Supplementary Information

Enzymatic Neutralization of the Chemical Warfare Agent VX: Evolution of Phosphotriesterase for Phosphorothiolate Hydrolysis[†]

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Construction of Active Site libraries by Overlap Extension. Mutagenic primers contained an NNS codon at the position of interest and extended 15 bp to either side of this codon. The PTE gene was amplified in three segments using standard PCR techniques (10 ng template and 125 ng each primer in a 50 μL reaction using pfuTurbo polymerase). The first segment extended from the 5'-end of the gene to 15 bp beyond the first mutagenic position. The second segment extended from 15 bp upstream of the first mutagenic position to 15 bp downstream of the second mutagenic position. The third segment extended from 15 bp upstream of the second mutagenic site to the 3'-end of the gene. The 5' and 3' primers included NdeI and EcoRI restriction sites respectively. A second PCR reaction was performed using the generated segments as the template DNA. The three fragments were combined in equimolar ratio (500 ngtotal) and amplified for 30 cycles with *pfu*Turbo using the primers for the 5'- and 3'-ends of the PTE gene. The overlaps between the fragments allowed for the formation of a single product corresponding to the size of the complete PTE gene. The product and vector were then digested with NdeI and EcoRI, gel purified and ligated together.

Construction of Error Prone Library. Primer pairs used to amplify Loop-7 corresponding to the DNA sequence for residues 242-252 and 277-287. The reaction contained 20 ng template (CVQFL variant), 1 μM forward and reverse primers, 0.35 mM dATP, 0.4 mM dCTP, 0.2 mM dGTP, 1.35 mM dTTP, 1 mM $MgCl_2$, 1XGoGreen Taq Buffer (Promega, Madison WI) 1.5 mM $MnCl_2$ and 1 μL Go Taq in 50 μL reaction. Thermocycler program was 2 min initial denaturation at 95° C, followed by 30 cycles of 95° C for 45 s, 60° C for 1 min, 72° C for 3 min, and a final elongation at 72° C for 10 min. The remaining portions of the PTE gene were amplified using

standard PCR techniques with the reverse primers for the Loop-7 fragment and the 5' and 3' end primer, resulting in three overlapping fragments. The final gene product was constructed by the overlap extension technique as described above. The mutated gene was digested with NdeI and EcoRI and ligated into pET 20 b (40 µL reaction containing 60 ng vector DNA, 3X molecular excess of PTE gene product, 4 µL T4 DNA ligase buffer and 2 µL T4 DNA ligase (NEB). Sequencing confirmed an average of 6 base pair changes per gene in loop-7. The identities of mutants from this library are given in **Table S2**.

Enzyme Expression and Purification. BL21 (DE3) cells containing plasmid with wild-type or variant PTE were grown for ~8 hours in 5mL LB broth. 1 L cultures of Terrific Broth (12 g Tryptone, 24 g yeast extract, 4 mL glycerol, 2.3 g KH_2PO_4, 12.5 g K_2HPO_4 in 1 L H_20) supplemented with 1.0 mM $CoCl_2$ were inoculated with 1 mL of the growing culture. Cells were grown overnight at 30° C with shaking. Protein expression was induced by addition of 1.0 mM IPTG and expression proceeded for an additional 24 hours. Cells were harvested by centrifugation at 11,000 g for 10 minutes. Cell pellets were stored at -80° C prior to use. Cells from 1 L of culture were resuspended in 100 mL purification buffer (50 mM HEPES (pH 8.5), 100 µM $CoCl_2$). Cellular lysis was achieved by sonication on ice for a total of 20 minutes using a medium power setting. Cell debris was removed by centrifugation at 18,500 g for 10 minutes. Protamine sulfate (0.45 g in 20 mL purification buffer) was added dropwise and incubated for 20 minutes to remove nucleic acids. Precipitated materials were removed by centrifugation at 18,500 g for 10 minutes. Supernatant was brought to 60% saturation with ammonium sulfate and stirred in the cold for 30 minutes to precipitate PTE. Protein was removed from the supernatant by centrifugation at 18,500 g for 20 minutes. The supernatant was decanted and

the pellet re-dissolved in 5 mL purification buffer. Up to 5 mL of the protein solution was loaded on a Superdex 200 (16/60) preparatory size exclusion column on a GE Health Care (Piscataway, NJ) AKTA FLPC system. Peak fractions were collected and assayed for activity against paraoxon. Fractions with the most activity were further purified using a gravity-fed DEAE column pre-equilibrated in purification buffer.

Synthesis of DEVX. DEVX was made by the reaction of diethylchlorophosphate with N,N-diisopropylaminoethanthiol. 1.5 grams of N,N-diisopropylaminoethanthiol was added to 100 mL diethyl ether and cooled in a dry ice acetone bath and purged with N_2 gas. to this mixture 7.5 ml of a 2.5 M solution of butyryl lithium in hexanes was added. 1.5 g of diethylchlorophosphate was mixed with 30 mL diethyl ether in a separate flask purged with N_2 and cooled in a dry ice acetone bath. The cooled diethylchlorophosphate solution was then added to the thiol solution and the reaction stirred at room temperature for 3 hr. The reaction was then brought to 400 mL with ethyl ether and extracted with water to remove side products. Product was then extracted into the aqueous phase with 0.5 M HCl and ethyl acetate. The aqueous phase was neutralized with sodium bicarbonate and extracted with chloroform. The organic phase was dried over $MgSO_4$ filtered and evaporated yielding the desired product as a pure oil.

^{1}H NMR (300 MHz, $CDCl_3$): 4.05-4.174 (4H, m, $OC\underline{H}_2CH_3$), 3.20-2.50 (6H, m, $SC\underline{H}_2C\underline{H}_2N(C\underline{H})_2$), 1.41-1.36 (6H, t, J=6.9Hz, $OCH_2C\underline{H}_3$), 1.05-1.03 (12H, d, J=4.8 Hz, $CH(C\underline{H}_3)_2$

^{31}P NMR (121.4 MHz $CDCl_3$): 29.77 ppm.

Purification of racemic VX. VX samples were Chemical Agent Standard Analytical Reference Material (CASARM) and were of the highest purity available. typically 99.9 +/- 5.4 weight % by oxidation-reduction titration, traceable to National Institute of Standards and Technology through 0.1 N iodine solution SRM 136e. However, as received, the VX gave high background readings at 412 nm at the concentrations required for kinetic analysis and therefore required further purification as follows: 80.1 µL neat VX was added to 120 µL isopropyl alcohol (to aid in dissolution), then added to 800 µL of 3 mM DTNB in 50 mM HEPES, pH 8.0. To this solution was added approximately 1 gram of Dowex® 1X4 chloride form beads (Sigma-Aldrich) and agitated gently for several minutes until the beads turned red. The VX was subsequently decanted and added to more beads until essentially all the yellow color was removed to the beads and the VX solution was almost colorless. A standard curve was then generated using 6, 30, 60 and 96 µM dilutions of VX, reacted to completion enzymatically. VX concentration in the bead-treated solution was determined by linear regression analysis using the standard curve from the direct dilutions of VX.

NMR Data Acquisition: All spectra were recorded on non-spinning samples at 25 ± 2 °C with a Varian Unity INOVA 600 spectrometer (600 MHz ^{1}H operating frequency) fitted with a triple resonance, *z*-gradient probe. Routine ^{1}H free induction decay (FID) data sets of 16,384 complex points were collected as summations of eight or 16 acquisitions recorded with 10 ppm spectral windows, 90º pulse widths of 12 µsec, and 2 sec relaxation delays before archiving to computer disk. FID data sets were apodized with a line broadening factor of 0.3 Hz before

Fourier transformation into spectra, manual phase correction into pure absorption mode, and chemical shift referencing to external tetramethylsilane.

^{31}P FID data sets of 65,536 complex points were collected as summations of 32 acquisitions using 100 ppm spectral windows and 90º pulse widths of 30 μsec. All ^{31}P data acquisitions incorporated inverse-gated ^{1}H decoupling (decoupling only during FID acquisition) with a low power composite pulse sequence to increase signal-to-noise ratios without signal enhancements from ^{1}H-^{31}P nuclear Overhauser effects.[1] Spin-lattice relaxation times (T_1) for the VX ^{31}P signal and that for the O-ethyl methylphosphonate (EMP) hydrolysis product were measured with the inversion recovery pulse sequence [180º-τ-90º-acquisition] incorporating nine randomized τ delays. For quantitative ^{31}P spectra, data sets were collected with relaxation delays >5T_1 for all ^{31}P signals in the spectra (~12 sec) to allow complete signal relaxation, and the spectrometer carrier frequency was centered between the VX substrate signal (*ca.* 57 ppm) and that of the O-ethyl,methylphosphonate (EMP) hydrolysis product (*ca.* 23 ppm) to minimize off-resonance effects. The ^{31}P{^{1}H} (^{1}H decoupled, ^{31}P observe) data sets were apodized with a 5 Hz line broadening factor before Fourier transformation into spectra and manual phase correction into pure absorption mode. ^{31}P chemical shift values in spectra were referenced to external 85% phosphoric acid at -0.73 ppm.[2]

NMR Observation of Enzymatic Hydrolysis of VX: The enzymatic hydrolysis of VX in the presence of PTE enzymes was observed by using NMR spectroscopy to follow VX disappearance, or the appearance of its EMP hydrolysis product, over time. Enzymatic reactions were initiated by adding 0.1-25.0 μL of a single enzyme solution to a 1 mL aliquot of a racemic VX solution and briefly mixing before transferring to a NMR sample tube. This was

immediately placed into the NMR spectrometer, and quantitative $^{31}P\{^{1}H\}$ FID data sets were acquired at 7.5 min time intervals over 20-75 min. Enzymatic hydrolysis rates were calculated directly from the integral values of the quantitative $^{31}P\{^{1}H\}$ signals, and included subtraction of the measured spontaneous rate (~55 μmole hr^{-1}) determined in separate experiments. The VX signal intensity decreases throughout the entire time course of the experiment until 75.0 min., where ≥99% of the intensity has disappeared (**Figure S2**). EMP signal intensity increases over this same time frame, and at 75 min., it is the only signal observed in the spectrum.

References

1. Shaka, A. J.; Keeler, J.; Freeman, J. R. *J. Magn. Reson.* **1983**, *53*, 313.

2. Batley, M.; Redmond, J. W. *J. Magn. Reson.* **1982**, *49*, 172.

Table S1. Mutations present in additional variants identified.

Variant	Mutations present
WT	Wild type
ARN	A80V/K185R/I274N
QF	H254Q/H257F
QF.1	H254Q/H257F/F306W/ Y309H
LQF	F132L/H254Q/H257F
VQF	F132V/H254Q/H257F
QF.a	W131H/F132L/H254Q/H257F
QF.b	W131H/F132I/H254Q/ H257F
LQF.1	F132L/H254Q/H257L
LQF.2	F132L/H254R/H257A
LQF.3	F132L/H254R/H257L
LQF.4	F132L/H254R/H257Y
LQF.a	F132L/H254Q/H257F/ L271V
LQF.b	F132L/H254Q/H257F/ L271M
LQF.c	F132L/H254Q/H257F/ L271A
LQFL	F132L/H254Q/H257F/ S308L
LQF.d	F132L/H254Q/H257F/L271R/S308N
VQFL	F132V/H254Q/H257F/S308L
CVQFL	I106C/F132V/H254Q/H257F/S308L
VQFL.1	I106G/F132V/H254Q/H257F/S308L
VQFL.2	I106S/F132V/H254Q/H257F/S308L
VQFL.3	I106A/F132V/H254Q/H257F/L303T/S308L
VRN-VQFL	A80V/F132V/K185R/H254Q/H257F/I274N/S308L
VRNGS-VQFL	A80V/F132V/K185R/D208G/H254Q/H257F/I274N/S308L/R319S
L7ep-1	F132V/H254S/H257W/A266T/L271P/S308L
L7ep-2	I106C/F132V/H254R/H257F/N265D/A270D/L272M/S276T/S308L
L7ep-3	I106C/F132V/H254Q/H257Y/A270V/L272M/S308L
L7ep-4	I106C/F132V/H254Q/H257F/I260N/D264N/I274N/S308L
L7ep-5	I106C/F132V/H254Q/H257Y/E264G/S308L
L7ep-6	I106C/F132V/H254Q/H257F/A266E/S269T/S308L
L7ep-7	I106C/F132V/H254Q/H257F/I260V/S269T/S308L
L7ep-8	I106C/F132V/H254Q/H257Y/I260V/S308L
L7ep-9	I106C/F132V/H254Q/H257F/S269T/I274T/S308L
L7ep-10	I106C/F132V/H254Q/H257Y/E263K/S308L
L7ep-11	I106C/F132V/H254Q/H257Y/A266R/S308L
L7ep-12	I106C/F132V/H254Q/H257F/S269T/I274S/S308L
L7ep-2a	I106C/F132V/H254R/H257F/N265D/A270D/L272M/I274T/S276T/S308L
L7ep-2b	I106C/F132V/H254R/H257F/N265D/A270D/I274N/S276T/S308L
L7ep-2c	I106C/F132V/H254Q/H257F/N265D/A270D/L272M/S276T/S308L
L7ep-2d	I106C/F132V/H254R/H257F/N265D/A270D/L272M/I274S/S276T/S308L
L7ep-2e	I106C/F132V/H254R/H257F/N265D/A270D/I274P/S276T/S308L
L7ep-2f	I106C/F132V/H254R/H257F/N265D/A270D/I274S/S276T/S308L
L7ep-2g	I106C/F132V/H254R/H257F/N265D/A270D/I274Q/S276T/S308L
L7ep-2h	I106C/F132V/H254R/H257F/N265D/A270D/L272M/S276H/S308L
L7ep-2i	I106C/F132V/H254R/H257F/N265D/A270D/L272M/S276S/S308L
L7ep-2j	I106C/F132V/H254R/H257F/N265D/A270D/L272M/S276P/S308L
L7ep-3a	I106C/F132V/H254Q/H257Y/A270V/L272M/I274N/S308L
L7ep-3b	I106C/F132V/H254Q/H257Y/A270D/L272M/S308L
L7ep-3c	I106C/F132V/H254Q/H257Y/N265D/L272M/S308L
L7ep-3d	I106C/F132V/H254Q/H257Y/A270V/L272M/I274T/S308L

Table S2: Activity of additional PTE Variants with DEVX.

Variant	k_{cat}(s^{-1})	K_m(mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
QF.1	0.67 ± 0.01	2.2 ± 0.1	(3.05 ± 0.01)x10^2
QF.a	1.23 ± 0.02	2.3 ± 0.1	(5.35 ± 0.02)x10^2
QF.b	0.7 ± 0.2	1.9 ± 0.1	(3.9 ± 0.1)x10^3
LQF.1	10.1 ± 0.2	2.32 ± 0.08	(4.4 ± 0.2)x10^3
LQF.2	4.2 ± 0.2	4.1 ± 0.3	(1.02 ± 0.08)x10^3
LQF.3	8.4 ± 0.3	3.0 ± 0.2	(2.8 ± 0.2)x10^3
LQF.4	21.7 ± 0.4	2.91 ± 0.09	(7.5 ± 0.2)x10^3
LQF.a	9.4 ± 0.2	1.62 ± 0.07	(5.8 ± 0.3)x10^3
LQF.b	14.5 ± 0.4	2.8 ± 0.1	(5.2 ± 0.3)x10^3
LQF.c	25.2 ± 0.8	2.8 ± 0.2	(9.2 ± 0.6)x10^3
LQF.d	4.1 ± 0.3	13 ± 1	(3.1 ± 0.4)10x^2
VQFL.1	24.9 ± 0.5	2.0 ± 0.1	(1.23 ± 0.05)x10^4
VQFL.2	20.4 ± 0.6	2.6 ± 0.1	(7.9 ± 0.5)x10^3
VQFL.3	0.93 ± 0.03	2.3 ± 0.1	(4.1 ± 0.3)x10^2
L7ep-4	18.8 ± 0.3	0.89 ± 0.03	(2.11 ± 0.08)x10^4
L7er-5	13.4 ± 0.1	1.06 ± 0.02	(1.26 ± 0.03)x10^4
L7ep-6	17.2 ± 0.2	0.63±0.02	(2.73 ± 0.08)x10^4
L7ep-7	18.4±0.2	0.66±0.02	(2.81 ± 0.09)x10^4
L7ep-8	16.2±0.3	1.11±0.04	(1.45 ± 0.05)x10^4
L7ep-9	16.3±0.2	0.54±0.02	(3.0 ± 0.1)x10^4
L7ep-10	9.6±0.1	1.23±0.03	(7.8 ± 0.2)x10^3
L7ep-11	17.1±0.2	1.21±0.03	(1.42 ± 0.04)x10^4
L7ep-12	5.74±0.08	0.50±0.02	(7.8 ± 0.2)x10^3
L72p-2c	16.1±0.6	1.6±0.1	(9.8±0.8)x10^3
L7ep-2d	94±3	1.7±01	(5.4±0.3)x10^4
L7ep-2e	44±2	1.24±0.09	(3.6±0.3)x10^4
L7ep-2f	80±2	1.58±0.08	(5.0±0.3)x10^4
L7ep-2g	80±2	1.58±0.08	(5.3±0.4)x10^4
L7ep-2h	82±2	0.94±0.05	(8.7±0.3)x10^4
L7ep-2i	44±1	0.88±0.04	(5.0±0.3)x104
L7ep-2j	31±1	0.8±0.05	(4.0±0.3)x10^4
L7ep-3c	35.2±0.5	0.79±0.02	(4.5±0.1) x 10^4
L73p-3d	25.2±0.5	0.58±0.03	(4.4±0.2) x 10^4

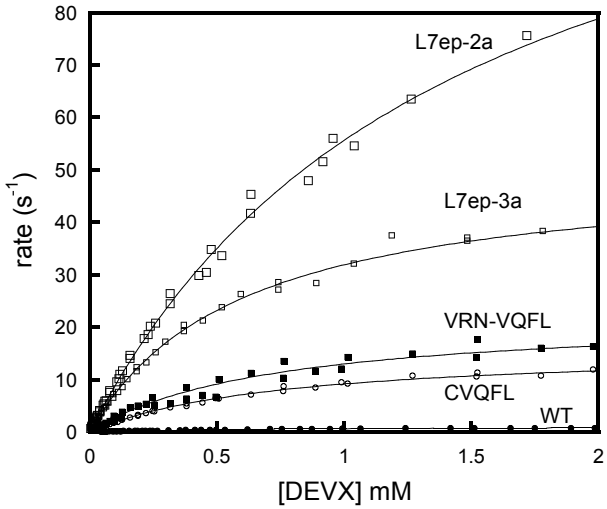

Figure S1. Representative Michaelis-Menton plots for the hydrolysis of DEVX by wild-type and evolved variants of PTE. Reaction conditions were 50 mM Hepes (pH 8), 100 µM $CoCl_2$, 0.3 mM DTNB in a total volume of 250 µL at 30° C. Reactions were initiated by addition of appropriately diluted enzyme. Enzyme concentrations in the reactions were; wild-type = 54 nM, CVQFL = 5.0 nM, VRN-VQFL = 6.29 nM, L7ep-3a = 1.88 nM, and L7ep-2a 2.26 nM. Solid line represents the fit of the data to equation 1.

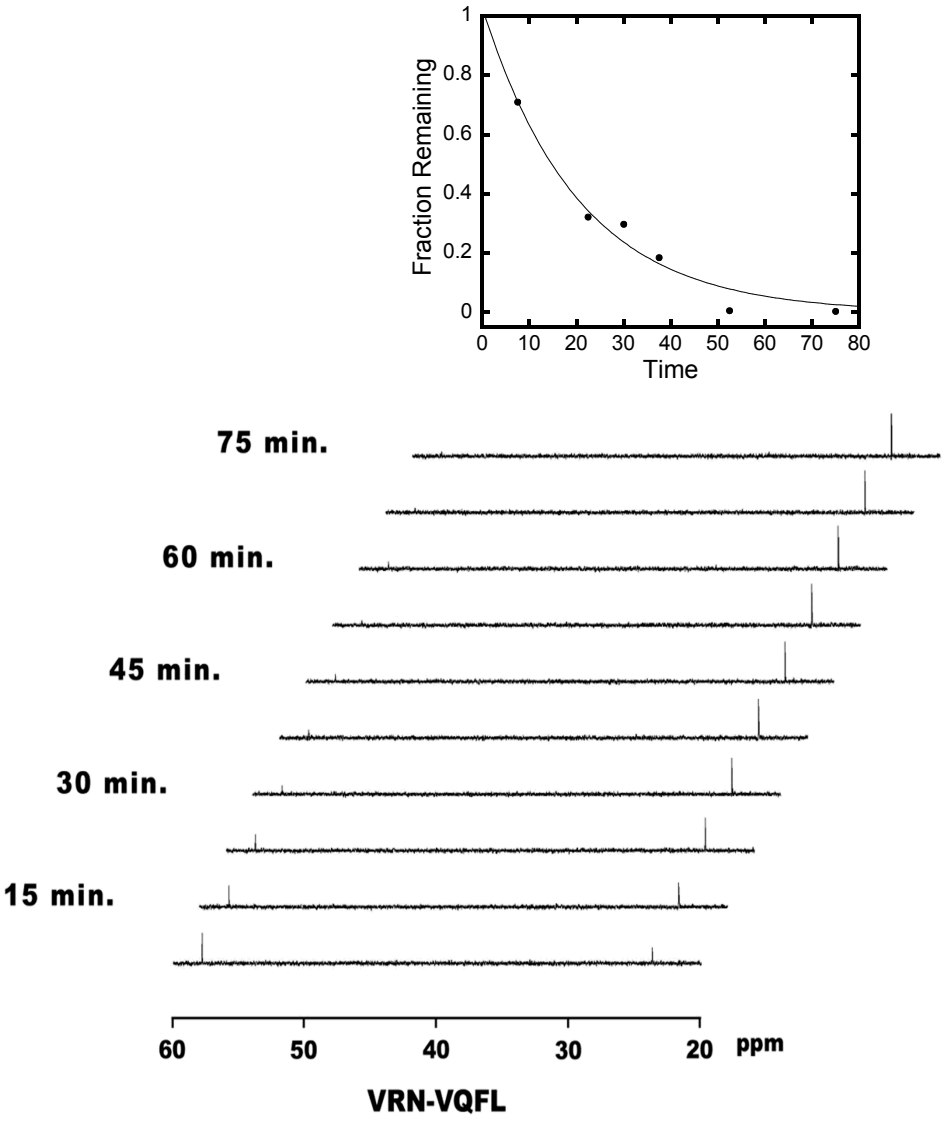

Figure S2: The hydrolysis of racemic VX by the VRN-VQFL variant observed by quantitative $^{31}P\{^{1}H\}$ NMR spectroscopy. The signal for the VX substrate is at *ca.* 57 ppm and that for the O-ethyl, methylphosphonic acid (EMPA) product is at *ca.* 23 ppm. Insets above show fraction VX remaining as a function of time as calculated from the integrated NMR signal. Data is fit to single exponential decay.