, the filtrate was

condensed to dryness at reduced pressure. The residue was then dissolved in water (10 mL) and acidified to pH 4. The solution was then washed with ethyl ether (3 x 10 mL) and condensed to dryness to yield compound **7** (0.73 g) in a yield of 63%.
Sodium ethyl 4-nitrophenyl phosphate **2**: ^{1}H NMR (D_2O, ppm): 8.15 (2H, d, J = 9 Hz): 7.18 (2H, d, J = 9 Hz); 3.96-3.82 (2H, m); 1.08-1.12 (3H, m). ^{31}P NMR (D_2O, ppm): -12.7. MS ($M\text{-}Na^+$, ESI, unit mass): found 246.0; calculated 246.0.

Sodium 4-nitrophenyl ethylphosphonate **3**: ^{1}H NMR (D_2O, ppm): 8.16-8.12 (2H, m): 7.23-7.19 (2H, m); 1.73-1.59 (2H, m); 1.10-0.95 (3H, m). ^{31}P NMR (D_2O, ppm): 32.4. MS ($M\text{-}Na^+$, ESI, unit mass): found 230.0; calculated 230.0.

Sodium 4-nitrophenyl methylphosphonate **4**: ^{1}H NMR (D_2O, ppm): 8.16 (2H, d, J = 9.1 Hz); 7.23 (2H, dd, $J_{H\text{-}H}$ = 9.1 Hz, $J_{P\text{-}H}$ = 1.0 Hz); 1.41 (3H, d, J = 16.8 Hz). ^{31}P NMR (D_2O, ppm): 28.4. MS ($M\text{-}Na^+$, ESI, unit mass): found 216.0; calculated 216.0.

Sodium 4-nitrophenyl methyl phosphate **5**: ^{1}H NMR (D_2O, ppm): 8.19 (2H, d, J = 9.3 Hz); 7.27 (2H, d, J = 9.3 Hz); 3.64 (3H, d, J = 11.2 Hz); ^{31}P NMR (D_2O, ppm): -0.34. MS ($M\text{-}Na^+$, ESI, unit mass): found 232.0; calculated 231.9.

Sodium 4-nitrophenyl phenyl phosphate **7**: ^{1}H NMR (D_2O, ppm): 8.15 (2H, d, J = 8.6 Hz), 7.35-7.20 (4H, m), 7.16-7.06 (3H, m). ^{31}P NMR (D_2O, ppm): -6.72. MS ($M\text{-}Na^+$, ESI, unit mass): found 294.0; calculated 294.0.

Sodium 4-nitrophenyl phenylphosphonate **8**: ^{1}H NMR (D_2O, ppm): 7.86-7.80 (2H, m); 7.70-7.58 (2H, m); 7.44-7.30 (3H, m); 7.02-7.92 (2H, m). ^{31}P (D_2O, ppm): 16.1. MS ($M\text{-}Na^+$, ESI, unit mass): found 278.0; calculated 278.0.

Sodium isopropyl 4-nitrophenyl phosphate **6**: 4-Nitrophenyl phosphorodichloridate (6.70 g) in ethyl ether (400 mL) was stirred and cooled to 0 ^{o}C in an ice/water bath. Into this solution was added isopropanol (1 eq) followed by dropwise addition of triethylamine (1 eq) in ethyl ether (30 mL) in 30 min. The cooling bath was removed and the reaction mixture was stirred for another 2

hours. 4-nitrophenol (1 eq.) was added directly into the reaction mixture followed by slow addition of triethylamine (1 eq.). The reaction mixture was stirred for another 5 hours. The reaction mixture was then washed three times with water (300, 200, 100 mL). The organic solution was dried with sodium sulfate and condensed to dryness. The residue was then applied to silica gel chromatography. A general workup yield bis(4-nitrophenyl) isopropyl phosphate (3.50 g, 15 %). Hydrolysis of the phosphate with sodium hydroxide in acetone following the procedure described above gave compound **6** in 74% yield. ^{1}H NMR (D_2O, ppm): 8.20 (2H, d, J = 9.0 Hz): 7.24 (2H, d, J = 9.0 Hz); 4.46-4.40 (1H, m); 1.20 (6H, d, J = 6.5 Hz). ^{31}P NMR (D_2O, ppm): -2.41. MS (M-Na^+, ESI, unit mass): found 260.0; calculated 260.0.

Sodium dimethyl phosphate **9**: A suspension of dimethyl chlorophosphate (5.2 g) in ethyl ether (50 mL) and sodium hydroxide (2 eq. 10 M aq.) was stirred at room temperature for 8 hours. After removal of the solvent under reduced pressure, the residue was washed with ethyl ether and dried under reduced pressure to yield the desired product (4.9 g. 92%). ^{1}H NMR (D_2O, ppm): 3.55 (6H, d, J = 10.5 Hz). ^{31}P NMR (D_2O, ppm): 6.4. MS (M-Na^+, ESI, unit mass): found 125.0; calculated 125.0.

Compounds **11**, **12**, **13**, **14**, **15** were prepared with one general method: The corresponding alcohol (20-40 mmol, ethanol, 2-propanol, 2-methyl propan-1-ol, 3,3-dimethyl butan-2-ol or cyclohexanol) in ethyl ether (0.2 M) were cooled with a dry ice/acetone bath and treated with butyl lithium (1.0 eq., 10 M in hexane). The solution or suspension was stirred for 10 minutes, and then mixed with a solution of phenyl methyl phosphonochloridate (20-40 mmol) in ethyl ether (0.2 M). The mixture was stirred for another 10 minutes and the cooling bath was removed and that the reaction temperature rose room temperature. After the reaction was completed in about 2 hours, the reaction mixture was filtered and the solid was washed with ethyl ether. The solution was condensed to dryness under reduced pressure. The residue was then fractionated via silica gel chromatography via elution with a mixture of ethyl acetate and chloroform in a ratio of 2 to 1. The UV active fractions were collected and condensed to dryness under reduced pressure to yield the pure phosphonate of the corresponding alcohol. All of the products were characterized by ^{1}H, ^{31}P NMR and MS. A solution of the product was dissolved in acetic acid (0.1 M) and hydrogenized by PtO_2 (5% mol) to remove the phenyl group. The reaction was

completed in 8-24 hours. After removal of the catalyst by filtration through a celite pad, the solvent was removed under reduce pressure to yield the corresponding alkyl hydrogen phosphonate quantitatively. The corresponding acid in water (0.1 M) was neutralized with aqueous sodium hydroxide (10 M) to pH around 7. After removal of water, the sodium salt of the desired phosphonate was obtained.

Sodium ethyl methylphosphonate **11**: ^{1}H NMR (D_2O, ppm): 3.78 (2H, pent., J = 7.2 Hz), 1.15 (3H, d, J = 15.85 Hz), 1.12 (3H, t, J = 7.15 Hz). ^{31}P NMR (D_2O, ppm): 30.12. MS (M-Na^+, ESI, unit mass): found: 123.0; calculated: 123.0.

Sodium isopropyl methylphosphonate **12**: ^{1}H NMR (D_2O, ppm): 4.32 (1H, m); 1.15 (3H, d, J_{H-P} = 16.5 Hz); 1.13 (6H, d, J_{H-H} = 6.21 Hz). ^{31}P NMR ($CDCl_3$, ppm): 26.37. MS (M-Na^+, ESI, unit mass): 137.0; calculated: 137.0.

Sodium isobutyl methylphosphonate **13**: ^{1}H NMR (D_2O, ppm): 3.48 (2H, dd, J_{H-H} = J_{H-P} =6.7 Hz) 1.73 (1H, sept. J = 6.7 Hz); 1.15 (3H, d, J_{H-P} = 16.3 Hz), 0.79 (6H, d, J_{H-H} = 6.7 Hz). ^{31}P NMR (D_2O, ppm): 30.05. MS (M-Na^+, ESI, Unit Mass): found 115.1; calculated 115.0.

Sodium pinacolyl methylphosphonate **14**: ^{1}H NMR (D_2O, ppm): 3.83 (1H, dq, J_{H-P} = 9.05 Hz, J_{H-H} = 6.50 Hz), 1.15 (3H, d, J_{H-P} = 16.30 Hz), 1.07 (3H, d, J_{H-H} = 6.50 Hz), 0.77 (9H, s). ^{31}P NMR (D_2O, ppm): 28.59. MS (M-Na^+, ESI, unit mass): found 179.1; calculated 179.1.

Sodium cyclohexyl methylphosphonate **15**: ^{1}H NMR (D_2O, ppm): 3.97 (1H, m), 1.90-0.94 (10H, m), 1.15 (3H, d, J_{H-P} = 16.30 Hz). ^{31}P NMR (D_2O, ppm): 30.12. MS (M-Na^+, ESI, unit mass): found 177.1; calculated 177.1.

Diethyl 4-nitrophenyl phosphate (paraoxon) **17**: Diethyl chlorophosphate (3.45 gr. 20 mmol) was stirred and mixed with 4-nitrophenol (1.0 eq.) in ethyl ether. After addition of triethylamine (1.1 eq.), the mixture was stirred for 8 hours. The reaction mixture was filtered and the mother solution was condensed to dryness under reduced pressure. The residue was applied to silica gel chromatography. A general work up yielded the pure paraoxon (4.40 g. 80%).

Ethyl methyl 4-nitrophenyl phosphate **18**: Ethyl phosphorodichloridate (3.26 g. 20 mmol) in ethyl ether (100 mL) was stirred and cooled to -78 ^{o}C in a dry ice/acetone bath. Into the solution was added lithium methoxide (1.0 eq.) which was prepared by addition of butyl lithium (1.eq, 10 M in hexane) in to a solution of methanol (1.0 eq) in ethyl ether (30 mL) and cooled by the dry ice/acetone bath. The bath was removed after mixing and the reaction mixture was stirred at room temperature for another one hour. 4-Nitrophenol (1.0 eq.) was added into the reaction mixture followed by slow addition of another portion of triethylamine (1.0 eq.). The reaction mixture was stirred at room temperature for another 6 hours. After removal of the precipitate by filtration, the mother solution was condensed to dryness. The residue was applied to silica gel chromatography. A general workup yielded compound **18** (2.87 g, 55%). ^{1}H NMR ($CDCl_3$, ppm): 8.26 (2H, d, J = 9.3 Hz); 7.40 (2H, d, J =9.3 Hz); 4.35-4.23 (2H, m); 3.91 (3 H, d, J = 11.5 Hz); 1.40 (3H, dd, $J_{H\text{-}H}$ = 7.0 Hz, $J_{H\text{-}P}$ = 1.0 Hz). ^{31}P NMR ($CDCl_3$, ppm); -5.31. MS ($M+H^+$, ESI, unit mass): found 262.1; calculated 262.1.

Dimethyl 4-nitrophenyl phosphate **19**: This compound was made in the same procedure as that described for preparation of **17** by substituting dimethyl phosphorochloridate for diethyl phosphorochloridate. ^{1}H NMR ($CDCl_3$, ppm): 8.24 (2H, d, J = 9.3 Hz); 7.38 (2H, d, J = 9.3 Hz); 3.9 (3H, d, J = 11.4 Hz). ^{31}P NMR ($CDCl_3$, ppm): -4.1. MS ($M+H^+$, ESI, Unit Mass): found 248.0; calculated 248.0.

S-2-(diisopropylamino)ethyl O-ethyl O-hydrogen phosphorothioate **22**: Ethyl phosphorodichloridate (3.26 gr. 20 mmol) in ethyl ether (100 mL) was stirred and cooled to -78 ^{o}C in a dry ice/acetone bath. Into the solution was added lithium *t*-butoxide (1.0 eq.) which was prepared by addition of butyl lithium (1.eq, 10 M in heaxe) in to a solution of *t*-butanol (1.0 eq) in ethyl ether (30 mL) and cooled by the dry ice/acetone bath. The bath was removed after mixing and the reaction mixture was stirred at room temperature for another one hour. The mixture of 2-(diisopropylamino)ethanethiol (1.0 eq.) and butyl lithium (1.0 eq., 10 M in hexane) in ethyl ether 30 mL was added into the reaction mixture which was stirred over night. After filtration the solution was condensed to dryness. The residue was applied to silica gel chromatography to yield pure O-*tert*-butyl S-2-(diisopropylamino)ethyl O-ethyl

phosphorothioate (2.93 gr. Yield, 45%). ^{1}H NMR ($CDCl_3$, ppm): 4.27-4.00 (2H, m); 3.11-2.95 (2H, m); 2.89-2.67 (4H, m), 1.58 (9H, s); 1.38 (3H, t, J = 7.0 Hz); 1.04 (12H, d, J = 6.6 Hz). ^{31}P NMR ($CDCl_3$, ppm). 24.44. MS (ESI, unit mass, $M+H^+$): found: 326.0; calculated: 326.2. A portion of this product (1.50 g.) was dissolved in CH_2Cl_2 (5 mL). Into this solution was added CF_3COOH (2 mL) and the solution was stirred room temperature for 2 hours. Removal of the solvent and CF_3COOH by reduced pressure yielded pure S-2-(diisopropylamino)ethyl O-ethyl O-hydrogen phosphorothioate **22** in a quantitative yield. ^{1}H NMR ($CDCl_3$, ppm): 4.16-4.01 (2H, m), 3.74-3.60 (2H, m); 3.34-3.25 (2H, m); 3.17-3.04 (2H, m); 1.51 (6H, d, J = 6.6 Hz); 1.43 (6H, d, J = 6.6 Hz); 1.33 (3H, t, J = 7.1 Hz). ^{31}P NMR ($CDCl_3$, ppm): 17. 80. MS (ESI, unit mass, $M+H^+$): found: 270.1; calculated: 270.1.

4-Acetylphenyl diethyl phosphate **24**: This compound was prepared by following the procedure described for compound **17** and substituting 4-acetylphenyl for 4-nitrophenyl in a yield of 83%. ^{1}H NMR ($CDCl_3$, ppm): 7.96 (2H, d, J = 8.7 Hz); 7.30 (2H, d, J = 8.7 Hz); 4.30-4.17 (4H, m); 2.58 (3H, s); 1.36 (6H, t, J = 7.0 Hz). ^{31}P NMR ($CDCl_3$, ppm): -6.2. MS ($M+H^+$, ESI, Unit Mass): found 273.1; calculated 273.6.

4-Acetylphenyl isobutyl methylphophonate **25**: Into a stirred solution of isobutyl alcohol (0.74 g, 10 mmol) in ethyl ether (50 mL) cooled in a dry ice-acetone bath was added butyl lithium (1.0 ml, 10 M in hexane). Into this resulting suspension was added a solution of methylphosphonic dichloride (1.33 g., 10 mmol) in ethyl ether (50 mL). The cooling bath was then removed and the mixture was stirred at room temperature for 1.5 hour. Into the mixture was then added 4-acetyl phenol (1.36 g. 10 mmol) followed by triethyl amine (1.2 g, 12 mmol). The mixture was stirred for another 4 hours and filtered. The mother solution was condensed to dryness and the residue was applied to silica gel chromatography to yield pure 4-acetylphenyl isobutyl methylphosphonate **25** (2.35 g., 87%). ^{1}H NMR ($CDCl_3$, ppm): 7.98 (2H, d, J = 9.0 Hz); 7.33 (2H, dd, J_{H-H} = 9.0 Hz), J_{P-H} = 1.0 Hz); 4.01-3.82 (2H, m); 2.61 (3H, s); 1.96 (1H, sept, J = 6.7 Hz); 1.70 (3H, d, J = 17. 8 Hz); 0.96 (6H, d, J = 6.7 Hz). ^{31}P NMR ($CDCl_3$, ppm): 28.4. MS ($M+H^+$, ESI, unit mass): found 271.1; calculated 271.1.

S-2-(ethylthio)ethyl O,O-dimethyl phosphorothioate (**20**): A solution of 2-(ethylthio)ethanethiol (1.22 g. 10 mmol) in ethyl ether (50 mL) was cooled by a dry ice/acetone bath for 5 min. In to the solution was added butyl lithium (4 mL, 2.5 M in hexane). Into the resulting suspension was added a solution of dimethyl chlorophosphate in ethyl ether (50 mL), which was cooled by a dry ice/acetone bath be addition. The mixture was then stirred at room temperature for 3 hours. After removal of precipitate from the reaction mixture by filtration, the solution was condensed to dryness. The residue was applied to silica gel flash chromatography with a mixture of ethyl acetate and hexanes of a 1:1 ratio as solvent. Fractions containing the product was collected and condensed to dryness to yield the desired product (colorless oil, 0.180 g, 8%). ^{1}H NMR ($CDCl_3$, ppm): 3.84 (6H, d, J = 12.6 Hz); 3.11-3.00 (2H, m); 2.88-2.83 (2H, m); 2.62 (2H, q, J = 7.4 Hz); 1.31 (3H, t, J = 7.4 Hz). ^{31}P NMR ($CDCl_3$, ppm): 31.623. MS ($M+H^+$, ESI, unit mass): found 231.03; calculated 231.03.

S-2-(ethylthio)ethyl O,O-diethyl phosphorothioate (**26**): This compound (2.01 g, 78%) was prepared by substituting diethyl chlorophosphate for dimethyl chlorophosphate and following the same procedure described for compound 25. ^{1}H NMR ($CDCl_3$, ppm): 4.29-4.10 (4H, m); 3.10-2.99 (2H, m); 2.88-2.82 (2H, m); 2.61 (2H, q, J = 7.4 Hz); 1.39 (6H, t, J = 7.10 Hz); 1.30 (3H, t, J = 7.40 Hz). ^{31}P NMR ($CDCl_3$, ppm):28.1. MS ($M+H^+$, ESI, unit mass): found 259.06; calculated 259.06.

Sodium O-ethyl S-2-(ethylthio)ethyl phosphorothiate (**27**): To *t*-butyl ethyl S-2-(ethylthio)ethyl phosphorothiate (130 mg.) was added trifluoroacetic acid (0.50 g.) and the mixture was stirred at room temperature for 10 min. After removal of trifluoroacetic acid under reduced pressure, the crude product was dissolved in water 5 ml and titrated to pH 7. The solution was washed with ethyl ether 4X10 mL and condensed to dryness to yield the product as colorless syrup (88.7 mg. 77%). ^{1}H NMR (D_2O, ppm): 3.79 (2H, q, J = 7.4 Hz); 2.80-2.73 (2H, m); 2.70-2.65 (2H, m); 2.44 (2H, q, J = 7.4 Hz); 1.09 (3H, t, J = 7.40 Hz); 1.06 (3H, t, J = 7.40 Hz). ^{31}P NMR (D_2O, ppm):21.6. MS ($M-Na^+$, ESI, unit mass): found 228.96; calculated 229.01.