Inhibitory Potency against Human Acetylcholinesterase and Enzymatic Hydrolysis of Fluorogenic Nerve Agent Mimics by Human Paraoxonase 1 and Squid Diisopropyl Fluorophosphatase

Marc-Michael Blum,[‡,§] Christopher M. Timperley,[||] Gareth R. Williams,[||] Horst Thiermann,[‡] and Franz Worek*[,‡]

Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, D-80937 Munich, Germany, Institute of Biophysical Chemistry, J. W. Goethe University Frankfurt, Max-von-Laue-Strasse 9, D-60438 Frankfurt, Germany, and Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire SP4 0JQ, U.K.

Received November 7, 2007; Revised Manuscript Received February 19, 2008

ABSTRACT: A wide range of toxic organophosphorus pesticides and nerve agents is effectively hydrolyzed by the structurally related phosphotriesterase enzymes paraoxonase (PON1) from human plasma and diisopropyl fluorophosphatase (DFPase) from the squid *Loligo vulgaris*. Both enzymes have potential use as medical countermeasures and decontaminants. Enhanced enzymatic activity, stereochemical preference, and substrate variety are still the focus of ongoing research. Derivatives of pesticides and nerve agents bearing a fluorogenic leaving group were introduced for high-throughput screening of mutant libraries recently. We report the inhibitory potency of fluorogenic organophosphorus compounds with three different leaving groups [3-chloro-7-oxy-4-methylcoumarin, 7-oxy-4-methylcoumarin, 7-oxy-4-(trifluoromethyl)-coumarin] toward human acetylcholinesterase (AChE) and report kinetic data for the enzymatic hydrolysis of these compounds by PON1 and DFPase. This is the first report of the hydrolysis of a substrate bearing a P−O bond to the leaving group by DFPase (its activity was believed to be restricted to cleavage of P−F and P−CN bonds). The reactivity of the enzymes toward the substrates is explained on the basis of structural reasoning and computational docking studies. We demonstrate that fluorogenic organophosphorus compounds can serve as valuable models for enzyme screening but also show that differences and limitations exist and have to be taken into account. The importance of using protein from human sources to obtain toxicological data for potential *in vivo* use is highlighted.

Organophosphorus (OP)[1] compounds still have to be considered a major problem in toxicology. The use of OP pesticides for pest control and for attempting suicide causes large numbers of intoxications and several hundreds of thousands of fatalities per year especially in developing countries (*1, 2*). In addition, the availability and use of highly toxic OP nerve agents poses a pertinent hazard to the population (*3, 4*). The main mechanism of action of OP compounds is an inhibition of acetylcholinesterase (AChE) by phosphylation (denotes phosphorylation and phosphonylation) of the active site serine leading to an inactive enzyme species (*5, 6*). The failure of inhibited AChE to hydrolyze the neurotransmitter acetylcholine results in an accumulation of the neurotransmitter followed by an overstimulation of cholinergic receptors, a massive disturbance of numerous body functions, and finally death by respiratory failure (*7, 8*).

Up to now, standard treatment of OP poisoning includes the administration of muscarinic antagonists (e.g., atropine) and an oxime to reactivate inhibited AChE (*9*). However, certain OP compounds can produce AChE complexes resistant to oxime reactivation, limiting therapeutic efficacy (*9–12*).

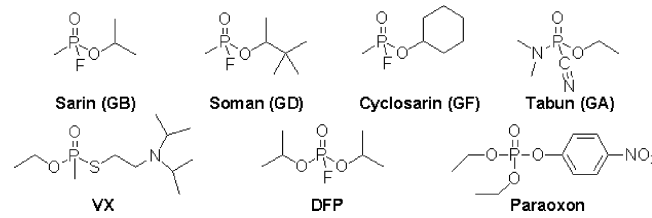


FIGURE 1: Structures of nerve agents, diisopropyl fluorophosphate (DFP), and the pesticide paraoxon.

In the past decades a number of enzymes have been identified that can hydrolyze OP pesticides and nerve agents. Among others, serum paraoxonase PON1 and diisopropyl fluorophosphatase (DFPase) from the squid *Loligo vulgaris* have been studied extensively (*13–16*). PON1 is present in human plasma and could be used in advance of exposure to nerve agents or for medical treatment of individuals exposed to OP compounds without causing adverse immune reactions (*17, 18*). Human PON1 has a catalytic activity against the pesticide paraoxon (Figure 1) and the nerve agents sarin (GB), soman (GD), and cyclosarin (GF) but shows only minimal activity against the nerve agent VX (*19–21*). Squid DFPase was shown to effectively catalyze the hydrolysis of P−F bonds (DFP, GB, GD, GF) and P−CN bonds (GA)

* Corresponding author: phone, +49-89-3168-2930; fax, +49-89-3168-2333; e-mail, franzworek@bundeswehr.org.
[‡] Bundeswehr Institute of Pharmacology and Toxicology, Munich.
[§] Institute of Biophysical Chemistry, University of Frankfurt.
[||] Defence Science and Technology Laboratory, Porton Down.

[1] Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; DFP, diisopropyl fluorophosphate; DFPase, diisopropyl fluorophosphatase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; HDL, high-density lipoprotein; OP, organophosphorus; PON, paraoxonase; PTE, phosphotriesterase.

10.1021/bi702222x CCC: $40.75
Published on Web 04/09/2008

but has no known activity for the cleavage of P−O and P−S bonds (*22*). Both enzymes have been characterized structurally by X-ray crystallography. The structure of DFPase was solved at an atomic resolution of 0.85 Å (PDB code 1PJX), and neutron diffraction is currently employed to investigate protonation states and water structure in the central tunnel of the enzyme (*23, 24*). The structure of an engineered mammalian PON1 was refined to a resolution of 2.2 Å (PDB code 1V04) (*25*). DFPase and PON share structural similarities. Both enzymes show a 6-fold β-propeller fold and contain two calcium ions with one of the ions located in the active site and crucial for catalysis. But also major differences exist. While PON exhibits major hydrolyzing activity against lactones and esters, DFPase lacks activity against these substrates. In this context it was found that different amino acid residues mediate the OP hydrolyzing activity and the esterase/lactonase activity of PON (*26*).

Besides the limited spectrum of OP compounds effectively hydrolyzed by PON1 and DFPase these enzymes exhibit a distinct stereospecificity, i.e., a low catalytic efficacy with the more toxic OP stereoisomers (*27, 28*). It should be noted, however, that the stereocenter at the phosphorus atom of these OP compounds undergoes fluoride-catalyzed racemization in aqueous solution, and in combination with the reduced activity of the enzyme against the more toxic stereoisomer, complete detoxification can be achieved, although at reduced speed which can be crucial in case of medical applications (*29*).

In view of these limitations, ongoing research is directed to the development of more effective OP hydrolyzing enzymes with reversed stereoselectivity and/or higher enzymatic activity (*20, 30, 31*). This necessitates the screening of huge numbers of mutants and large gene libraries, which may be facilitated by simple chromogenic or fluorogenic enzyme assays (*32*). Various pesticide and nerve agent analogues bearing a fluorescent 3-chloro-7-oxy-4-methylcoumarin moiety were described recently (*33*). Inhibition kinetics of these compounds with bovine erythrocyte AChE and the efficiency of hydrolysis of these fluorogenic OP by a phosphotriesterase (PTE) from *Pseudomonas diminuta* and a recombinant version of engineered mammalian PON were determined.

In the present work we investigated the ability of human PON1 and recombinant DFPase to hydrolyze a number of analogues bearing a 7-oxy-4-methylcoumarin or 7-oxy-4-(trifluoromethyl)coumarin leaving group (*34*). As a result of these tests we report the first hydrolysis of an OP compound bearing a P−O bond to the leaving group catalyzed by squid DFPase and propose an explanation for the different observed activities based on protein structure. In addition, the inhibitory potency of these fluorogenic OP compounds was determined with human erythrocyte AChE. The use of more easily obtainable enzymes (both PON and AChE) from nonhuman sources for reasons of practicality and convenience is understandable and can serve as a valuable model. However, it is only by using human enzymes *in vitro* that data may be obtained to assess the toxic potency of the fluorogenic OP compounds and the rates of PON-catalyzed hydrolysis that might occur in the human body, and as we will show, some significant differences exist.

MATERIALS AND METHODS

Materials. The 26 fluorescent OP compounds (Figure 1) were prepared as described before (*33, 34*). Acetylthiocholine iodide (ATCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Triton X-100, and Cibacron Blue 3GA agarose gel (type 3000-CL) were obtained from Sigma (Deisenhofen, Germany), and all other chemicals were from Merck Eurolab GmbH.

Stock solutions of the fluorescent OP compounds (1% v/v; Table 1) were prepared in DMSO, stored at ambient temperature, and appropriately diluted in distilled water just before the experiment.

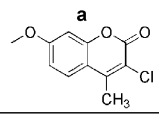
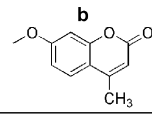
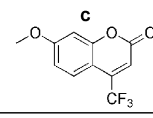
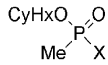
Preparation of Erythrocyte Ghosts. Human hemoglobin-free erythrocyte ghosts in 0.1 M phosphate buffer, pH 7.4, were prepared as described before (*35*). Aliquots of the erythrocyte ghosts with an AChE activity adjusted to that found in whole blood were stored at –60 °C until use. Prior to use, aliquots were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenator (Bandelin Electronic, Berlin, Germany), three times for 5 s with 30 s intervals, to achieve a homogeneous matrix for the kinetic studies.

PON and DFPase Preparation. Human PON1 was purified from outdated human plasma (Bundeswehr Blood Transfusion Service, Koblenz, Germany) following the general method by Gan et al. (*36*) and the modified version of Josse et al. (*37*). A typical procedure started from 1 L of human plasma. All work was carried out at room temperature unless otherwise noted. $CaCl_2$ solution (1 M) was added to a final calcium concentration of 10 mM to induce fibrin clotting. The clot developed over a period of 2 h and was removed by centrifugation at 5000*g* for 20 min at 4 °C. The cleared plasma was loaded on a column of Cibacron Blue 3GA agarose gel using 50 mM Tris buffer (pH 8.0) containing 1 mM $CaCl_2$ and 3 M NaCl. Elution of hydrophobic proteins including PON was initiated using the same buffer complemented with 0.1% Triton X-100 and 1% sodium deoxycholate. Fractions were assayed for PON activity and pooled. Further purification was achieved by ion-exchange chromatography on Q-Sepharose fast-flow resin (Amersham) employing 25 mM Tris buffer (pH 8.0) containing 0.1% Triton X-100 and 1 mM $CaCl_2$. Proteins were separated by a NaCl gradient running from 0 to 0.35 M. The eluting protein solution was fractionated, and protein solution not containing PON activity was discarded. As the ion-exchange chromatography step normally employed a rather large column volume, the step was repeated the following day after overnight dialysis against NaCl-free buffer with a smaller column and Q-Sepharose high-performance resin (Amersham) using the same buffer and gradient conditions. Finally, the protein solution was submitted to a Vivaspin concentrator (10 kDa cutoff), and salt was removed by washing with 25 mM Tris buffer (pH 8.0) containing 0.1% Triton X-100 and 1 mM $CaCl_2$.

His-tagged DFPase was expressed in *Escherichia coli* BL21 cells and purified according to the method of Hartleib and Rüterjans (*15*) with the His tag removed by thrombin digestion.

Determination of AChE Activity. AChE activities were measured spectrophotometrically (Cary 3Bio; Varian, Darmstadt, Germany) with a modified Ellman assay (*38, 39*). The assay mixture (3.16 mL) contained 0.45 mM ATCh as

Table 1: Inhibition Kinetics with Human AChE (pH 8.0, 37°C)

	Structure[a]	k_i (M^{-1} min^{-1})[b]			Parent OP compound[c]	
		a 	b 	c 	Name	k_i (M^{-1} min^{-1})
1		ø	n.a.	n.a.	Methylparathion	ø
2		ø	ø	ø	Parathion	ø
3		$4.1 \pm 0.3 \times 10^5$ (2.9)	n.a.	n.a.	Methylparaoxon	1.2×10^6
4		$1.2 \pm 0.1 \times 10^6$ (2.9)	$6.9 \pm 0.2 \times 10^5$ (4.8)	$2.4 \pm 0.4 \times 10^6$ (1.4)	Paraoxon	3.3×10^6
5		$5.0 \pm 0.0 \times 10^5$ (0.26)	n.a	n.a	DFP	1.3×10^5
6		ø	n.a	n.a	Dimefox	n.t.
7		ø	ø	ø	Tabun (GA)	1.8×10^7
8		$9.1 \pm 0.9 \times 10^6$ (10.9)	$4.8 \pm 0.3 \times 10^6$ (20.6)	$6.8 \pm 0.1 \times 10^6$ (14.6)	VX and Ethylsarin	9.9×10^7
9		$1.1 \pm 0.1 \times 10^6$ (34.2)	n.a.	$9.4 \pm 1.3 \times 10^5$ (38.4)	Sarin	3.6×10^7
10		$7.2 \pm 0.5 \times 10^7$ (6.3)	$6.2 \pm 0.7 \times 10^7$ (7.5)	$2.3 \pm 0.1 \times 10^7$ (20.3)	Russ. VX (VR) and i-Butylsarin	4.6×10^8
11		$1.7 \pm 0.1 \times 10^6$ (116.2)	$1.0 \pm 0.1 \times 10^7$ (19.3)	$8.9 \pm 0.5 \times 10^5$ (216.9)	Soman (GD)	1.9×10^8
12		$5.4 \pm 0.5 \times 10^7$ (7.8)	n.a.	$2.2 \pm 0.5 \times 10^7$ (18.8)	Cyclosarin (GF)	4.2×10^8

[a] Pin = pinacolyl; CyHx = cyclohexyl. [b] n.a. = compound not available; ø = activity below detection limit. Numbers in parentheses give the k_i ratio between the parent OP and the fluorogenic OP compound. [c] n.t. = not tested.

substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37 °C.

Determination of Inhibition Rate Constants of Fluorescent OP Compounds with Human AChE. Ten microliters erythrocyte ghosts and 5 μL of diluted OP inhibitor were added to a cuvette containing phosphate buffer, DTNB, and ATCh (final volume 3.165 mL). ATCh hydrolysis was continuously monitored over up to 30 min. The recorded curves were analyzed by nonlinear regression analysis for the determination of the first-order rate constant k_1 (min^{-1}) and used for the further determination of $k_i = k_2/K_d$ based on the following equation according to (*40, 41*)

$$1/k_1 = (K_d/k_2)\{1/([XI](1-\alpha)) + k_2\}$$

where K_d is the dissociation constant, k_2 is the unimolecular phosphylation rate constant, [XI] is the concentration of the inhibiting OP compound, and α is $[S]/(K_M + [S])$, where [S] is substrate concentration and K_M is the Michaelis constant. Under the conditions employed α was 0.833.

A detailed derivation of this equation can be found in the work of Hart and O'Brien (*42*). From the plot of $1/k_1$ against $1/([XI](1-\alpha))$ K_d is determined as the reciprocal value of the intercept on the *x*-axis and k_2 as the reciprocal value of the intercept on the *y*-axis.

Hydrolysis of Fluorescent OP Compounds by huPON/DFPase. The hydrolysis of fluorescent OP compounds by human PON1 was followed by the change in fluorescence using a Tecan Freedom EVO robotic workstation with a Tecan Saphire microplate reader (Tecan, Crailsheim, Germany) at 460, 410, and 510 nm with compounds bearing a 3-chloro-7-oxy-4-methylcoumarin, 7-oxy-4-methylcoumarin,

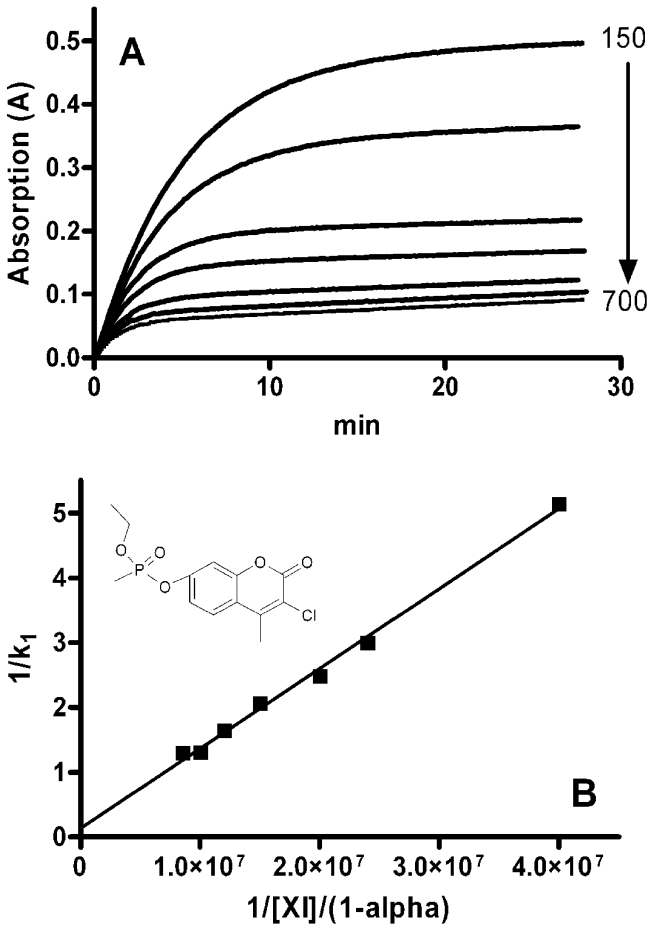


FIGURE 2: Inhibition kinetics of human AChE inhibited by compound **8a** (Table 1). Enzyme was incubated with buffer, substrate, and **8a** as indicated (in nM final concentration), acetylthiocholine (ATCh) hydrolysis was continuously monitored (A), and the slopes of the tangents (k_1, min^{-1}) were calculated. $1/k_1$ was plotted against 1/[XI] $(1 - \alpha)$ (B). [XI] is the inhibitor concentration, whereas α stands for $[S]/(K_M + [S])$, where [S] is the substrate concentration and K_M is the Michaelis constant. The second-order inhibition constant was calculated according to Aurbek et al. (*41*).

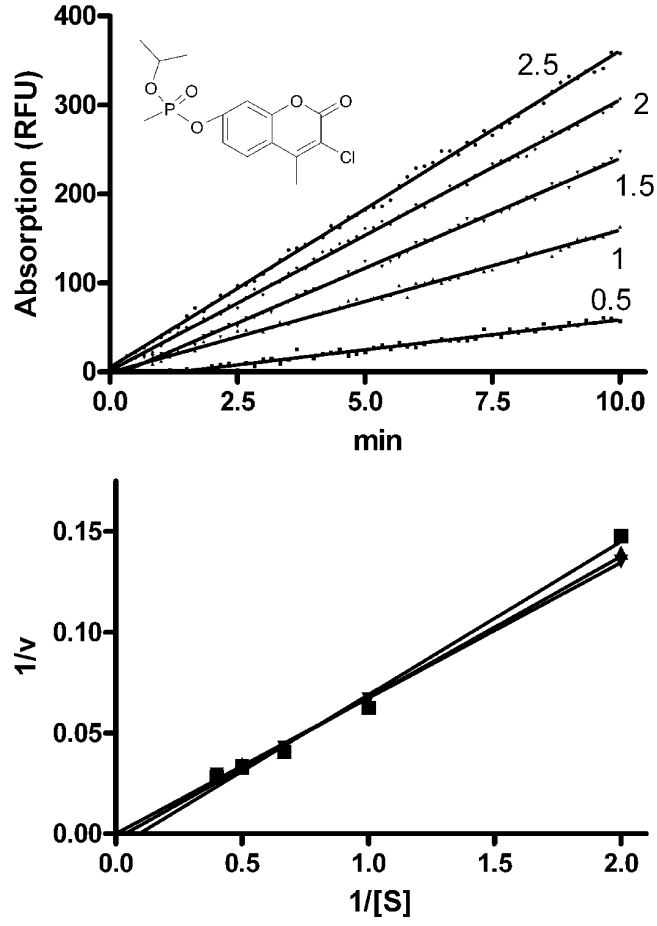


FIGURE 3: Hydrolysis of fluorogenic OP compounds by human PON1. Compound **9a** (0.5–2.5 μM) was incubated with PON1, and the change in fluorescence was followed (top). The slopes were calculated and were plotted as $1/v$ (min^{-1}) vs 1/[S] (μM) for the calculation of k_{cat}/K_M from the slope of the line resulting in a value of 1.4 ± 10^7 M^{-1} min^{-1} (bottom). Results are mean values of three independent experiments.

and 7-oxy-4-(trifluoromethyl)coumarin leaving group, respectively. Human PON1 (0.5 mg/mL) was diluted 2200-fold in 25 mM Tris buffer containing 1 mM $CaCl_2$ and 0.1% Triton X-100 (pH 8.0) and was equilibrated to 37 °C. PON1 (220 μL) solution were transferred to a microplate (BD Falcon Optilux; BD Bioscience, Franklin Lakes, NJ), and the reaction was started by adding 10 μL of OP substrate (0.5, 1.0, 1.5, 2.0, 2.5 μM final concentration). Parallel solutions of substrates in buffer without PON1 served as controls for spontaneous hydrolysis. The kinetic parameter k_{cat}/K_M was obtained by fitting the initial rate (v) at each OP concentration $[S]_0$ to the Michaelis–Menten equation ($v = [E]_0[S]_0k_{cat}/[S]_0 + K_M$) (*33*) (Figure 3). This approach is valid as $[S]_0 \ll K_M$. It should be noted that the individual determination of k_{cat} and K_M was not possible due to the very limited solubility of the fluorogenic OP compounds in water that did not allow using them in concentrations approaching the K_M.

In addition, the hydrolysis of the fluorogenic OP compounds (2.5 μM) by DFPase (3.3 μg/mL in 10 mM Tris and 2 mM $CaCl_2$, pH 7.5) was tested by applying the described procedure.

The analysis of the data was performed with Prism Version 4.00 (GraphPad Software, San Diego, CA).

Computational Docking. Docking of OP compounds in the active site of PON and DFPase was carried out using the software AUTODOCK4 (*43*) with low-energy conformers of the ligands obtained with the CORINA (*44*) program. Per docking run 100 individual results were computed and clustered by similar orientation of the ligands in the enzyme active sites. Dockings were carried out both with a rigid enzyme receptor and with a model in which some of the active site amino acid residues were treated as rotationally flexible. In the case of PON those residues comprise the side chains of amino acids L69, H115, F222, H285, and F292. For DFPase amino acid side chains of residues E37, I72, M90, and Y144 were included. Protein structures were obtained from the Protein Data Bank (PON, 1V04; DFPase, 1E1A).

RESULTS AND DISCUSSION

Inhibition of Human AChE by OP Compounds. Table 1 shows the tested OP compounds and their inhibitory potency toward human AChE. k_i values are given for the fluorogenic compounds as well as for the corresponding parent OP compounds. Except for the DFP analogue **5a**, the inhibitory

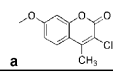
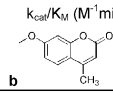
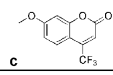
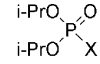
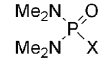
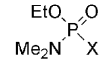
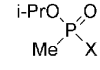
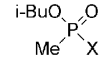
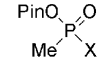
potency of the analogues was substantially lower than that of the parent compounds. Analogues **1a**, **2a**−**c**, and **7a**−**c** did not inhibit AChE, although in other cases altering the coumarin leaving group (e.g., **11a**−**c**) did modify inhibitory potency. The inhibition of human AChE followed pseudo-first-order kinetics in all cases (Figure 2). As already pointed out, OP compounds bearing four different residues at the phosphorus atom do exist as stereoisomers with the phosphorus atom forming the chiral center. They also exhibit different toxicities. The fluorogenic compounds **7**–**12** used here were synthesized and employed as racemic mixtures, and compound **11a**−**c** also bear a second stereocenter in the pinacolyl side chain. Inhibition constants therefore reflect the inhibitory potency of an equal mixture of all possible stereoisomers.

Analogues **1**, **2**, **6**, and **7** did not inhibit human AChE in agreement with findings for bovine AChE (*33*). OP compounds such as **1** and **2** in which the phosphoryl oxygen is replaced by sulfur are known to be unable to act as inhibitors due to the different electronic environment at the phosphorus atom that results from the reduced electronegativity and greater polarizability of sulfur compared to oxygen. The toxicity of these compounds *in vivo* is due to a conversion where the P═S moiety is replaced by P═O. Compounds **6** and **7** bear dialkylamino residues at the phosphorus atom. These groups donate electron density to the phosphorus, making it less susceptible for nucleophilic attack. The dimethylamino group in tabun, for example, is responsible for the relatively high stability of the compound in water. In contrast, diisopropyl phosphorocyanidate, which is a derivative of DFP with the fluorine atom replaced by a cyano group, is readily hydrolyzed in water within a few minutes (*45*).

A comparison of the inhibition data obtained from OP compounds with the 3-chloro-7-oxy-4-methylcoumarin leaving group for both human AChE and bovine AChE (*33*) reveals quite remarkable differences. The most striking example is the fluorogenic analogue of soman (GD, compound **11a**). Using bovine AChE only a relatively low k_i of 5.7×10^4 M^{-1} min^{-1} was reported for the fluorogenic analogue, and this was almost identical with the k_i of the parent compound. In contrast to this, a much higher k_i was found with the human enzyme. The calculated k_i was 1.7×10^6 M^{-1} min^{-1}, 2 orders of magnitude lower than the k_i for the parent compound that was determined to be 1.9×10^8 M^{-1} min^{-1}. This is in good agreement with the k_i value for recombinant human AChE obtained by Amitai et al. using a similar fluorogenic pinacolyl OP compound (*46*). For all of the nerve agents and their analogues higher k_i values were determined with human AChE compared to bovine AChE. Although it was shown that both human and bovine AChE share high resemblance in sequence (*47*), these findings are indicative of subtle but important differences between the two enzymes that can lead to significantly different results with inhibiting compounds.

Hydrolysis of OP Compounds by huPON and DFPase. Both human PON1 and DFPase were tested with all available OP compounds (Figure 3). Only a portion of the tested fluorogenic OP compounds was hydrolyzed by human PON1 (Table 2). PON1 did not catalyze the hydrolysis of compounds **1**–**3**, **5**–**7**, and **11**. The inability of PON1 to hydrolyze compounds with a thiophosphoryl group (**1**, **2**) and phosphoramidates (**6**, **7**) can be explained by the electronic environment at the phosphorus atom. Both the sulfur and nitrogen atoms render the central phosphorus atom more electron rich and therefore less susceptible for nucleophilic attack. Electronic effects cannot explain the results obtained with the other substrates, and explanations for their reactivity or the lack thereof must focus on structural aspects of the enzymes involved.

Table 2: Hydrolysis of OP Compounds by Human Plasma PON1 (pH 7.4, 37 °C)

	Structure[a]	k_{cat}/K_M ($M^{-1}min^{-1}$)[b]		
		a 	**b** 	**c** 
1		ø	n.a.	n.a.
2		ø	ø	ø
3		$6.3 \pm 1.6 \times 10^5$	n.a.	n.a.
4		$8.2 \pm 0.0 \times 10^5$	ø	$5.5 \pm 0.1 \times 10^6$
5		ø	n.a.	n.a
6		ø	n.a.	n.a.
7		ø	ø	ø
8		$2.9 \pm 0.1 \times 10^7$	$1.5 \pm 0.1 \times 10^7$	$5.6 \pm 0.1 \times 10^8$
9		$1.4 \pm 0.1 \times 10^7$	n.a.	$6.6 \pm 0.1 \times 10^7$
10		$1.9 \pm 0.1 \times 10^7$	$6.6 \pm 0.7 \times 10^6$	$1.1 \pm 0.2 \times 10^8$
11		ø	ø	ø
12		$2.9 \pm 1.1 \times 10^6$	n.a	$1.0 \pm 0.1 \times 10^7$
	Paraoxon	(p-nitrophenlate leaving group) $9.2 \pm 0.3 \times 10^6$		

[a] Pin = pinacolyl; CyHx = cyclohexyl. [b] n.a. = not available; ø = no activity.

Compounds **4a** and **4c** are derivatives of the pesticide paraoxon. Comparison of their hydrolysis by PON with the parent compound reveals slightly lower k_{cat}/K_M values although the value for **4c** comes very close to the one for paraoxon itself. The fact that the differences between the parent compound and the derivatives are small shows that fluorogenic OP compounds can indeed serve as useful model compounds.

Comparison of different coumarin leaving groups indicates that compounds bearing a 7-oxy-4-(trifluoromethyl)coumarin group were more susceptible toward hydrolysis by PON1 than compounds with 3-chloro-7-oxy-4-methylcoumarin and 7-oxy-4-methylcoumarin moieties. The pK_a value can serve as a good indicator for the ability of the three coumarin derivatives to act as leaving groups. The pK_a values of 7-oxy-

4-methylcoumarin (7.8) and 7-oxy-4-(trifluoromethyl)coumarin (7.3) have been published (*34*). The lower value for 7-oxy-4-(trifluoromethyl)coumarin is indicative of better leaving group properties. It has to be noted, however, that other factors such as the free enthalpy of solvation of the leaving group anion can have a significant influence as well.

Even though DFPase is inactive against compounds with larger leaving groups like VX, incubation of the different fluorogenic substrates with the squid enzyme did reveal that DFPase is able to hydrolyze some of these compounds. Compared with human PON1 the number of reactive substrates is significantly smaller. Only compounds bearing the 7-oxy-4-(trifluoromethyl)coumarin leaving group and one rather large alkoxy side chain at the phosphorus atom could be hydrolyzed. These are the derivatives of Russian VX **10c**, soman **11c**, and cyclosarin **12c**. The corresponding k_{cat}/K_M (M^{-1} min^{-1}) values were 6.5×10^6, 6.3×10^6, and 1.8×10^7. Interestingly, DFPase was able to hydrolyze the fluorogenic soman derivative **11c** while PON was inactive against **11a**−**c**. This is also in accordance with data reported by Briseño-Roa et al. (*33*) for soman derivatives with fluorogenic leaving groups hydrolyzed by an engineered variant of PON with low k_{cat}/K_M values <100 M^{-1} min^{-1}. Amitai et al. also report the synthesis of nerve agent analogues with a different fluorogenic leaving group including a soman derivative but, unfortunately, only report data on the hydrolysis of a cyclosarin derivative by PON (*46*). These data, however, show that the stereochemical preference of PON for one stereoisomer did not change from the preference found with the parent nerve agent cyclosarin.

Hydrolytic Activity and Enzyme Structure. To be able to serve as model compounds for screening of enzyme mutants for enhanced activity and stereoselectivity, the kinetics of hydrolysis for the fluorogenic analogues must be comparable with those for the parent nerve agents. As the experiments with human PON1 have shown, it is possible to hydrolyze all compounds derived from nerve agents except those based on tabun and soman. While this result was to be expected for tabun, it was rather surprising in the case of the soman analogue as PON shows good activity against this agent (*20*). The second surprise concerns DFPase. Up to now, squid DFPase was always associated with the cleavage of P−F and P−CN bonds and was thought to be unable to hydrolyze P−O bonds. Interestingly, DFPase was found to be active against the soman analogue **11c** and compounds **10c** and **12c**, all of which contained an oxy-4-(trifluoromethyl)coumarin leaving group. The fact that squid DFPase has been shown to hydrolyze certain P−O leaving groups might shed light on its natural function, which to date remains elusive: P−O groups are prevalent in living systems unlike synthetic P−F and P−CN bonds. As structures of DFPase and PON are available from the Protein Data Bank, computational docking was employed to investigate steric constraints for substrate binding with the aim to formulate general trends and rules for these two similar enzymes (Figure 4).

A new reaction mechanism for DFPase was recently proposed on the basis of data from isotope labeling, structural, and kinetic results (*14*). In this mechanism, calcium-coordinating aspartate D229 acts as the nucleophile attacking the phosphorus atom in line with the leaving group of the substrate to generate a phospho-enzyme intermediate which is then hydrolyzed to generate the products and regenerate the enzyme. The possibility

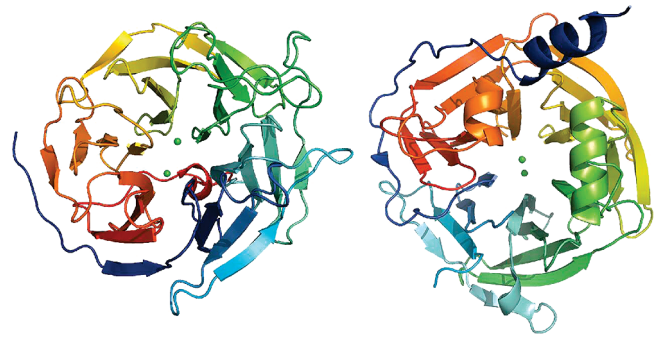


FIGURE 4: Tertiary structure of DFPase (left) and PON (right). Both proteins are 6-fold β-propellers containing two calcium ions. The extra helices on top of PON are thought to act as anchors of the protein in high-density lipoprotein (HDL) particles.

of a similar mechanism was highlighted for PON with aspartate D269 acting as the nucleophile. This proposal was based on structural comparison with DFPase and findings by Khersonsky and Tawfik (*26*) that certain mutations of the H115-H134 His-His dyad leave the phosphotriesterase activity intact but extinguish the esterase and lactonase activity of paraoxonase. This His-His dyad was originally proposed to act as the water-activating moiety in the phosphotriesterase mechanism of PON (*25*). Therefore, there is currently no proposed mechanism for the phosphotriesterase mechanism of PON apart from the mechanism for DFPase proposed on the basis of structural analogy. Isotope labeling experiments such as those conducted with DFPase were repeated with PON, but due to the rapid inactivation of PON upon lyophilization of the enzyme the results were inconclusive. Both D229 in DFPase and D269 in PON1 are ligands of the catalytic calcium ion. One would expect a significant drop in nucleophilicity of an aspartate residue upon binding to an earth-alkaline metal ion, but several reports in the literature show that this kind of mechanism is indeed possible. Examples include the phosphatase mechanism in soluble epoxide hydrolase (*48, 49*) or metal-dependent phosphatase activities of enzymes belonging to the haloacid dehalogenase (HAD) superfamily (*50*) with the magnesium-dependent human phosphoserine phosphatase (*51*) and β-phosphoglucomutase (*52*) as specific examples.

Two conditions have to be met by an OP substrate bound to the active sites of the two enzymes. First, the phosphoryl oxygen of the substrate has to be coordinated to the calcium ion in the active site. Second, the orientation of the substrate must enable the nucleophile D229/D269 to attack the phosphorus atom in line with the leaving group.

In the case of PON the bulky leaving groups of the fluorogenic OP compounds are easily accommodated as the active site provides sufficient space opposite of D269 in the direction of the His-His dyad. On the other hand, the space for other phosphorus-bound groups is rather restricted. Accommodating two groups that are larger than ethoxy groups while preserving a reactive conformation seems impossible as indicated by the docking of the DFP derivative **5c** (Supporting Information S1). Compounds **8**–**12** bearing a methyl and an alkoxy group can fit into the active site with the exception of compound **11** derived from soman. In the case of the cyclosarin derivative **12c**, it is obvious that the sterical limits of the active site are reached (Figure 5).

We have also docked the two stereoisomers of the parent OP compound cyclosarin. Only R_P-cyclosarin is oriented

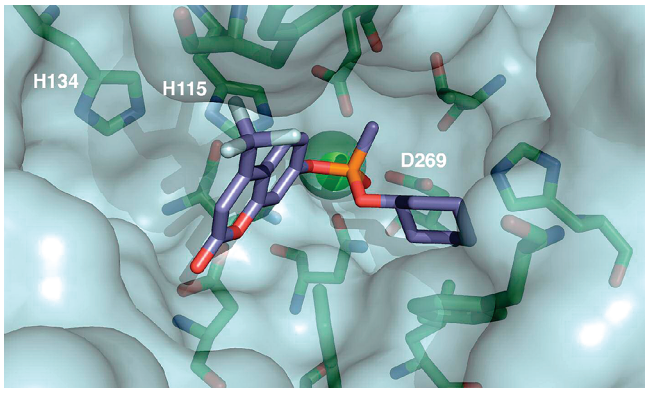


FIGURE 5: Cyclosarin derivative **12c** docked into the active site of PON. The leaving group is positioned in a way that an in-line attack of D269 on the phosphorus atom is possible. Attack of a nucleophile from the area of the His-His dyad would require the leaving group to take the position of the cyclohexyl ring. For steric reasons this is not possible.

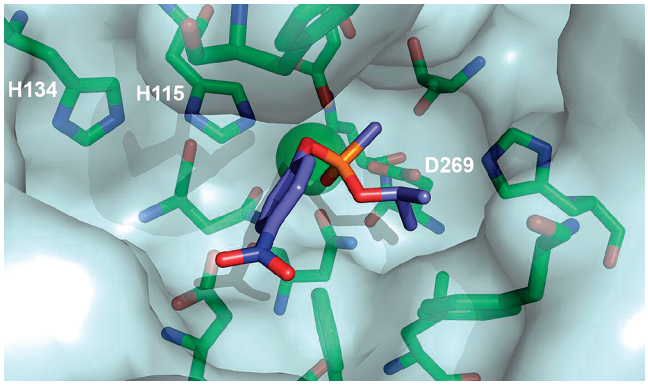


FIGURE 6: Sarin analogue, bearing a *p*-nitrophenolate leaving group with configuration R_P at the phosphorus atom, docked into the active site of PON. The molecule is oriented properly for attack by D269 while no such orientation was found by docking the S_P enantiomer.

properly for an in-line attack by D269. To allow S_P-cyclosarin a similar orientation, more space would be needed in the area of amino acid residues V346 and L69. This is in agreement with the findings of Amitai et al. (*20*) that mutants V346A and L69A exhibit higher activity against cyclosarin. Amitai et al. also reported that experiments with a sarin analogue in which the fluoride leaving group was replaced by the *p*-nitrophenolate group of paraoxon revealed that the more toxic stereoisomer was not hydrolyzed over extended periods of time while the less toxic stereoisomer was hydrolyzed with measurable speed. The V346A mutant, however, was able to hydrolyze both stereoisomers. According to Benschop and De Jong (*52*) the less toxic enantiomer of sarin is likely to have the R_P configuration. Docking the *p*-nitrophenolate analogue of S_P-sarin did not reveal a single orientation in which the phosphoryl oxygen is coordinated with the catalytic calcium ion while for the analogue of R_P-sarin this is indeed the case and the resulting orientation is properly positioned for an attack by D269 (Figure 6).

Although the pinacolyl group has the same number of carbon atoms as the cyclohexyl group, the different shape leads to steric crowding (Supporting Information S2). DFPase only hydrolyzes those compounds with rather bulky alkoxy side chains on the phosphorus atom. As the docking of the cyclosarin derivative **12c** shows, this creates favorable interactions of the cyclohexyl ring with the hydrophobic parts

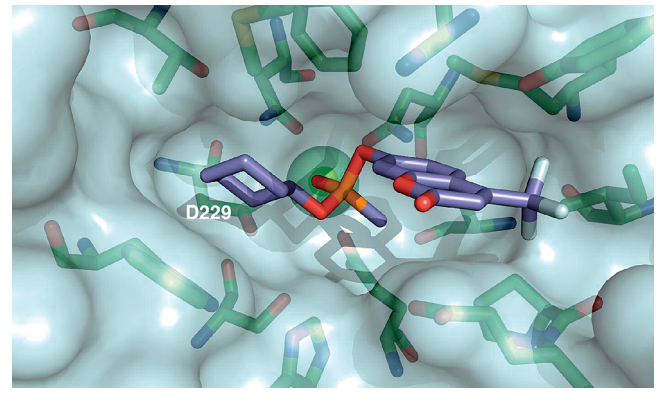


FIGURE 7: Cyclosarin derivative **12c** docked into the active site of DFPase. The orientation of the substrate allows a nucleophilic attack of D229. The bulky cyclohexyl group just fits into the area of the active site on the left and generates strong hydrophobic interactions with the surrounding side chains of the enzyme.

of the active site, helping to orient and fix **12c** in a reactive conformation (Figure 7). A similar orientation could be obtained for the soman derivative **11c** but only for one of the four possible stereoisomers (Supporting Information S3). For compounds **8**–**12** docking of the R_P stereoisomer leads to better results regarding reactive conformations compared to the S_P stereoisomer.

The assumption of a rigid receptor molecule in the docking experiments is a simplification that can lead to false results. Effects such as induced fit are not accounted for. While the complex of DFPase with *O*,*O*-dicyclopentylphosphoroamidate displayed only marginal movement of amino acid side chains in the active site compared to the wild-type structure (*14*) and a rigid receptor appears to be a reasonable model for this enzyme, there are no structural data available for PON in complex with a ligand other than inorganic phosphate. The latest version of the docking software AUTODOCK (*42*) allows the treatment of amino acid side chains in the receptor as rotationally flexible. Dockings were repeated with the flexible model. For PON1 the side chains of amino acids L69, H115, F222, H285, and F292, all being part of the active site cleft, were treated as flexible. For DFPase side chains of E37, I72, and M90 were chosen as flexible as they are interacting with the large coumarin-based leaving groups of the OP compounds. The orientation of the OP compounds docked with the lowest energy in the flexible model corresponds with the orientation in the rigid model, and only marginal deviations were observed.

CONCLUSIONS

Organophosphorus compounds with fluorogenic leaving groups allow the rapid determination of reaction kinetics with OP hydrolyzing enzymes using highly automated laboratory equipment. But in order to be able to serve as mimics for pesticides and nerve agents, it is important that the screening for more efficient enzymes does come up with mutants that are more reactive not only with respect to the fluorogenic OP derivatives but also for the parent OP compounds. This condition is not easy to meet as the coumarin-based leaving groups differ significantly in size and chemical properties from the leaving groups of the parent compounds. While experimental results with human serum PON1 show encouraging results, this is not the case for DFPase, an enzyme

that is in general not able to tolerate large leaving groups. It was shown, for example, that the fluorogenic derivatives of the best substrate, DFP, were not hydrolyzed. On the basis of our findings it becomes clear that fluorogenic OP compounds can serve as valuable test substrates for rapid automated screening of mutant libraries but the limitations of the model have to be kept in mind. It seems necessary to combine the kinetic results with structural information about the involved enzymes in order to be able to judge the usefulness of the obtained data.

Even though the limits of fluorogenic OP compounds as test substrates were shown in the case of squid DFPase, we were able to demonstrate for the first time that DFPase is able to hydrolyze compounds bearing a P−O bond to the leaving group if certain conditions are met. Most importantly, a large side chain on the phosphorus atom is needed to create the binding forces to orient the substrate in a reactive position. This is an encouraging result for further protein engineering with DFPase in order to generate mutants with a wider substrate range.

Limitations exist not only for substrate derivatives but also for the choice of enzymes. We were able to show that some significant differences exist between human and bovine AChE with respect to the inhibitory potency of OP compounds. Again, it is necessary to know the differences and limitations when using enzymes from other sources. A delicate choice: either choose human enzyme (in case of AChE) and the parent OP compounds or select OP derivatives that are easy to screen and an enzyme that is more readily available. While the first choice will generate relevant and exact data, one will also have to deal with more complicated and time-consuming assays as well as highly toxic and regulated OP compounds. The second choice will generate data more easily, but the limitations of the models must be known in order to assess the relevance of the obtained results.

ACKNOWLEDGMENT

The authors are indebted to Krystal E. Casey for the synthesis of several fluorogenic OP compounds, to Bodo Pfeiffer for skilful technical assistance, and to Janet Wetherell for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Docked conformations of compounds **5c** and **11c** into the active site of PON and of compound **11c** into the active site of DFPase. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Bertolote, J. M., Fleischmann, A., Eddleston, M., and Gunnell, D. (2006) Deaths from pesticide poisoning: a global response. *Br. J. Psychiatr. 189*, 201–203.
2. Eddleston, M., and Phillips, M. R. (2004) Self poisoning with pesticides. *Br. Med. J. 328*, 42–44.
3. Bismuth, C., Borron, S. W., Baud, F. J., and Barriot, P. (2004) Chemical weapons: documented use and compounds on the horizon. *Toxicol. Lett. 149*, 11–18.
4. Evison, D., Hinsley, D., and Rice, P. (2002) Chemical weapons. *Br. Med. J. 324*, 332–335.
5. Silman, I., and Sussman, J. L. (2005) Acetylcholinesterase: "classical" and "non-classical" functions and pharmacology. *Curr. Opin. Pharmacol. 5*, 293–302.
6. Holmstedt, B. (1959) Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacol. Rev. 11*, 567–688.
7. Eddleston, M., Mohamed, F., Davies, J. O. J., Eyer, P., Worek, F., Sheriff, M. H. R., and Buckley, N. A. (2006) Respiratory failure in acute organophosphorus pesticide self-poisoning. *Q. J. Med. 99*, 513–522.
8. Sakai, F., Dal Ri, H., Erdmann, W. D., and Schmidt, G. (1958) Respiratory paralysis by parathion or paraoxon and antagonistic influences. *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol. 234*, 210–219.
9. Eyer, P. (2003) The role of oximes in the management of organophosphorus pesticide poisoning. *Toxicol. Rev. 22*, 165–190.
10. Thiermann, H., Szinicz, L., Eyer, F., Worek, F., Eyer, P., Felgenhauer, N., and Zilker, T. (1999) Modern strategies in therapy of organophosphate poisoning. *Toxicol. Lett. 107*, 233–239.
11. Worek, F., Eyer, F., Aurbek, N., Szinicz, L., and Thiermann, H. (2007) Recent advances in evaluation of oxime efficacy in nerve agent poisoning by in vitro analysis. *Toxicol. Appl. Pharmacol. 219*, 226–234.
12. Worek, F., Thiermann, H., Szinicz, L., and Eyer, P. (2004) Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. *Biochem. Pharmacol. 68*, 2237–2248.
13. Mackness, B., Durrington, P. N., and Mackness, M. I. (1998) Human serum paraoxonase. *Gen. Pharmacol. 31*, 329–336.
14. Blum, M. M., Löhr, F., Richardt, A., Rüterjans, H., and Chen, J. C. H. (2006) Binding of a designed substrate analogue to diisopropyl fluorophosphatase: implications for the phosphotriesterase mechanism. *J. Am. Chem. Soc. 128*, 12750–12757.
15. Hartleib, J., and Rüterjans, H. (2001) High-yield expression, purification, and characterization of the recombinant diisopropylfluorophosphatase from *Loligo vulgaris*. *Protein Expression Purif. 21*, 210–219.
16. Hoskin, F. C. G., and Long, R. J. (1972) Purification of a DFP-hydrolyzing enzyme from squid head ganglion. *Arch. Biochem. Biophys. 150*, 548–555.
17. Draganov, D. I., and LaDu, B. N. (2004) Pharmacogenetics of paraoxonases: a brief review. *Naunyn-Schmiedeberg's Arch. Pharmacol. 369*, 78–88.
18. Li, B., Sedlacek, M., Manaharan, I., Boopathy, R., Duysen, E. G., Masson, P., and Lockridge, O. (2005) Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem. Pharmacol. 70*, 1673–1684.
19. Josse, D., Lockridge, O., Xie, W., Bartels, C. F., Schopfer, L. M., and Masson, P. (2001) The active site of human paraoxonase (PON1). *J. Appl. Toxicol. 21* (Suppl. 1), S7–S11.
20. Amitai, G., Gaidukov, L., Adani, R., Yishay, S., Yacov, G., Taitlboim, S., Lindenbaum, M., Bel, P., Tawfik, D. S., and Meshulam, H. (2006) Enhanced stereoselective hydrolysis of toxic organophosphates by directly evolved variants of mammalian serum paraoxonase. *FEBS J. 273*, 1906–1919.
21. Broomfield, C. A., Morris, B. C., Anderson, R., Josse, D., and Masson, P. (2000) Kinetics of nerve agent hydrolysis by a human plasma enzyme, in *CBMTS III Conference Proceedings*, Spiez, Switzerland.
22. Hartleib, J., and Rüterjans, H. (2001) Insights into the reaction mechanism of the diisopropyl fluorophosphatase from *Loligo vulgaris* by means of kinetic studies, chemical modification and site-directed mutagenesis. *Biochim. Biophys. Acta 1546*, 312–324.
23. Koepke, J., Scharff, E. I., Lücke, C., Rüterjans, H., and Fritzsch, G. (2003) Statistical analysis of crystallographic data obtained from squid ganglion DFPase at 0.85 Å resolution. *Acta Crystallogr. D 59*, 1744–1754.
24. Blum, M. M., Koglin, A., Rüterjans, H., Schoenborn, B., Langan, P., and Chen, J. C. H. (2007) Preliminary time-of-flight neutron diffraction study on diisopropyl fluorophosphatase (DFPase) from *Loligo vulgaris*. *Acta Crystallogr. F 63*, 42–45.
25. Harel, M., Aharoni, A., Gaidukov, L., Brumshtein, B., Khersonsky, O., Meged, R., Dvir, H., Ravelli, R. B. G., McCarthy, A., Toker, L., Silman, I., Sussman, J. L., and Tawfik, D. S. (2004) Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat. Struct. Mol. Biol. 11*, 412–419.
26. Khersonsky, O., and Tawfik, D. S. (2006) The histidine 115-histidine 134 dyad mediates the lactonase activity of mammalian serum paraoxonases. *J. Biol. Chem. 281*, 7649–7656.
27. Ooms, A. J. J., and Boter, H. L. (1965) Stereospecificity of hydrolytic enzymes in their reaction with optically active organophosphorus compounds. II. The inhibition of aliesterase,

acetylesterase, chymotrypsin and trypsin by S-alkyl para-nitrophenyl methylphosphonothiolates. *Biochem. Pharmacol. 14*, 1839–1846.
28. Yeung, D. T., Smith, J. R., Sweeney, R. E., Lenz, D. E., and Cerasoli, D. M. (2007) Direct detection of stereospecific soman hydrolysis by wild-type human serum paraoxonase. *FEBS J. 274*, 1183–1191.
29. Christen, P. J., and van den Muysenberg, J. A. (1965) The enzymatic isolation and fluoride catalysed racemisation of optically active Sarin. *Biochim. Biophys. Acta 110*, 217–220.
30. Gopal, S., Rastogi, V., Ashman, W., and Mulbry, W. (2000) Mutagenesis of organophosphorus hydrolase to enhance hydrolysis of the nerve agent VX. *Biochem. Biophys. Res. Commun. 279*, 516–519.
31. Chen-Goodspeed, M., Sogorb, M. A., Wu, F., and Raushel, F. M. (2001) Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues. *Biochemistry 40*, 1332–1339.
32. Aharoni, A., Griffiths, A. D., and Tawfik, D. S. (2005) High-throughput screens and selections of enzyme-encoding genes. *Curr. Opin. Chem. Biol. 9*, 210–216.
33. Briseno-Roa, L., Hill, J., Notman, S., Sellers, D., Smith, A. P., Timperley, C. M., Wetherell, J., Williams, N. H., Williams, G. R., Fersht, A. R., and Griffiths, A. D. (2006) Analogues with fluorescent leaving groups for screening and selection of enzymes that efficiently hydrolyze organophosphorus nerve agents. *J. Med. Chem. 49*, 246–255.
34. Timperley, C. M., Casey, K. E., Notman, S., Sellers, D. J., Williams, N. E., Williams, N. H., and Williams, G. R. (2006) Synthesis and anticholinesterase activity of some new fluorogenic analogues of organophosphorus nerve agents. *J. Fluorine Chem. 127*, 1554–1563.
35. Worek, F., Reiter, G., Eyer, P., and Szinicz, L. (2002) Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. *Arch. Toxicol. 76*, 523–529.
36. Gan, K. N., Smolen, A., Eckerson, H. W., and LaDu, B. N. (1991) Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab. Dispos. 19*, 100–106.
37. Josse, D., Xie, W., Renault, F., Rochu, D., Schopfer, L. M., Masson, P., and Lockridge, O. (1999) Identification of residues essential for human paraoxonase (PON1) arylesterase/organophosphatase activities. *Biochemistry 38*, 2816–2825.
38. Worek, F., Mast, U., Kiderlen, D., Diepold, C., and Eyer, P. (1999) Improved determination of acetylcholinesterase activity in human whole blood. *Clin. Chim. Acta 288*, 73–90.
39. Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., and Reiner, E. (2003) Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal. Biochem. 312*, 224–227.
40. Forsberg, A., and Puu, G. (1984) Kinetics for the inhibition of acetylcholinesterase from the electric eel by some organophosphates and carbamates. *Eur. J. Biochem. 140*, 153–156.
41. Aurbek, N., Thiermann, H., Szinicz, L., Eyer, P., and Worek, F. (2006) Analysis of inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds with human and pig acetylcholinesterase. *Toxicology 224*, 91–99.
42. Hart, G. J., and O'Brien, R. D. (1973) Recording spectrophotometric method for determination of dissociation and phosphorylation constants for the inhibition of acetylcholinesterase by organophosphates in the presence of substrate. *Biochemistry 12*, 2940–2945. Huey, R., Morris, G. M., Olson, A. J., and Goodsell, D. S. (2007) A semiempirical free energy force field with charge-based desolvation. *J. Comput. Chem. 28*, 1145–1152.
43. Sadowski, J., and Gasteiger, J. (1993) From atoms and bonds to three-dimensional atomic coordinates: Automatic model builders. *Chem. Rev. 93*, 2567–2581.
44. Hudson, R. F., and Greenhaigh, R. (1969) Influence of the leaving group on the rate of phosphorylation. *J. Chem. Soc. B,* 325–329.
45. Amitai, G., Adani, R., Yacov, G., Yishay, S., Teitlboim, S., Tveria, L., Limanovich, O., Kushnir, M., and Meshulam, H. (2007) Asymmetric fluorogenic organophosphates for the development of active organophosphate hydrolases with reversed stereoselectivity. *Toxicology 233*, 187–198.
46. Mendelson, I., Kronman, C., Ariel, N., Shafferman, A., and Velan, B. (1998) Bovine acetylcholinesterase: cloning, expression and characterization. *Biochem. J. 334*, 251–259.
47. De Vivo, M., Ensing, B., and Klein, M. L. (2005) Computational study of phosphatase activity in soluble epoxide hydrolase: high efficiency through a water bridge mediated proton shuttle. *J. Am. Chem. Soc. 127*, 11226–11227.
48. De Vivo, M., Ensing, B., Dal Peraro, M., Gomez, G. A., Christianson, D. W., and Klein, M. L. (2007) Proton shuttles and phosphatase activity in soluble epoxide hydrolase. *J. Am. Chem. Soc. 129*, 387–394.
49. Allen, K. N., and Dunaway-Mariano, D. (2004) Phosphoryl group transfer: evolution of a catalytic scaffold. *Trends Biochem. Sci. 29*, 495–503.
50. Peeraer, Y., Rabijns, A., Gollet, J. F., Van Schaftingen, E., and De Ranter, C. (2004) How calcium inhibits the magnesium-dependent enzyme human phosphoserine phosphatase. *Eur. J. Biochem. 271*, 3421–3427.
51. Lahiri, S. D., Zhang, G, Dunaway-Mariano, D., and Allen, K. N. (2003) The pentacovalent phosphorus intermediate of a phosphoryl transfer reaction. *Science 299*, 2067–2071.
52. Benschop, H. P., and De Jong, L. P. A. (1988) Nerve agent stereoisomers: Analysis, isolation, and toxicology. *Acc. Chem. Res. 21*, 368–374.

BI702222X