Articles

Stereochemical Constraints on the Substrate Specificity of Phosphotriesterase†

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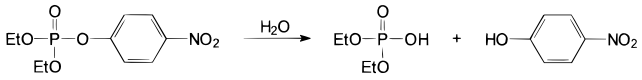
ABSTRACT: A series of achiral, chiral, and racemic mixtures of paraoxon analogues containing various combinations of methyl, ethyl, isopropyl, or phenyl substituents were synthesized as probes of the stereochemical constraints within the active site of phosphotriesterase. The kinetic constants for these paraoxon analogues with the enzyme varied significantly with the size of substituents surrounding the phosphorus center. These results indicate that binding and catalysis depend significantly on the relative size and orientation of the two subsites that must accommodate the coordination of the alkyl or aryl substituents within the enzyme active site. Individual enantiomers of paraoxon analogues were also synthesized and the stereochemical specificity for phosphotriesterase determined. In general, the kinetic constants, k_{cat} and k_{cat}/K_m, for the (−)-enantiomers of these phosphotriesters were 1−2 orders of magnitude greater than the (+)-enantiomers. In every case, the preferred isomer is of the S_P-configuration. For example, the k_{cat}/K_m for S_P-(−)-ethyl phenyl *p*-nitrophenyl phosphate is 1.8×10^8 M^{-1} s^{-1} but is only 1.8×10^6 M^{-1} s^{-1} for the R_P-(+)-isomer. These results suggest that one enantiomer is positioned for hydrolysis more favorably than the other enantiomer. The inactivation of acetylcholinesterase with the same series of organophosphate nerve agents was also measured. The stereoisomer that more rapidly inactivates human acetylcholinesterase is hydrolyzed more slowly than its enantiomer by the phosphotriesterase.

Phosphotriesterase (PTE)[1] from *Pseudomonas diminuta* catalyzes the hydrolysis of a wide variety of organophosphorus nerve agents (*1*, *2*). The organophosphate nerve agents are highly toxic materials because they react rapidly with acetylcholinesterase (AChE),[1] an enzyme critical for nerve function. The mechanism for the inactivation of AChE induced by phosphotriesters (or the related phosphonates) has been found to be the irreversible phosphorylation (or phosphonylation) of an active site serine residue and subsequent impairment of catalytic activity (*3*, *4*). Although naturally occurring substrates are not known for this enzyme, PTE treats many of these highly toxic nerve agents as true substrates rather than as inhibitors or inactivators. The hydrolysis of the insecticide paraoxon is shown below in Scheme 1.

PTE catalyzes the hydrolysis of substrates such as paraoxon with a remarkably high efficiency. The turnover numbers (k_{cat}) for the best substrates approach 10^4 s^{-1}, and k_{cat}/K_m is nearly 10^8 $M^{-1}s^{-1}$, a value that is close to the diffusion-controlled limit (*5*). The active site of the enzyme consists of a binuclear metal center and the native enzyme

Scheme 1



requires two Zn^{2+} ions for maximal catalytic activity. However, the enzyme is equally active with other divalent cations such as Co^{2+}, Cd^{2+}, Ni^{2+}, and Mn^{2+} (*5*). The three-dimensional crystal structure of the Zn^{2+}/Zn^{2+}-substituted enzyme with a bound substrate analogue has shown that the two metal ions are ligated to the protein by four histidines and an aspartate and are bridged together with a hydroxide from solvent and a modified lysine residue (*6*). The reaction mechanism has been proposed to proceed via a single in-line displacement of the leaving group by the metal-bound hydroxide, which attacks the phosphorus center with net inversion of configuration (*7*).

Phosphonofluoridates and phosphonothiolates such as sarin, soman, and VX are potent acetylcholinesterase inactivators and have been stockpiled as chemical warfare agents. Many of these compounds have a stereogenic phosphorus center. The individual stereoisomers differ greatly in their inhibitory and toxicological properties (ref *8* and references therein). For example, all four stereoisomers of soman are potent inhibitors of AChE, but the enzyme exhibits a 10^4-fold preference for the (S_P)-enantiomers over the (R_P)-enantiomers (*9*, *10*). Toxicity studies with mice indicate that the (S_P)-soman enantiomers are 10^2-fold more potent than the (R_P)-enantiomers (*9*). These results demonstrate that the

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[1] Abbreviations: PTE, phosphotriesterase; AChE, acetylcholinesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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biological activity of nerve agents and their reaction rates with AChE are very much dependent on the size and nature of the substituents, chirality, and asymmetric enzyme environment.

Among numerous enzymes capable of catalyzing the hydrolysis of organophosphorus compounds, PTE from *P. diminuta* has attracted a heightened interest. The enzyme has a broad substrate specificity and has exhibited significant potential for the detoxification and treatment of organophosphorus nerve agents (*1, 11*). The X-ray crystal structure of Zn^{2+}-PTE with a bound substrate analogue, diethyl *p*-methylbenzylphosphonate, has exposed the very hydrophobic environment of the active site. It has also been shown that PTE will only hydrolyze the (S_P)-enantiomer of ethyl *p*-nitrophenyl phenyl thiophosphonate (EPN) (*7*). This latter result indicates that the substrate selectivity exhibited by PTE is highly dependent on the absolute configuration at the phosphorus center. A full appreciation of the stereochemical constraints within the active site of PTE is critical for a greater understanding of the hydrophobic and electrostatic interactions between the protein and substrate. The stereochemical selectivity of this enzyme has now been probed with enantiomeric pairs of isomers that differ in their capacity for hydrophobic and steric interactions within the enzyme active site. Paraoxon analogues in which one or both of the two ethoxy substituents were replaced with various combinations of methyl, isopropyl, or phenyl groups were utilized as potential substrates for PTE. These compounds were tested as reporters of the stereochemical determinants governing the substrate specificity of PTE and the results compared with the stereoselective inhibition of human recombinant AChE. These investigations have unveiled evidence for steric and chiral constraints within the binding site that govern phosphotriester reactivity. These results have proven to be of significant value in the redesign of the PTE active site in an effort to alter and enhance the substrate specificity of the native enzyme.

MATERIALS AND METHODS

Materials. Dimethyl *p*-nitrophenyl phosphate (**I**) and paraoxon (diethyl *p*-nitrophenyl phosphate) (**II**) were purchased from Chem Service and Sigma, respectively. Acetylthiocholine chloride and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were products of Sigma. Unless otherwise stated, all other chemicals were obtained from Aldrich. Solvents used for the synthesis of phosphotriesters were freshly prepared according to the procedures of Perrin and Armarego (*12*). Diisopropyl (**III**), diphenyl (**IV**), and the racemic phosphotriesters (**V**−**VIII**) in which *p*-nitrophenol is the leaving group were synthesized from *p*-nitrophenyl phosphorodichloridate and the corresponding alcohols by the general method of Steurbaut et al. (*13*). The structures of all compounds were confirmed by ^{1}H and ^{13}C NMR spectroscopy. The mass spectral data were consistent with the expected molecular weights of all compounds. The wild-type phosphotriesterase was purified and reconstituted with Zn^{2+}, Co^{2+}, and Cd^{2+} as described previously (*5*). Human recombinant acetylcholinesterase (AChE) was obtained from Sigma and used without further purification.

Methods. ^{1}H and ^{13}C NMR spectra were obtained on a Varian XL-200E spectrometer. ^{31}P NMR spectra were obtained on a Varian Unity 300 spectrometer. Specific rotations were measured using a Jasco DIP-360 digital polarimeter. Reaction rates were determined spectrophotometrically at 25 °C with a Gilford model 260 spectrophotometer.

Chemical Hydrolysis of Phosphotriesters. The rates of chemical hydrolysis for the prochiral and racemic phosphotriesters **I**−**VIII** (50 μM) were determined at 25 °C by monitoring the release of 4-nitrophenolate at 400 nm upon addition of KOH to a final concentration of 1.0 M. Owing to the low solubility of compounds, **IV**, **VII**, and **VIII** in water, samples of the phosphotriesters were prepared in a final concentration of 20% methanol in water for **IV** and 5% for **VII** and **VIII**. The pseudo-first-order rate constants were obtained by a fit of the data to eq 1 where A is the concentration of the substrate, k is the pseudo-first-order rate constant, and t is time.

$$A = A_o e^{-kt} \quad (1)$$

Enzymatic Hydrolysis of Phosphotriesters. The kinetic parameters, k_{cat}/K_m and k_{cat}, for PTE were determined for the prochiral, chiral, and racemic phosphotriesters. The assays were conducted in 100 mM CHES (pH 9.0) at 25 °C, and the reaction rates were determined by monitoring the release of 4-nitrophenolate at 400 nm ($\epsilon = 1.7 \times 10^4$ M^{-1} cm^{-1}). The same ratios of methanol/water mixtures were used for the preparation of samples of **IV**, **VII,** and **VIII** as described above. The kinetic constants were determined from a fit of the data to eq 2, where v is the initial velocity, k_{cat} is the turnover number, K_m is the Michaelis constant, and A is the substrate concentration. The standard errors were all less than 20% of the reported values.

$$v/E_t = k_{cat} A/(K_m + A) \quad (2)$$

*Synthesis of Optically Active Phosphotriesters **V**−**VII***. The two enantiomers of phosphotriester **V** (ethyl methyl *p*-nitrophenyl phosphate) were synthesized according to a modification of the method of Koizumi et al. (*14*). A solution of L-proline methyl ester (1.5 g, 11.6 mol) in 10 mL of dry pyridine was added dropwise to a stirred solution of phenyl phosphodichloridate (1.5 g, 7.11 mol) in 30 mL of dry pyridine at 0 °C under N_2. After stirring at room temperature for 4 h, an excess of anhydrous ethanol was added dropwise and the stirring continued overnight. The mixture was extracted with ether, and the combined ethereal phases were washed with H_2O, dried over magnesium sulfate, filtered, and concentrated to give a pale yellow viscous oil (2.8 g). The two diastereomers were separated by flash column chromatography on silica gel using benzene-ethyl acetate (80:20) as the eluent, and each diastereomer of the ethyl phenyl phosphoramidate was isolated. Each diastereomer (500 mg, 1.43 mmol) was refluxed with 18 mL of 1 M H_2SO_4-methanol with stirring for 4 h. The reaction mixture was diluted with H_2O and extracted with ether. The combined organic layers were washed with 0.1 N HCl, H_2O, and dilute $NaHCO_3$, dried over $MgSO_4$, filtered, and then concentrated. Purification of the crude product by flash column chromatography using 30% ethyl acetate in hexanes as the eluent afforded 222 mg (72%) and 252 mg (82%) of optically active (+)- and (−)-ethyl methyl phenyl phosphate, respectively.

Each enantiomer (215 mg, 1.0 mmol) was treated with 0.2 mL of concentrated sulfuric acid at 0 °C, and then, ammonium nitrate (119 mg, 1.5 mmol) dissolved in 0.2 mL of concentrated sulfuric acid was added dropwise. After 20 min, the reaction was quenched and extracted with ether. The combined organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated. Purification by flash column chromatography using 40% EtOAc in hexanes as the eluent afforded 211 mg (81%) and 156 mg (60%) of optically active (+)- and (−)-ethyl methyl *p*-nitrophenyl phosphate **V**. Nitration of the phenoxy ligand of (+)- and (−)-ethyl methyl phenyl phosphate yielded the (+)- and (−)-isomers of **V**, respectively. The specific optical rotations, $[\alpha]_D^{25}$, for the two enantiomers of **V** were found to be +4.1 (*c* 5.6) and −4.0 (*c* 5.5). The individual isomers of compounds **VI** and **VII** (methyl isopropyl *p*-nitrophenyl phosphate and ethyl phenyl *p*-nitrophenyl phosphate) were prepared from *p*-nitrophenyl phosphodichloridate using a modification of the method described above. The specific optical rotations for the two enantiomers of **VI** and **VII** were determined to be +5.9 (*c* 5.0), −5.5(*c* 4.0) and +3.0 (*c* 6.3), −3.2 (*c* 4.5), respectively.

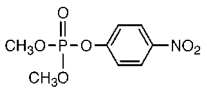
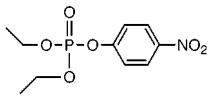
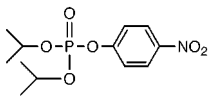
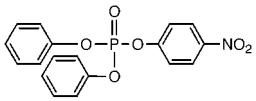
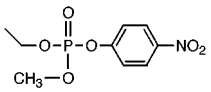
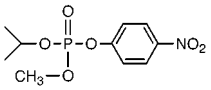
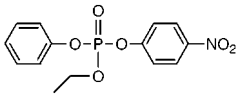
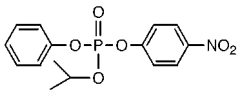
*Enzymatic Preparation of Optically Active Phosphotriester **VIII***. To obtain the (+)-enantiomer of compound **VIII** by means of stereospecific hydrolysis of one isomer of the racemate, 6.1 mM racemic **VIII** (782 mg) in 50 mM CHES (pH 9.0) containing 20% MeOH was incubated with PTE. The solution was stirred at room temperature, and the reaction was monitored spectrophotometrically. When the reaction was half-complete, the solution was acidified to pH <2 with 6.0 N HCl. The acidic solution was extracted with chloroform three times, and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The resulting crude product was further purified by flash column chromatography using 40% ethyl acetate and hexane to afford 256 mg (66%) of (+)-**VIII**. The specific optical rotation was determined to be +2.5 (*c* 6.5).

Correlation of Absolute Stereochemistry. To assign the stereochemistry at phosphorus in the phosphotriesters **V**−**VIII**, chiral phosphotriesters whose absolute configurations are known were synthesized according to the procedure of Nakayama and Thompson (*15*). The representative procedure for the synthesis of chiral ethyl methyl phenyl phosphate via displacement by sodium methoxide of (+)-**VII** is as follows: to a stirred solution of (+)-**VII** (54 mg, 0.167 mmol) was slowly added sodium methoxide (1 equiv mol) in 2 mL of dry THF under N_2. After the reaction mixture was stirred for 3 h, it was poured into saturated $NaHCO_3$, extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated. Purification of the crude product by flash column chromatography using 30% ethyl acetate in hexanes gave 13 mg (36%) of (−)-ethyl methyl phenyl phosphate [$[\alpha]_D$ −2.2 (*c* 2.5)]. A solution of (+)-**VIII** in THF was treated with sodium methoxide to give (−)-isopropyl methyl phenyl phosphate. This product was subsequently nitrated with ammonium nitrate in sulfuric acid to afford (−)-**VI**.

Determination of the Rate Constant of Acetylcholinesterase Inactivation. Human recombinant acetylcholinesterase (5 units) was incubated at 25 °C in a 1.0 mL solution containing 0.1 M phosphate buffer, pH 7.0, and 1 mg/mL BSA in the presence of 500 nM phosphotriester. After various times of incubation, a 20 μL aliquot was assayed with 1.0 mM acetylthiocholine and 0.3 mM DTNB in 0.1 M phosphate buffer, pH 7.0, at 25 °C according to Ellman et al. (*16*). The pseudo-first-order rate constants were obtained by a fit of the data to eq 1.

*Inactivation Constants for Inactivation of AChE by Enantiomers of **VI***. Human recombinant acetylcholinesterase (5 units) was incubated at 25 °C in a solution containing 0.1 M phosphate buffer, pH 7.0, with 1 mg/mL BSA and various concentrations of (+)- and (−)-**VI**. Aliquots were removed at various time intervals and assayed for residual enzyme activity as described above.

Table 1: Chemical Hydrolysis of Phosphotriesters by 1.0 N KOH

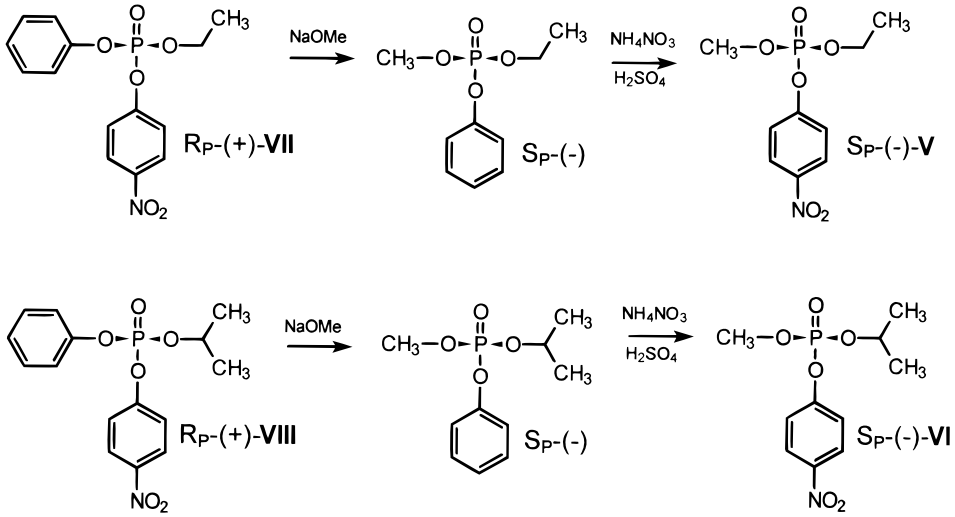
	compd	k_{obs} (min^{-1})
I		2.2
II		0.69
III		0.07
IV		35[a]
(±)-**V**		1.4
(±)-**VI**		0.47
(±)-**VII**		4.3[b]
(±)-**VIII**		1.5[b]

[a] Determined in 20% MeOH solution. [b] Determined in 5% MeOH solution.

RESULTS

Chemical Hydrolysis of Paraoxon Analogues. All of the paraoxon analogues synthesized for this investigation were hydrolyzed with KOH in order to determine the inherent chemical stability of these compounds with a small nucleophile such as hydroxide. The pseudo-first-order rate constants for the hydrolysis of these compounds with 1.0 M KOH are listed in Table 1. The rate constants were found to decrease with an increase in the alkyl chain length and degree of branching for either of the two substituents attached to the phosphorus core. The substitution of one or more phenyl substituents resulted in an increase in rate. The negative steric effect of the more bulky phenyl substituent is apparently offset by an inductive enhancement at the phosphorus center. The relative reaction rates for the eight compounds listed in Table 1 can be predicted quite closely by taking the product

Scheme 2



of the individual substituent effects. The trend for these effects is phenyl > methyl > ethyl > *i*-propyl with relative substituent constants of 20:5:3:1, respectively.

Assignment of Absolute Configuration. Many of the compounds prepared for this investigation have never been isolated in optically pure form. Assignment of the relative and absolute stereochemistry of these organophosphate triesters was required in order to correlate the relative reaction rates of individual enantiomers with the detailed three-dimensional structure of the enzyme active site. Hall and Inch (*17*) have shown that the absolute stereochemistry of (−)-ethyl methyl phenyl phosphate is of the (S_P)-configuration. When the para position of the phenoxy group of S_P-(−)-ethyl methyl phenyl phosphate [$[\alpha]_D$ −2.9 (*c* 3.6)] is nitrated, the product is compound **V** [$[\alpha]_D$ −4.0 (*c* 5.5)]. Since the nitration of this compound does not perturb the chirality at the phosphorus center, then the product of this reaction must also be of the (S_P)-configuration. Treatment of (+)-**VII** with sodium methoxide gave (−)-ethyl methyl phenyl phosphate which has the S_p configuration at the phosphorus center (*17*). Since the methoxide displacement of the *p*-nitrophenoxy group from (+)-ethyl phenyl *p*-nitrophenyl phosphate (**VII**) proceeds with net inversion of configuration at phosphorus (*15*), then (+)-**VII** must be of the (R_P)-configuration. Similarly, (+)-isopropyl phenyl *p*-nitrophenyl phosphate (**VIII**) was converted into (−)-isopropyl methyl phenyl phosphate with $[\alpha]_D$ −3.2 (*c* 4.2) upon treatment with methoxide to displace the *p*-nitrophenolate. Nitration of (−)-isopropyl methyl phenyl phosphate provided (−)-isopropyl methyl *p*-nitrophenyl phosphate (**VI**) with $[\alpha]_D$ −5.8 (*c* 0.9). The *p*-nitrophenolate of (+)-**VIII** is displaced by methoxide with inversion of configuration, and the nitration of the resulting product occurs without alteration of stereochemistry at the phosphorus center to produce (−)-**VI**. These results demonstrate that (+)-isopropyl phenyl *p*-nitrophenyl phosphate (**VIII**) has the same relative stereochemistry at the phosphorus center as (+)-isopropyl methyl *p*-nitrophenyl phosphate (**VI**). These relationships are presented graphically in Scheme 2.

Enzymatic Hydrolysis of Racemic Phosphotriesters. Figure 1 illustrates the time course for the hydrolysis of a racemic mixture of isopropyl methyl *p*-nitrophenyl phosphate (**VI**). This figure clearly shows that one of the two enantiomers

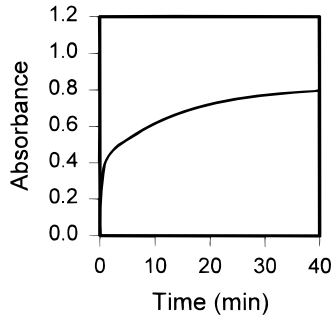


FIGURE 1: Time course for the enzymatic hydrolysis of 50 μM of racemic isopropyl methyl *p*-nitrophenyl phosphate (**VI**) at pH 9.0 and 25 °C. The reaction was monitored at 400 nm. The ratio of first-order rate constants for the fast and slow isomer of **VI** is 43. The total absorbance change for the fast phase is 0.41 while the change in absorbance for the slow phase is 0.43.

in the racemic mixture is hydrolyzed significantly faster than the other enantiomer. Measurement of the specific rotation of the material after ∼50% of the original phosphotriester had been consumed indicated that $[\alpha]_D = +6.0$. This result demonstrates that phosphotriesterase favors the hydrolysis of (−)-**VI**. At a concentration of 50 μM the (−)-**VI** isomer is hydrolyzed 43-fold faster than is the (+)-**VI** isomer. The substrate specificity and reactivity of PTE have therefore been addressed using both enantiomers of various dialkyl and alkyl aryl phosphotriesters.

Hydrolysis of Paraoxon Analogues by Phosphotriesterase. The substrate and stereochemical specificity of the bacterial phosphotriesterase was determined with a modest library of organophosphate triesters containing *p*-nitrophenol as the leaving group. The values of K_m, k_{cat}, and k_{cat}/K_m for the hydrolysis of prochiral, chiral and racemic versions of the organophosphate triesters **I**−**VIII** are listed in Table 2 for PTE substituted with Zn^{2+}, Co^{2+}, or Cd^{2+}. The enzyme is sensitive to the size and polarity of the two substituents attached to the phosphorus center. In every case, the S_P-isomer is hydrolyzed significantly faster than is the R_P-isomer.

Inactivation of AChE by Organophosphate Triesters. Achiral and chiral enantiomers of the organophosphate triesters were used to inactivate the recombinant human acetylcholinesterase. The pseudo-first-order rate constants for

Table 2: Kinetic Constants Determined for the Hydrolysis of Prochiral, Chiral, and Racemic Phosphotriesters Catalyzed by Metal-Substituted Phosphotriesterase

	Zn−PTE			Co−PTE			Cd−PTE		
substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
I	1050	7500	7.2×10^6	1450	18 200	1.2×10^7	7500	7900	1.1×10^6
II	35	2280	6.2×10^7	110	7080	6.4×10^7	440	5800	1.3×10^7
III	47	170	3.5×10^6	46	220	4.8×10^6	510	460	9.1×10^5
IV[a]	230	3700	1.6×10^7	210	3370	1.6×10^7	110	120	1.1×10^6
R_P-(+)-**V**	160	4000	2.5×10^7	430	13 800	3.2×10^7	1620	6700	4.2×10^6
S_P-(−)-**V**	160	5800	3.7×10^7	420	14 500	3.4×10^7	690	4100	5.9×10^6
(±)-**V**	180	4900	2.7×10^7	530	14 000	2.7×10^7	1100	5400	4.8×10^6
R_P-(+)-**VI**	490	180	3.6×10^5	590	500	8.5×10^5	2800	430	1.5×10^5
S_P-(−)-**VI**	43	2000	4.7×10^7	260	7120	2.7×10^7	630	3900	6.2×10^6
(±)**VI**	150	2000	1.4×10^7	460	5090	1.1×10^7	700	1670	2.4×10^6
R_P-(+)-**VII**[b]	170	310	1.8×10^6	270	1010	3.7×10^6	640	320	5.1×10^5
S_P-(−)-**VII**[b]	18	3100	1.8×10^8	130	9530	7.6×10^7	150	3600	2.4×10^7
(±)-**VII**[b]	34	2700	8.0×10^7	210	10 600	5.1×10^7	510	3200	6.2×10^6
R_P-(+)-**VIII**[b]	110	100	9.5×10^5	120	230	1.9×10^6	650	270	4.1×10^5
(±)-**VIII**[b]	15	820	5.5×10^7	22	970	4.4×10^7	550	2200	4.1×10^6

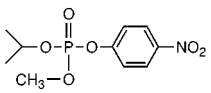
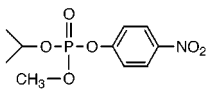
[a] Determined in 20% MeOH solution. [b] Determined in 5% MeOH solution.

Table 3: Inactivation of Recombinant Human Acetylcholinesterase by Phosphotriesters[a]

compd	k (min^{-1})
I	0.15
II	0.80
III	0.087
IV	0.19
R_P-(+)-**V**	1.6
S_P-(−)-**V**	0.12
R_P-(+)-**VI**	0.39
S_P-(−)-**VI**	0.10
R_P-(+)-**VII**	0.44
S_P-(−)-**VII**	0.16
R_P-(+)-**VIII**	0.16

[a] pH 7.0 and 25 °C using 500 nM of inactivator.

Table 4: Inactivation of Human AChE by Isopropyl Methyl *p*-Nitrophenyl Phosphate

	compd	k_{inact} (min^{-1})	K_I (μM)
R_P-(+)-**VI**		5.3 ± 0.6	5.5 ± 1.0
S_P-(−)-**VI**		13.3 ± 2.8	108 ± 33

inactivation of AChE for the organophosphate triesters **I**−**VIII** at an inhibitor concentration of 500 nM are listed in Table 3. In every case, the R_P-isomers are more potent inactivators of AChE than are the S_P-isomers. The two isomers of compound **VI** were also used to inactivate the human AChE. The concentration of both isomers was varied to obtain the maximum rate constant for inactivation (k_{inact}) and the concentration of inactivator that gives 50% of this rate (K_I). The R_P-isomer is a significantly better inactivator of the human AChE than the S_P-isomer. The results are summarized in Table 4.

DISCUSSION

Substrate Specificity. The bacterial phosphotriesterase is able to accept a wide range of substituents attached to the central phosphorus core. For the prochiral set of substrates (**I**−**IV**) substitutions have successfully been made with methyl, ethyl, isopropyl, and phenyl groups. For all of the metal-substituted versions of PTE tested (Zn^{2+}, Co^{2+}, and Cd^{2+}), the k_{cat} values decrease in the series methyl > ethyl > isopropyl. However, the relative positioning of the phenyl substituent in this series is quite variable and dependent on the specific metal-substituted form of PTE that is utilized. For example, the k_{cat} for substrate **IV** with Cd−PTE is 50-fold *lower* than with paraoxon (**II**), but 1.6-fold *greater* than found for paraoxon with the Zn-substituted enzyme. It would thus appear that the specific metal ion bound to the active site of PTE can significantly influence the substrate specificity. The k_{cat} values for the racemic substrates (**V**−**VIII**) with unmatched pairs of substituents are intermediate in magnitude when compared with the values exhibited by the prochiral substrates. The K_m values for the dimethyl substituted analogue (**I**) are significantly higher than for any of the other substrate analogues tested. This observation suggests that a significant fraction of the binding interactions for a substrate such as paraoxon are contributed through the two ethyl groups. The diminished binding affinity of the methyl group is probably correlated with the elevated levels of k_{cat} observed with the dimethyl substituted substrate (**I**).

Stereochemical Specificity. For the racemic pairs of substrates (**V**−**VIII**), one enantiomer is invariably a better substrate than the other enantiomer. For example, the k_{cat} and K_m values for the S_P-isomer of ethyl phenyl *p*-nitrophenyl phosphate (**VII**) with the Zn-substituted PTE are each an order of magnitude more favorable than those exhibited by the R_P-isomer. Thus, the k_{cat}/K_m value is 100-fold greater for the S_P-isomer than for the R_P-isomer. Similar differences are also observed for the two isomers of **VI** and **VIII,** while much smaller differences in reactivity are apparent when comparing the methyl and ethyl substituents of compound **V**.

The absolute stereochemistry of the preferred isomer of **VII** is of the S_P configuration, and the moderately preferred substrate for **V** is also of the S_P configuration. In each case the larger of the two substituents is positioned in the *pro-S* position (using paraoxon as a model). This stereochemical preference is also reflected with the single isomer of the insecticide EPN that is hydrolyzed by the bacterial phos-

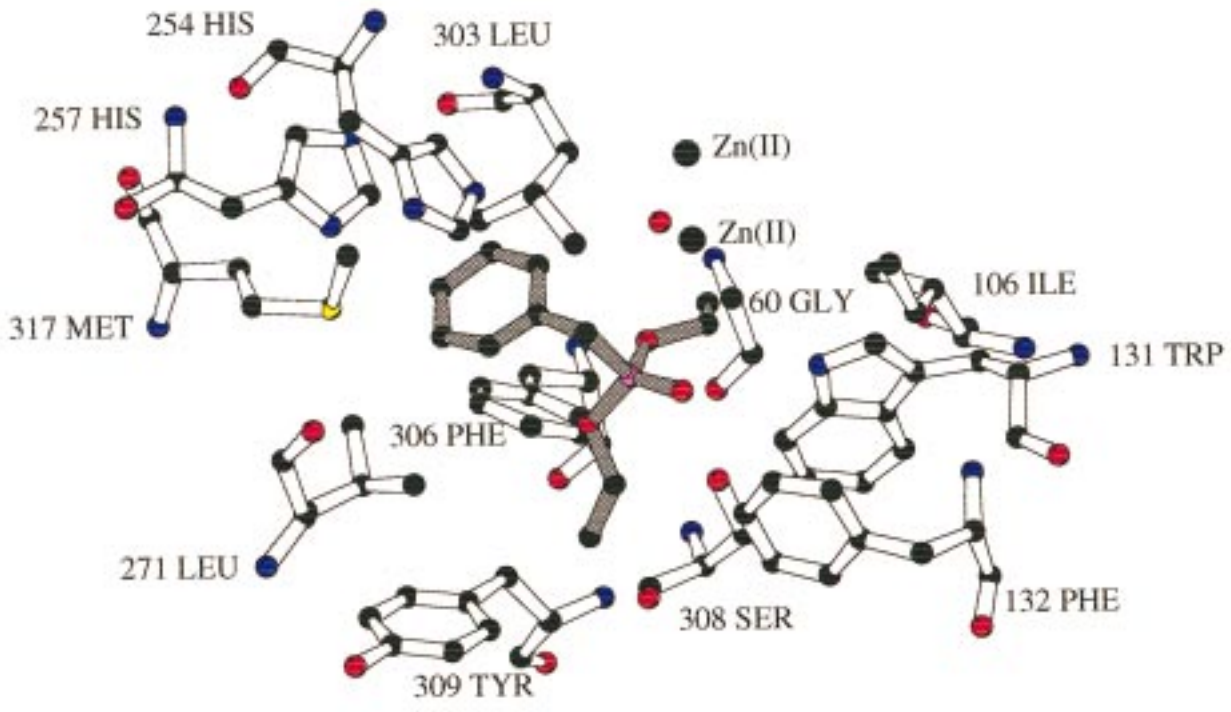
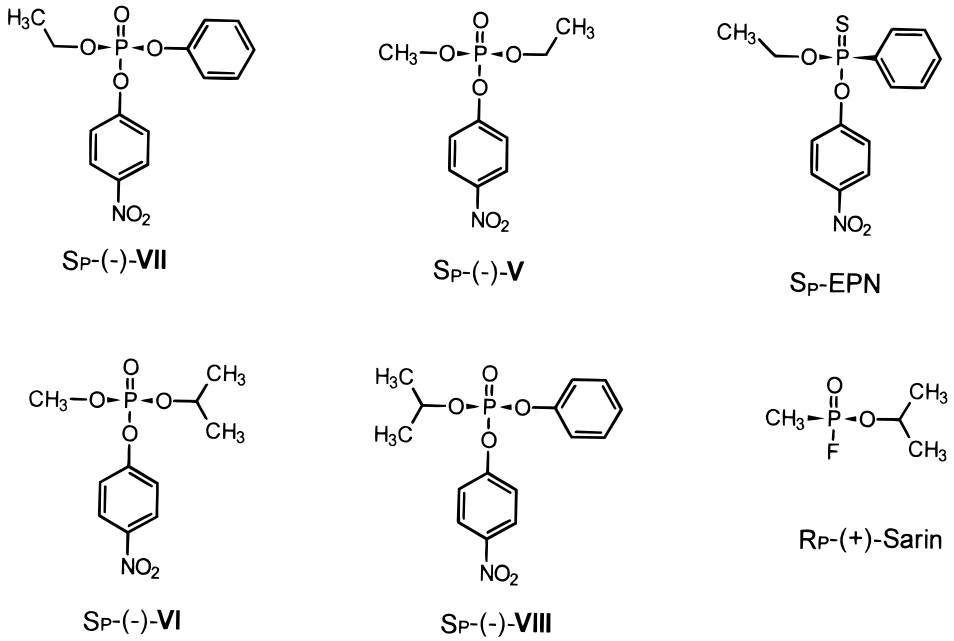


FIGURE 2: Representation of the active site of phosphotriesterase in the presence of a bound substrate analogue (*6*). For clarity the residues interacting directly with either of the two divalent cations have been omitted.

Scheme 3



photriesterase. The only isomer of EPN to be hydrolyzed is of the S_P-configuration. When the *p*-nitrophenol leaving group and phosphoryl sulfur of this isomer are orientated in the same way as they are found in S_P-(**VII**), the phenyl and ethoxy substituents of EPN are positioned in the same relative orientation as the phenoxy and ethoxy substituents in S_P-(**VII**). The relative orientations are visualized in Scheme 3. It has also been demonstrated that the preferred isomer of compound **VI** has the same relative configuration as the preferred isomer of **VIII**. Given the demonstrated preference of PTE for the larger and bulkier substituent to occupy the *pro-S* position, it can be safely concluded that (−)-(**VI**) and (−)-(**VIII**) must be of the S_P-configuration as presented in Scheme 3.

Substrate-Binding Environment. The subsites for the binding of substrates to the active site of PTE are highly hydrophobic. In the crystal structure of PTE with a bound substrate analogue, three distinct hydrophobic subsites of PTE have been shown to contribute to the binding of the groups attached to the phosphorus of the substrate analogue (*6*). The methylbenzyl group of the inhibitor is embedded in a hydrophobic pocket that consists of His-257, Leu-271, Phe-306, and Met-317. One of the prochiral ethoxy groups is positioned within the pocket defined by Trp-131, Ile-106,

Leu-303, Phe-306, and Ser-308. The other prochiral ethoxy substituent binds to a region that consists of Trp-131, Phe-132, Leu-271, Phe-306, and Tyr-309. This structure has shown that the substrate analogue for PTE is bound at the active site in close proximity to the binuclear metal center (*18*). The phosphoryl oxygen atom of the substrate analogue is 3.5 Å from the more solvent-exposed zinc ion and in a position that would accept attack from the hydroxide bound to the more buried metal ion. The phosphonate analogue used in the crystallographic study is nonhydrolyzable, and the *pro-S* ethoxy group of the inhibitor appears to occupy the position expected for the leaving group of a true substrate. A representation of the active site with the bound substrate analogue is presented in Figure 2.

The observed stereoselectivity displayed by PTE for the chiral enantiomers of compounds **V**−**VIII** must reflect the size of the subsites that can accommodate the binding of the substituents liganded to the central phosphorus atom. This view dictates that the subsite, roughly defined by residues His-257, Leu-271, Phe-306, and Met-317, is better able to accept the large and bulkier substituents than the subsite defined by the side chains of Trp-131, Ile-106, Leu-303, Phe-306, and Ser-308. Since electrostatic interactions are apparently of minor importance, then it seems reasonable to conclude that the physical size of the hydrophobic cavities dictates the substrate and stereochemical preferences of PTE. It is likely that these specificities can be systematically altered by judicious changes in the amino acid side chains that define these subsites. In fact, in preliminary experiments, we have found that mutation of a glycine residue found within the subsite that prefers the smaller ligands to an alanine residue increases the selectivity for the preferred isomer by another 2 orders of magnitude. We interpret these results to be consistent with a reduction in the size of this subsite that enforces an even greater preference for the small ligand. Further experiments in this area are in progress.

Inactivation of AChE with Enantiomeric Phosphotriesters. The present study indicates that the (R_P)-(+)-enantiomers of the series **V**−**VIII** of phosphotriesters are more potent inactivators of AChE than are the (S_P)-(−)-enantiomers. These results show that enantiomeric preference of PTE and AChE for chiral phosphotriesters are *opposite*. Thus, PTE is a more efficient catalyst with the least toxic enantiomer of these organophosphate nerve agents. It has previously been demonstrated that (S_p)-(−)-sarin inactivates AChE at rates that are 3 orders of magnitude faster than (R_P)-(+)-sarin (*8*). The absolute configuration of (S_p)-(−)-sarin can be correlated with the absolute configuration of (R_P)-(+)-**VI** if the two leaving groups (fluoride and *p*-nitrophenol) are overlaid (see Scheme 3). If this is accepted, PTE is expected to hydrolyze (R_P)-(+)-sarin faster than (S_p)-(−)-sarin. A similar comparison can be made with the inactivation of AChE with the individual enantiomers of isopropyl methyl phosphonyl thiocholine. With the native mouse enzyme, the second-order rate constant for inactivation of AChE by the S_P-isomer of this compound is 2 orders of magnitude larger than that found for the R_P-isomer (*19*). The absolute configuration of (S_P)-isopropyl methyl phosphonyl thiocholine correlates with the absolute configuration of (R_P)-(+)-**VI** if the two leaving groups (thiocholine and *p*-nitrophenol) are overlaid.

These studies provide valuable information on the intricate relationship between structure and function of PTE as well as factors that contribute to the determination of stereochemical constraints on substrate specificity. This study may have practical applications in the redesign of mutants with improved activity toward organophosphorus pesticides and nerve gases.

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