



Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2083–2091

BIOORGANIC &
MEDICINAL
CHEMISTRY

Stereoselective Detoxification of Chiral Sarin and Soman Analogues by Phosphotriesterase

Wen-Shan Li, Karin T. Lum, Misty Chen-Goodspeed,
Miguel A. Sogorb and Frank M. Raushel*

Department of Chemistry, Texas A&M University, PO Box 30012, College Station, TX 77842-3012, USA

Received 10 January 2001; accepted 27 March 2001

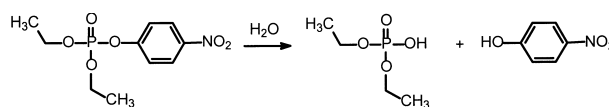
Abstract—The catalytic activity of the bacterial phosphotriesterase (PTE) toward a series of chiral analogues of the chemical warfare agents sarin and soman was measured. Chemical procedures were developed for the chiral syntheses of the S_P - and R_P -enantiomers of *O*-isopropyl *p*-nitrophenyl methylphosphonate (sarin analogue) in high enantiomeric excess. The R_P -enantiomer of the sarin analogue ($k_{\text{cat}} = 2600 \text{ s}^{-1}$) was the preferred substrate for the wild-type PTE relative to the corresponding S_P -enantiomer ($k_{\text{cat}} = 290 \text{ s}^{-1}$). The observed stereoselectivity was reversed using the PTE mutant, I106A/F132A/H254Y where the k_{cat} values for the R_P - and S_P -enantiomers were 410 and 4200 s^{-1} , respectively. A chemo-enzymatic procedure was developed for the chiral synthesis of the four stereoisomers of *O*-pinacolyl *p*-nitrophenyl methylphosphonate (soman analogue) with high diastereomeric excess. The $R_P R_C$ -stereoisomer of the soman analogue was the preferred substrate for PTE. The k_{cat} values for the soman analogues were measured as follows: $R_P R_C$, 48 s^{-1} ; $R_P S_C$, 4.8 s^{-1} ; $S_P R_C$, 0.3 s^{-1} , and $S_P S_C$, 0.04 s^{-1} . With the I106A/F132A/H254Y mutant of PTE the stereoselectivity toward the chiral phosphorus center was reversed. With the triple mutant the k_{cat} values for the soman analogues were found to be as follows: $R_P R_C$, 0.3 s^{-1} ; $R_P S_C$, 0.3 s^{-1} ; $S_P R_C$, 11 s^{-1} , and $S_P S_C$, 2.1 s^{-1} . Prior investigations have demonstrated that the S_P -enantiomers of sarin and soman are significantly more toxic than the R_P -enantiomers. This investigation has demonstrated that mutants of the wild-type PTE can be readily constructed with enhanced catalytic activities toward the most toxic stereoisomers of sarin and soman. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Activated organophosphates triesters and organophosphonate diesters are very toxic materials because of their inherent ability to inhibit the enzyme acetylcholinesterase (AChE). This property has been exploited over the last half century through the development of numerous commercial insecticides for agricultural and household uses. For example, in the USA today, there are approximately 40 organophosphate insecticides registered for use by the Environmental Protection Agency. Each year approximately 100 million pounds of insecticides, including acephate, malathion, and chlorpyrifos are applied to the environment for the control of a variety of insects and other pests.¹ The organophosphate insecticides inactivate AChE through the irreversible phosphorylation of an active site serine residue. The persistence of organophosphate insecticides in the soil is significantly diminished through microbial degradation. One of the primary

bacterial detoxification mechanisms for organophosphate esters is enzymatic hydrolysis.

The phosphotriesterase (PTE) from *Pseudomonas diminuta* is the best-characterized enzyme for the hydrolytic detoxification of organophosphate nerve agents.² This enzyme is a zinc metalloprotein, which catalyzes the hydrolysis of a wide variety of organophosphates and related phosphonates with a high catalytic turnover and broad substrate specificity.³ For example, the k_{cat} and k_{cat}/K_m values for the insecticide paraoxon are approximately 10^4 s^{-1} and $10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively.⁴ The hydrolysis of paraoxon is illustrated in Scheme 1. The X-ray crystal structure of PTE, determined by the Holden laboratory, has shown that the protein folds in a 'TIM-barrel' motif that is embedded with a binuclear zinc center at the active site.^{5,6} The zinc metallo-center of PTE is homologous to the binuclear



Scheme 1.

*Corresponding author. Tel.: +1-979-845-3373; fax: +1-979-845-9452; e-mail: raushel@tamu.edu

Ni^{2+} -center found in urease.⁷ These enzymes are members of the amidohydrolase superfamily.⁸

It has previously been demonstrated that the bacterial PTE will hydrolyze, and thus detoxify, the military organophosphonate nerve agents sarin (**1**) and soman (**2**), although the turnover numbers for these compounds do not rival the rate constants for the enzymatic hydrolysis of insecticides such as paraoxon.^{9,10} Nevertheless, the catalytic properties of the wild-type PTE make it the leading candidate for the enzymatic detoxification of organophosphate nerve agents under a variety of field conditions. However, of particular concern is the differential toxicity for the stereoisomers of the nerve agents, sarin and soman. Sarin has a single chiral center at phosphorus while soman possesses an additional center of asymmetry within the pinacolyl substituent. The structures of these stereoisomers are illustrated in Scheme 2. It has been determined that the S_P -enantiomer of sarin (**1**) inactivates AChE $\sim 10^4$ times faster than the R_P -enantiomer.¹¹ For soman (**2**), the two S_P -diastereomers inactivate AChE $\sim 10^5$ times faster than the two R_P -diastereomers.¹²

The wild-type PTE is moderately stereoselective for the hydrolysis of chiral organophosphate triesters. All possible combinations of the substituents methyl, ethyl, isopropyl, and phenyl have been synthesized and characterized as substrates for PTE using the template depicted in Scheme 3.¹³ In every case the preferred stereoisomer is the one where the substituent 'Y' is bulkier than the substituent 'X'. The ratios of the experimental k_{cat}/K_m values for the S_P - and R_P -enantiomers in this series of stereoisomers ranged from 1 to 90.¹³ These results have been shown to be consistent with the physical dimensions of the subsites within the active site of PTE.

In this paper, we have examined the stereoselective properties of the wild-type PTE toward chiral analogues of sarin and soman. Since the individual stereoisomers of these nerve agents are not readily available, we have developed novel chemo-enzymatic syntheses of chiral analogues where the leaving group fluoride has been replaced with *p*-nitrophenol. This substitution provides for a convenient optical signal of the cleavage event. For the sarin analogue the relative rates of hydrolysis for the two enantiomers varied by an order of magnitude when the wild-type PTE was used as a catalyst. With the soman analogue, the relative rates of hydrolysis differed by nearly four orders of magnitude. A small number of mutations within the active site of PTE were

able to reverse the stereoselectivity exhibited by the wild-type enzyme.

Results

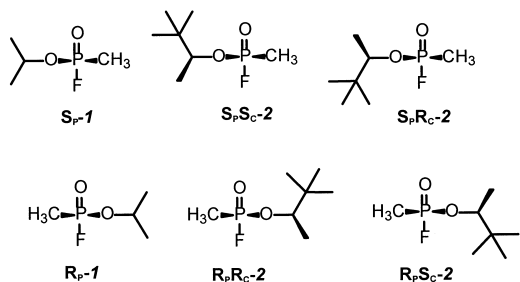
Preparation of the chiral sarin analogues

The two enantiomers of the sarin analogue (**8**) were prepared by an entirely chemical method and also through a kinetic resolution of a racemic mixture of both enantiomers using mutants of phosphotriesterase with modified catalytic properties. The key step in the chemical route was the fractional crystallization of the individual R_P - and S_P -isomers of intermediate **5** using the two enantiomers of 1-phenylethylamine.¹⁴ The absolute stereochemistry for each enantiomer of **5** was determined by X-ray crystallography using the known configuration of the chiral amine in the salt **6** as a point of reference. The chiral phosphonothioic acids were then converted via three chemical transformations of known stereochemical outcome to the sarin analogue targets. Kinetic assays with the wild-type PTE definitively established that the R_P -enantiomer was hydrolyzed about an order of magnitude faster than the S_P -enantiomer (see Table 1).

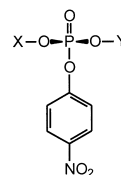
A library of active site mutants of PTE was surveyed in order to identify enzymes that were more stereoselective for the R_P -enantiomer than the wild-type enzyme. This same library was also probed for mutant enzymes that would prefer the S_P -enantiomer relative to the R_P -enantiomer. The G60A mutant was found to have an enhanced stereoselectivity for the R_P -isomer of the sarin analogue while the mutant I106A/F132A/H257Y possessed a preference for the S_P -isomer. The differing chiral preferences for these two mutants are graphically shown in Figure 1. In this experiment, G60A was able to hydrolyze only one stereoisomer at a significant rate of the racemic mixture while I106A/F132A/H257Y was able to hydrolyze the remaining stereoisomer. This discovery permitted the utilization of these two isomers in the kinetic resolution of either stereoisomer from the racemic mixture. When the G60A mutant was used as the hydrolytic catalyst, the S_P -isomer was isolated in high yield with a very low contamination by the R_P -isomer. The R_P -isomer was isolated in high yield and excellent enantiomeric excess using the mutant I106A/F132A/H257Y.

Kinetic analysis of chiral sarin analogues

The purified enantiomers of the sarin analogues, S_P -**8** and R_P -**8**, in addition to the racemic mixture, were tested



Scheme 2.



Scheme 3.

Table 1. Kinetic parameters for PTE and selected mutants with analogues of soman and sarin^a

Substrate	Wild type			G60A			I106A/F132A/H257Y		
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
<i>R</i> _P / <i>S</i> _P - 8	1800±15	0.44±0.01	(4.5±0.2)×10 ⁶	2300±110	0.28±0.03	(8.5±0.5)×10 ⁶	3700±370	5.1±0.6	(7.3±0.1)×10 ⁵
<i>R</i> _P - 8	2600±69	0.31±0.02	(8.2±0.1)×10 ⁶	2900±200	0.27±0.02	(1.1±0.1)×10 ⁷	410±17	4.6±0.4	(9.0±0.1)×10 ⁴
<i>S</i> _P - 8	290±2	0.72±0.01	(4.1±0.1)×10 ⁵	58±2	0.80±0.1	(7.2±0.2)×10 ⁴	4200±130	1.6±0.1	(2.7±0.1)×10 ⁶
<i>R</i> _P <i>R</i> _C / <i>R</i> _P <i>S</i> _C - 11	44±2	1.1±0.01	(4.0±0.1)×10 ⁴	93±13	1.0±0.3	(9.3±1)×10 ⁴	4.6±0.6	1.6±0.4	(2.9±0.2)×10 ³
<i>S</i> _P <i>R</i> _C / <i>S</i> _P <i>S</i> _C - 11									
<i>R</i> _P <i>R</i> _C / <i>S</i> _P <i>R</i> _C - 11	40±7	1.1±0.3	(3.6±0.5)×10 ⁴	92±13	0.75±0.23	(1.2±0.2)×10 ⁵	5.6±0.6	0.98±0.3	(5.6±1)×10 ³
<i>R</i> _P <i>S</i> _C / <i>S</i> _P <i>S</i> _C - 11	9.6±0.4	1.0±0.1	(9.6±0.4)×10 ³	16±1	0.29±0.06	(5.3±1)×10 ⁴	1.1±0.1	0.93±0.3	(1.2±0.3)×10 ³
<i>R</i> _P <i>R</i> _C - 11	48±2	0.34±0.07	(1.6±0.2)×10 ⁵	120±11	0.8±0.2	(1.5±0.2)×10 ⁵	0.3±0.03	1.1±0.3	(2.8±0.5)×10 ²
<i>R</i> _P <i>S</i> _C - 11	4.8±0.06	0.42±0.02	(1.2±0.4)×10 ⁴	33±3	0.6±0.1	(5.6±0.6)×10 ⁴	0.3±0.05	4.5±1.1	(5.6±0.4)×10 ¹
<i>S</i> _P <i>R</i> _C - 11	0.3±0.03	0.78±0.17	(3.8±0.5)×10 ²	0.07±0.01	10±2	(7.0±0.6)×10 ⁰	11±1	1.2±0.1	(9.2±2)×10 ³
<i>S</i> _P <i>S</i> _C - 11	0.04±0.01	2.4±0.5	(1.6±0.1)×10 ¹	0.02±0.001	2.8±0.4	(7.1±0.4)×10 ⁰	2.1±0.2	1.7±0.2	(1.2±0.1)×10 ³

^aThe enzyme activity was measured in 50 mM CHES, pH 9.0 for the sarin analogues (**8**), and 50 mM CHES, pH 9.0, 10% methanol for the soman analogues (**11**).

as substrates for the wild-type enzyme and the two mutant enzymes, G60A and I106A/F132A/H257Y. For the wild-type enzyme the k_{cat} for the *R*_P-isomer is ~9 times faster than the *S*_P-isomer while $k_{\text{cat}}/K_{\text{a}}$ is 20 times more favorable. For the mutant G60A, the chiral preference for the *R*_P-isomer of **8** is enhanced relative to the wild-type enzyme. Thus, the ratios of k_{cat} and $k_{\text{cat}}/K_{\text{a}}$ for G60A are 50- and 140-fold more favorable, respectively, for the *R*_P-stereoisomer. In contrast, the k_{cat} for the triple mutant is 10 times greater for the *S*_P-isomer than the *R*_P-isomer while $k_{\text{cat}}/K_{\text{a}}$ is 30-fold higher for the same isomer. The actual kinetic constants are presented in Table 1.

Chemoenzymatic preparation of chiral soman analogues

The preparation of the chiral soman analogues (**11**) began with the chemical resolution of the chiral pinacol alcohol (**10**). The utilization of these alcohols to displace one of the *p*-nitrophenolic groups from intermediate **9** enabled the construction of two sets of

diastereomers. These two pairs of diastereomers were of a single configuration at the chiral carbon center but racemic at the phosphorus center (*S*_P*R*_C/*R*_P*R*_C-**11** and *S*_P*S*_C/*R*_P*S*_C-**11**). For each pair of diastereomers, the wild-type PTE hydrolyzed one of the stereoisomers at a significantly faster rate than the other (data not shown). By analogy with the known stereoselectivity exhibited by the wild-type enzyme for the sarin analogues (see above) and six paraoxon triesters,¹³ the more rapidly hydrolyzed stereoisomer within each pair of diastereomers was concluded to be of the *R*_P-configuration. This conclusion was supported by the preference for the same isomer when the mutant G60A was tested with these two pairs of diastereomers.

The differential rates of hydrolysis exhibited by G60A and I106A/F132A/H257Y for the two stereoisomers contained within the two mixtures of diastereomers are graphically presented in Figures 2 and 3. It is assumed in both cases that G60A is preferentially hydrolyzing

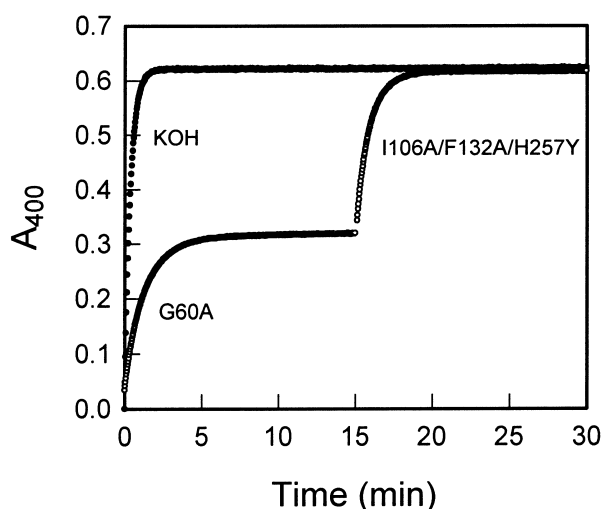


Figure 1. Time course for the hydrolysis of the racemic sarin analogue **8** using the mutant forms of PTE. The total concentration of the sarin analogue **8** (36 μM) was determined by the addition of KOH. The PTE mutant G60A was added at zero time to hydrolyze the *R*_P-enantiomer to completion. After 15 min, the I106A/F132A/H257Y mutant form of PTE was added to hydrolyze the *S*_P-enantiomer.

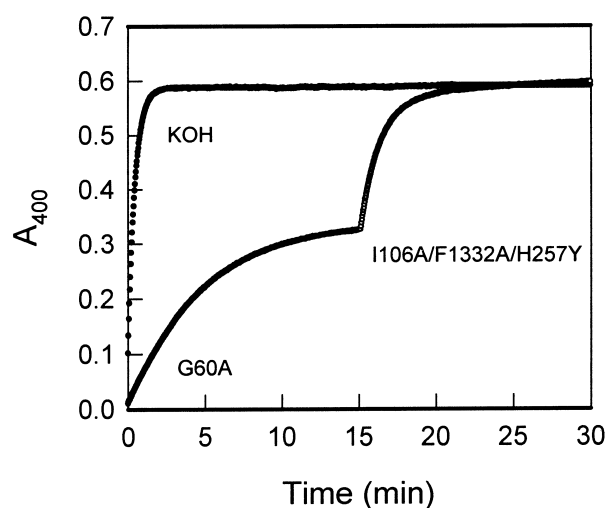


Figure 2. Time course for the hydrolysis of the diastereomeric mixture of the soman analogue *S*_P*R*_C-**11** and *R*_P*R*_C-**11** using the mutant forms of PTE. The total concentration of the soman analogue **11** (35 μM) was determined by the addition of KOH. The PTE mutant G60A was added at zero time to hydrolyze the *R*_P*R*_C-**11** enantiomer. After 15 min, the I106A/F132A/H257Y mutant form of PTE was added to hydrolyze the *S*_P*R*_C-**11** enantiomer.

the R_P -isomers while I106A/F132A/H257Y is preferentially hydrolyzing the S_P -isomers. These contrasting stereoselectivities made it technically feasible to use these two mutants as catalysts for the kinetic resolution of the two pairs of diastereomers into the four individual stereoisomers in high yield and excellent enantiomeric purity. The overall synthetic protocol is outlined in Scheme 5.

Kinetic analysis of chiral soman analogues

The four soman analogue stereoisomers were characterized as substrates for the wild-type PTE and the two mutant enzymes, G60A and I106A/F132A/H257Y. For the wild-type enzyme the catalytic selectivity as measured by k_{cat}/K_a is $R_P R_C : R_P S_C : S_P R_C : S_P S_C$ of 10,000:750:23:1. The same relative order of isomeric preferences was found for G60A where the relative rates were determined to be 21,000:8000:1:1. For the triple mutant, the relative stereoselectivity was found to be 5:1:164:21. The stereoselectivity of the G60A mutant for the R_P -isomer is more pronounced than the wild-type enzyme while the triple mutant is actually reversed but the rate differential between the fastest and slowest stereoisomers is diminished. The catalytic constants are presented in Table 1.

Discussion

Enzymatic hydrolysis of sarin analogues

The wild-type PTE and the selected mutants are able to catalyze the hydrolysis of both stereoisomers of the sarin analogue **8**. For the R_P -isomer, k_{cat} and k_{cat}/K_m with the wild type enzyme are 2600 s^{-1} and $8.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The magnitude of these values is approximately one third the size of the kinetic constants

obtained for the insecticide paraoxon. Thus, a substantial fraction of the inherent catalytic power within the native PTE is available for the hydrolysis of the R_P -isomer of the sarin analogue **8**. For the S_P -enantiomer of the sarin analogue **8**, the values of k_{cat} and k_{cat}/K_m (Table 1) are approximately an order of magnitude smaller. This stereoselectivity towards the R_P -enantiomer of **8** is consistent with the previously determined substrate specificity of PTE using a small library of organophosphate triesters.¹³ The catalytic preference for the hydrolysis of the sarin analogue R_P -**8** over S_P -**8** can also be rationalized structurally on the basis of the optimized size of the subsites for the binding of the *i*-propyl substituent relative to that for the methyl substituent of the two enantiomers.¹⁵

The wild-type PTE is able to hydrolyze the analogue of the most toxic stereoisomer of the nerve agent sarin. However, the rate of hydrolysis of this substrate is significantly slower than the turnover rate with the less toxic R_P -enantiomer of the sarin analogue. If PTE is going to achieve its full potential as an agent for the catalytic destruction of toxic nerve agents, then derivatives of this protein with altered kinetic properties must be constructed and more fully characterized. The catalytic properties of the triple mutant of PTE, I106A/F132A/H257Y, effectively demonstrate that structural variants of PTE can be readily constructed with significant enhancements to both k_{cat} and k_{cat}/K_m for the initially slower S_P -isomer of the sarin analogue **8**. This objective was met through a subtle alteration of the two binding subsites within the active site of PTE that dictate the catalytic and substrate specificity of this enzyme. Prior X-ray crystallographic analysis of PTE in the presence of a bound inhibitor by the Holden laboratory has localized those residues that dictate the structural dimensions of these binding subsites.¹⁵ In the example presented here, the *small* subsite was slightly enlarged through the mutation of Ile-106 and Phe-132 to alanine residues while the *large* subsite was diminished in size by the conversion of His-257 to a phenylalanine. These three changes *enhanced* the turnover rate of the initially slower S_P -isomer by approximately one order of magnitude and *reduced* the rate of turnover of the initially faster R_P -isomer by the same amount.

Enzymatic hydrolysis of soman analogues

All four analogues of the soman stereoisomers are substrates for the wild-type PTE. However, there is a rather large variance in the rate of substrate turnover that is very much dependent on the specific stereochemical configuration around the phosphorus center and the chiral pinacoyl substituent. The extreme values of k_{cat} differ by a factor of ~ 1000 while k_{cat}/K_m for the $R_P R_C$ -isomer of analogue **11** is four orders of magnitude greater than it is for the corresponding $S_P S_C$ -isomer. The two diastereomers with the R_P -configuration are better substrates than the two diastereomers with the S_P -configuration. Moreover, the R_C -isomer of the chiral pinacoyl substituent is preferred in both pairs of diastereomers by a factor of about 10 over the S_C -configuration.

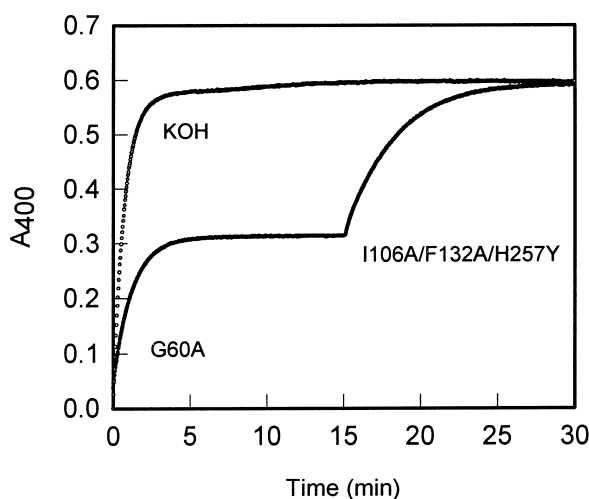


Figure 3. Time course for the hydrolysis of the diastereomeric mixture of the soman analogue $S_P S_C$ -**11** and $R_P S_C$ -**11** using the mutant forms of PTE. The total concentration of the soman analogue **11** ($35 \mu\text{M}$) was determined by the addition of KOH. The PTE mutant G60A was added at zero time to hydrolyze the $R_P S_C$ -**11** enantiomer. After 15 min, the I106A/F132A/H257Y mutant form of PTE was added to hydrolyze the $S_P S_C$ -**11** enantiomer.

A comparison in the rates of overall turnover for the soman analogue (**11**) with the sarin analogue (**8**) clearly shows that the pinacolyl substituent is tolerated less well within the active site of the wild-type enzyme than is the *i*-propyl group. For example, the R_P -isomer of **8** is hydrolyzed about two orders of magnitude faster than is the $R_P R_C$ -isomer of **11**. A similar difference is found in the corresponding values of k_{cat}/K_m . The data collected in this investigation also demonstrate that the wild-type PTE hydrolyzes the two stereoisomer of the most toxic analogues of soman significantly more slowly than the analogues of the two least toxic stereoisomers. If PTE is going to be an effective reagent for the catalytic destruction of soman then the rate of hydrolysis of the most toxic stereoisomers must be significantly enhanced through site-directed and combinatorial mutagenesis. For the $S_P S_C$ -enantiomer increases in k_{cat} and k_{cat}/K_m of nearly 4 orders of magnitude should be obtainable. The catalytic properties of the I106A/F132A/H257Y mutant with the analogues of the two S_P -diastereomers of **11** show that such gains in catalytic activity are likely to be achieved. This mutant has a 50-fold enhancement in the value of k_{cat} and a 75-fold increase in k_{cat}/K_m for the $S_P S_C$ -enantiomer of **11**.

Enzymatic hydrolysis of sarin and soman. In preliminary experiments conducted in collaboration with Drs. Steven Harvey and Joseph DeFrank of the Edgewood Chemical and Biological Center, the wild-type PTE, in addition to the I106A/F132A/H257Y and G60A mutant enzymes, has been shown to catalyze the hydrolysis of racemic mixtures of sarin and soman. The time courses have also been shown to be bi-phasic and thus consistent with differential turnover rates for the individual enantiomers. Turnover rates exceeded 1000 s^{-1} for the I106A/F132A/H257Y mutant PTE with racemic sarin. This value is 3 times larger than is the value of the wild-type enzyme but the catalytic constants for the individual enantiomers have not, as yet, been measured. For racemic soman, the G60A mutant has a turnover number that is greater than 10 s^{-1} and is approximately three times faster than the wild-type enzyme. The turnover numbers for the sarin and soman analogues measured in the investigation reported here are very similar to the preliminary values obtained for sarin and soman themselves. Therefore, the utilization of the chiral enantiomers in the development of novel mutants of PTE with enhanced catalytic activities will be productive. Experiments designed to sample a larger fraction of amino acid sequence space within the vicinity of the two binding subsites of PTE are currently in progress.

Conclusion

Chemical procedures were developed for the chiral syntheses of the S_P - and R_P -enantiomers of *O*-isopropyl *p*-nitrophenyl methylphosphonate (sarin analogue) in high enantiomeric excess. A chemo-enzymatic procedure was developed for the chiral synthesis of the four stereoisomers of *O*-pinacolyl *p*-nitrophenyl methylphosphonate (soman analogue) with high diastereomeric excess. This investigation has demonstrated that

mutants of the wild-type PTE can be readily constructed with enhanced catalytic activities toward the most toxic stereoisomers of sarin and soman.

Experimental

Materials

Methylphosphonothioic dichloride, R_C -1-phenylethylamine, S_C -1-phenylethylamine, phosphorus pentachloride, *m*-chloroperbenzoic acid (*m*-CPBA), racemic pinacolyl alcohol, phthalic anhydride, brucine, 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU) and R_C -mandelic acid were obtained from Aldrich. Restriction enzymes and T4 DNA ligase were obtained from Promega or New England Biolabs. Wizard Miniprep DNA Purification System was purchased from Promega. Gene Clean DNA purification kit was purchased from Bio 101. Oligonucleotide synthesis and DNA sequencing reactions were conducted by the Gene Technology Laboratory of Texas A&M University.

General methods

Optical rotations were obtained using a Jasco DIP-360 digital polarimeter. NMR spectra were obtained with a Varian Unity Plus 300, VXR-300 or XL-200E spectrometers. Proton chemical shifts (δ) are reported in parts per million (ppm) relative to the methine singlet at 7.26 ppm for the residual CHCl_3 in the deuteriochloroform, or the methyl pentet at 3.30 ppm for the residual CHD_2OD in the methanol- d_4 . Carbon chemical shifts are reported in parts per million relative to the internal ^{13}C signals in CDCl_3 (77.0 ppm) and $\text{CD}_3\text{OD}-d_4$ (49.0 ppm). ^{31}P NMR spectra were referenced using phosphoric acid as an external standard. Mass spectra (MS) were obtained on a VG 70-250 spectrometer using electron impact ionization (20–40 eV).

Site directed mutagenesis

Changes to the amino acid sequence of the phosphotriesterase enzyme were initiated by cassette mutagenesis of the *opd* gene within the plasmid pJW01.^{16,17} Unique restriction sites on either side of the codon to be mutated were identified and the targeted site was excised with the appropriate enzymes. The digested fragment was purified by agarose gel electrophoresis. The two mutagenic primers were annealed by incubating 20 μL of 1–2 μM of each oligonucleotide with 2 μL of T4 DNA ligase buffer at 72 °C for 5 min and then incubated at 25 °C for 1 h. The annealed oligonucleotides and restricted pJW01 plasmid were ligated with T4 DNA ligase at 16 °C overnight and then transformed into BL-21 cells. The mutated plasmids were sequenced to ensure that no other alterations were introduced during the mutagenic protocols.

Purification of enzymes

The mutants and the wild-type PTE were expressed in BL21 cells as previously described.¹⁸ PTE and the

mutant enzymes were purified according to the protocol of Omburo et al.³ SDS-PAGE indicated that the purified mutants were the same size as the wild-type PTE and were at least 95% pure. Apoenzyme was prepared according to an established procedure and then reconstituted with 5 equivalents of CoCl₂.³

Kinetic measurements and data analysis

The enzymatic hydrolysis of paraoxon and the substrate analogues was measured by monitoring the accumulation of *p*-nitrophenol at 400 nm ($\epsilon = 17,000 \text{ M}^{-1} \text{ cm}^{-1}$) and 25 °C using a Gilford Model 260 spectrophotometer. The reactions were conducted in 50 mM CHES buffer, pH 9.0, for paraoxon and the sarin analogues while 10% methanol was added to improve the solubility of the soman analogues. The kinetic constants were obtained by fitting the data to eq (1), where v is the initial velocity, V_m is the maximal velocity, A is the concentration of substrate, and K_a is the Michaelis constant.

$$v = V_m A / (K_a + A) \quad (1)$$

Synthesis of racemic *O*-isopropyl methylphosphonothioic acid (5). This synthesis of racemic **5** was conducted in two steps using a modification of the method of Hoffmann.¹⁹ Methylphosphonothioic dichloride (**3**) was reacted with one equivalent of *i*-propyl alcohol to provide racemic *O*-isopropyl methylphosphonochloridothionate (**4**) with a yield of 52%. (**4**): b.p. 45–46 °C/1.5 mm-Hg (lit.¹⁹ 41 °C/0.8 mm-Hg); ¹H NMR (300 MHz, CDCl₃) δ 1.36 (dd, $J = 6.2, 10.9 \text{ Hz}$, 6H), 2.25 (d, $J = 15.4 \text{ Hz}$, 3H), 5.04 (m, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 23.3 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 38.0 \text{ Hz}$), 23.5 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 38.0 \text{ Hz}$), 30.4 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 102.7 \text{ Hz}$), 73.3 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 8.0 \text{ Hz}$). This material was then hydrolyzed to yield the racemic *O*-isopropyl methylphosphonothioic acid (**5**) with an isolated yield of 37%. (**5**): ¹H NMR (300 MHz, CDCl₃) δ 1.29 (dd, $J = 6.1, 8.6 \text{ Hz}$, 6H), 1.81 (d, $J = 15.9 \text{ Hz}$, 3H), 4.83 (m, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 22.5 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 114.8 \text{ Hz}$), 23.7 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 15.3 \text{ Hz}$), 23.8 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 15.3 \text{ Hz}$), 71.5 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 7.1 \text{ Hz}$).

Preparation of *R*_C-1-phenylethylammonium *R*_P-*O*-isopropyl methylphosphonothioate (*R*_P-6**) and *S*_C-1-phenylethylammonium *S*_P-*O*-isopropyl methylphosphonothioate (*S*_P-**6**).** The chiral resolution of racemic *O*-isopropyl methylphosphonothioate (**5**) was obtained by fractional crystallization with the individual enantiomers of 1-phenylethylamine as previously described by Boter.¹⁴ The absolute configurations of these diastereomeric crystalline salts were determined by X-ray diffraction analysis in Table 2. The *R*_P-enantiomer of **5** crystallized with the *R*_C-enantiomer of 1-phenylethylamine with an isolated yield of 30% (mp 158–159 °C; $[\alpha]_D^{25} 10.5^\circ$). (*R*_P-**6**): ¹H NMR (300 MHz, CDCl₃) δ 1.11 (dd, $J = 6.2, 11.6 \text{ Hz}$, 6H), 1.21 (d, $J = 22.5 \text{ Hz}$, 3H), 1.67 (d, $J = 6.8 \text{ Hz}$, 3H), 4.38 (m, 1H), 4.49 (q, $J = 6.9 \text{ Hz}$, 1H, benzylic methine proton), 7.23–7.33 (m, 3H, aromatic protons), 7.48–7.51 (m, 2H, aromatic protons); ¹³C

NMR (75 MHz, CDCl₃) δ 21.5 (CH₃), 23.8 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 4.2 \text{ Hz}$), 24.3 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 4.2 \text{ Hz}$), 24.6 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 100.8 \text{ Hz}$), 51.4, 69.0 (d, $J(^{31}\text{P}, ^{13}\text{C}) = 5.5 \text{ Hz}$), 127.2, 128.4, 128.8, 139.0. The *S*_C-enantiomer of 1-phenylethylamine crystallized with the *S*_P-enantiomer of **5** with an isolated yield of 41% (mp 158–159 °C; $[\alpha]_D^{25} -10.8^\circ$). (*S*_P-**6**) shares the same NMR pattern as (*R*_P-**6**).

Synthesis of chiral *O*-isopropyl *p*-nitrophenyl methylphosphonothioate (7). The chiral methylthiophosphonates, *S*_P-**7** and *R*_P-**7**, were prepared from their corresponding amine salt precursors in three steps. The chiral amine salt *R*_P-**6** (1.6 g, 5.81 mmol) was suspended in 6 mL of benzene, cooled to 0 °C, and then 12.3 mL of 1 N NaOH was added dropwise. The reaction mixture was stirred at 0 °C until the amine salt was dissolved. The reaction mixture was then diluted with 20 mL of benzene and 40 mL of water. The layers were separated and the aqueous phase extracted with benzene. The combined aqueous phases were cooled to 0 °C, acidified to a pH < 2 with HCl, and then extracted three times with 20 mL of ether. The ether layers were combined, dried over anhydrous sodium sulfate, and concentrated in vacuo for 2 days to yield 0.79 g (88%) of the corresponding free acid (*R*_P-**5**). The enantiomeric purity of this compound was determined using the ³¹P NMR method of Mikolajczyk²⁰ and found to be $\geq 99\%$. An oven-dried, argon-purged, 25-mL round-bottomed flask was charged with 1.28 g (6.1 mmol) of PCl₅ and 6 mL of CCl₄ and then cooled to 0 °C. The anhydrous phosphonothioic acid *R*_P-**5** was dissolved in 6 mL of CCl₄ and added dropwise to the PCl₅ suspension. The reaction mixture was stirred at 0 °C for an additional 30 min and then filtered. The solution was concentrated to yield 0.69 g (77%) of the crude methylphosphonochloridothioate (*S*_P-**4**) with an inversion of configuration at the phosphorus center.²¹ With stirring, 1.12 mL of triethylamine (8 mmol), and then 0.56 g of *p*-nitrophenol (4 mmol) in 8 mL of anhydrous THF was slowly added to a mixture of *S*_P-**4** dissolved in 2 mL of anhydrous THF. After the addition of the *p*-nitrophenol, the reaction mixture was heated at 60 °C overnight. After cooling, the triethylamine hydrochloride was separated

Table 2. Crystallographic data for (*R*_P-**6**) and (*S*_P-**6**)

	(<i>R</i> _P - 6)	(<i>S</i> _P - 6)
Formula	C ₁₂ H ₂₂ NO ₂ PS	C ₁₂ H ₂₂ NO ₂ PS
Formula weight	275.34	275.34
Crystal system	Monoclinic	Monoclinic
Space group	P2 ₁	P2 ₁
Crystal size (mm)	0.30 × 0.30 × 0.20	0.30 × 0.20 × 0.20
a (Å)	11.128 (16)	11.131 (3)
b (Å)	6.656 (12)	6.652 (3)
c (Å)	11.365 (2)	11.365 (5)
α (°)	90	90
β (°)	108.99 (11)	108.98 (3)
γ (°)	90	90
V (Å ³)	795.9 (2)	795.8 (5)
Z	2	2
d (calculated density) (mg/m ³)	1.149	1.145
Absorption coefficient (mm ⁻¹)	0.296	0.296
No. of total reflections	2576	1563
No. of unique reflections	2466	1485

by filtration and washed with anhydrous THF. The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was chromatographed ($\text{EtOAc}/\text{hexane}=1:4$) to give a pale yellowish liquid (S_P -**7**) in 69% yield with an inversion of configuration at the phosphorus center.²² (S_P -**7**): ^1H NMR (300 MHz, CDCl_3) δ 1.29 (dd, $J=6.2, 18.7$ Hz, 6H), 1.99 (d, $J=15.4$ Hz, 3H), 4.88 (m, 1H), 7.31 (dd, $J=1.7$ and 9.1 Hz, 2H, protons at the 2- and 6-positions of the *p*-nitrophenoxy moiety), 8.22 (d, $J=9.1$ Hz, 2H, protons at the 3- and 5-positions of the *p*-nitrophenoxy moiety); ^{13}C NMR (75 MHz, CDCl_3) δ 23.4 (d, $J(^{31}\text{P}, ^{13}\text{C})=115.3$ Hz), 23.8 (d, $J(^{31}\text{P}, ^{13}\text{C})=2.1$ Hz), 23.9 (d, $J(^{31}\text{P}, ^{13}\text{C})=1.5$ Hz), 73.3 (d, $J(^{31}\text{P}, ^{13}\text{C})=7.1$ Hz), 122.5 (d, $J(^{31}\text{P}, ^{13}\text{C})=5.0$ Hz, C2/C6 position of the *p*-nitrophenoxy moiety), 125.5 (d, $J(^{31}\text{P}, ^{13}\text{C})=1.0$ Hz, C3/C5 position of the *p*-nitrophenoxy moiety), 144.9, 155.6 (d, $J(^{31}\text{P}, ^{13}\text{C})=9.6$ Hz, C1 position of the *p*-nitrophenoxy moiety); EI-MS m/z (relative intensity) 275 (M^+ , 35), 234 (60), 206 (24), 155 (69). The other enantiomer (R_P -**7**) was prepared in the same way. (R_P -**7**): EI-MS m/z (relative intensity) 275 (M^+ , 52), 234 (43), 206 (74), 155 (67).

Synthesis of chiral *O*-isopropyl *p*-nitrophenyl methylphosphonate (8**).** The chiral methyl phosphonates, S_P -**8** and R_P -**8**, were prepared from their corresponding thiophosphonates by oxidation with *m*-chloroperbenzoic acid with an overall retention of configuration at the phosphorus center.²³ A solution of *m*-CPBA (0.56 g, 3.27 mmol) in anhydrous dichloromethane (10 mL) was slowly added to a solution of R_P -**7** (0.5 g, 1.82 mmol) in anhydrous dichloromethane (15 mL). The solution was stirred for 1 h at room temperature and then concentrated under reduced pressure. The residue was chromatographed ($\text{EtOAc}/\text{hexane}=1:4\rightarrow 1:1$) to give a colorless liquid (0.39 g, 82%) of R_P -**8**. (R_P -**8**): ^1H NMR (300 MHz, CDCl_3) δ 1.24 (dd, $J=6.1, 27.8$ Hz, 6H), 1.64 (d, $J=17.8$ Hz, 3H), 4.80 (m, 1H), 7.36 (dd, $J=1.2$ and 9.3 Hz, 2H, protons at the 2- and 6-positions of the *p*-nitrophenoxy moiety), 8.20 (d, $J=8.8$ Hz, 2H, protons at the 3- and 5-positions of the *p*-nitrophenoxy moiety); ^{13}C NMR (75 MHz, CDCl_3) δ 12.3 (d, $J(^{31}\text{P}, ^{13}\text{C})=146.0$ Hz), 23.8 (d, $J(^{31}\text{P}, ^{13}\text{C})=11.3$ Hz), 23.9 (d, $J(^{31}\text{P}, ^{13}\text{C})=11.3$ Hz), 72.1 (d, $J(^{31}\text{P}, ^{13}\text{C})=7.0$ Hz), 121.0 (d, $J(^{31}\text{P}, ^{13}\text{C})=4.6$ Hz, C2/C6 position of the *p*-nitrophenoxy moiety), 125.6, 144.4, 155.6 (d, $J(^{31}\text{P}, ^{13}\text{C})=8.1$ Hz, C1 position of the *p*-nitrophenoxy moiety); EI-MS m/z (relative intensity) 259 (M^+ , 96), 244 (94), 217 (98), 201 (81), 139 (100). The enantiomer S_P -**8** was prepared with the same procedure starting with the R_P -**6** precursor. The mass spectral data are as follow: EI-MS m/z (relative intensity) 259 (M^+ , 81), 244 (80), 217 (84), 201 (78), 139 (100). Scheme 4 summarizes the overall synthetic strategy for the preparation of the individual enantiomers of **8**. The synthesis of racemic *O*-isopropyl *p*-nitrophenyl methylphosphonate (**8**) was obtained in two-steps (74% overall yield) via a bis-(*p*-nitrophenyl)methylphosphonate (**9**) intermediate as described by Green et al.²⁴

Enzymatic resolution of racemic *O*-isopropyl *p*-nitrophenyl methylphosphonate. To a solution of 5.0 mM racemic **8** (390 mg) in 0.5 M CHES buffer (pH 9.0)

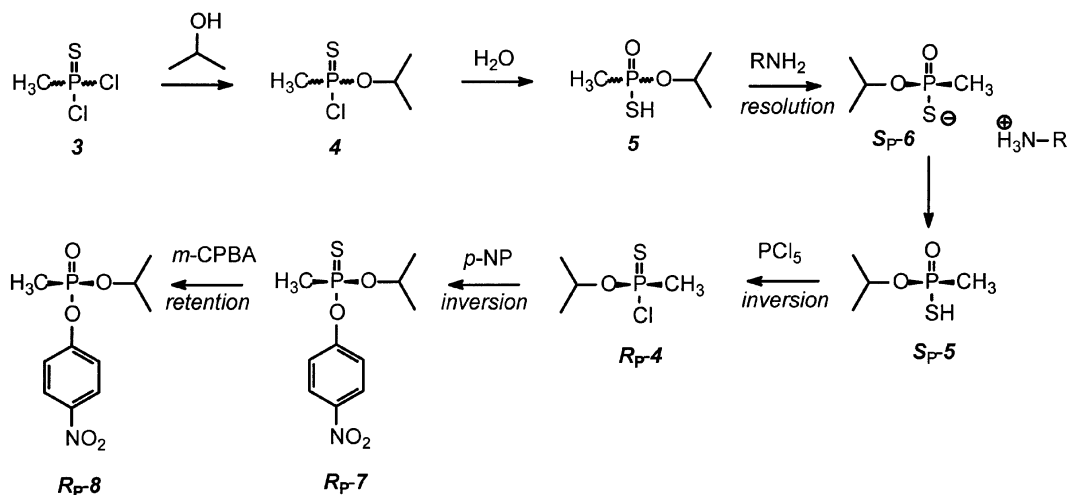
containing 30% methanol was added the PTE mutant G60A and the mixture was stirred at room temperature. The progress of the reaction was monitored spectrophotometrically at 400 nm. When the reaction was half-complete, the reaction was quenched by extraction with chloroform (3×80 mL). The combined organic layers were dried over Na_2SO_4 , filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (30% EtOAc and 70% hexane) to give 191 mg (95%) of S_P -**8** as a colorless liquid. The ratio of S_P -**8** to R_P -**8** in this preparation was found to be 99:1 as determined by chiral capillary electrophoresis.²⁵ When the PTE I106A/F132A/H257Y mutant was used as the enzymatic catalyst, the R_P -**8** isomer was obtained in 77% yield. Chiral capillary electrophoresis indicated a ratio of R_P -**8** to S_P -**8** of 93:7.

Synthesis of racemic *O*-pinacolyl *p*-nitrophenyl methylphosphonate. The racemic methylphosphonate **11** was obtained in two steps via a bis-(*p*-nitrophenyl) methylphosphonate intermediate (**9**) as described previously for the preparation of the racemic methylphosphonate **8**, except for the use of the racemic pinacolyl alcohol (**10**) instead of *i*-propanol. The isomer ratios were determined by chiral HPLC analysis to be $S_P S_C/R_P R_C/R_P S_C$ of 27:22:28:23.

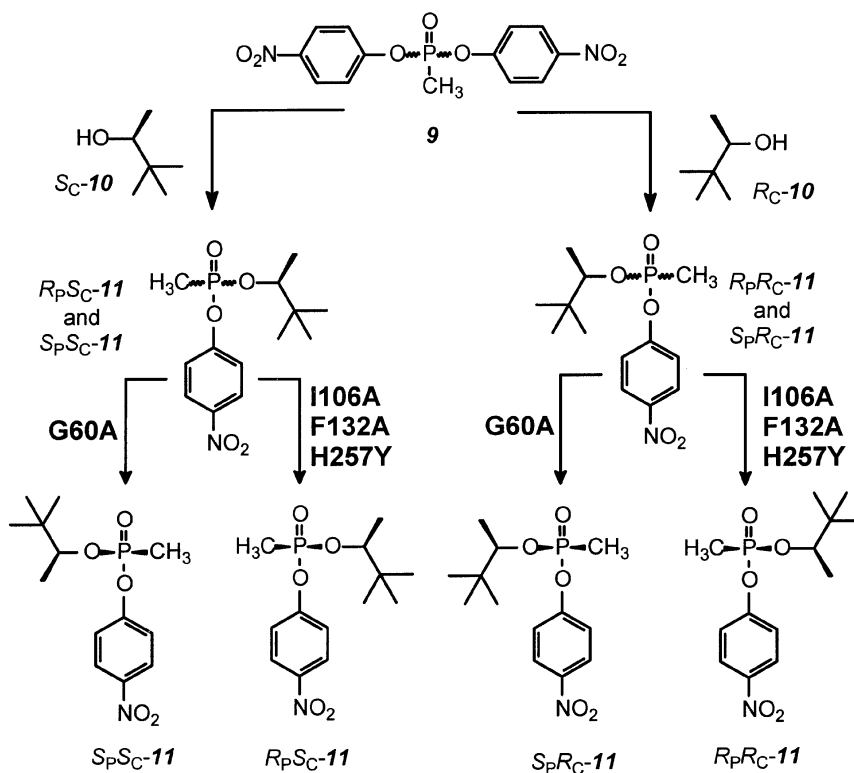
Synthesis of diastereomeric mixture of (S_P/R_P)-*O*-(S_C)-pinacolyl *p*-nitrophenyl methylphosphonate (11**).** The synthesis of the diastereomeric mixture of $S_P S_C$ -**11** and $R_P S_C$ -**11** utilized the chiral S_C -pinacolyl alcohol (**10**) to displace *p*-nitrophenol from bis-(*p*-nitrophenyl) methylphosphonate (**9**) in 82% overall yield using the method described by Green et al.²⁴ The S_C -pinacolyl alcohol (**10**) was prepared in five steps (11% yield) by repeated crystallization of the brucine salt of R_C/S_C -pinacolyl phthalate according to the procedure of Pickard and Kenyon.²⁶ Chiral HPLC analysis of the S_C -pair of diastereomers for compound **11** gave isomer ratios of $S_P S_C/S_P R_C/R_P R_C/R_P S_C$ of 50:3:3:44.

Synthesis of diastereomeric mixture of S_P/R_P -*O*- R_C -pinacolyl *p*-nitrophenyl methylphosphonate (11**).** The diastereomeric mixture of $S_P R_C$ -**11** and $R_P R_C$ -**11** was prepared in the same way as the mixture of $S_P S_C$ -**11** and $R_P S_C$ -**11** except for the utilization of R_C -pinacolyl alcohol. The R_C -pinacolyl alcohol (**10**) was obtained in three steps (8% yield) by crystallization of the R_C/S_C -pinacolyl- R_C -mandelate from $\text{MeOH}/\text{H}_2\text{O}$ according to the method of Benschop.¹² The diastereomeric mixture of $S_P R_C$ -**11** and $R_P R_C$ -**11** was prepared in a yield of 77%. Chiral HPLC analysis of the R_C -pair of diastereomers for compound **11** gave isomer ratios of $S_P S_C/S_P R_C/R_P R_C/R_P S_C$ of 4:40:55:1.

Enzymatic resolution of S_P/R_P -*O*- S_C -pinacolyl *p*-nitrophenyl methylphosphonate (11**).** The diastereomeric mixture of $S_P S_C$ -**11** and $R_P S_C$ -**11** was resolved enzymatically with the G60A and I106A/F132A/H257Y mutants of PTE. To a solution of 3.0 mM $S_P S_C$ -**11** and $R_P S_C$ -**11** (105 mg) in 0.5 M CHES (pH 9.0) containing 20% CH_3CN was added the G60A mutant. The mixture was stirred at room temperature and the progress of the



Scheme 4.



Scheme 5.

reaction was monitored by UV and NMR spectroscopy. The reaction was stopped by extracting the solution with chloroform (3×60 mL), and the combined organic layers were dried over Na_2SO_4 , filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (30% EtOAc and 70% hexane) to give 48 mg (91%) of $S_P S_C$ -11 as a colorless liquid. Chiral HPLC analysis of the enzymatically prepared $S_P S_C$ -isomer of compound 11 gave isomer ratios of $S_P S_C/S_P R_C/R_P R_C/R_P S_C$ of 98:2:0:0. ($S_P S_C$ -11): ^1H NMR (300 MHz, CDCl_3) δ 0.88 (s, 9H), 1.10 (d, $J=6.4$ Hz, 3H), 1.63 (d, $J=17.6$ Hz, 3H), 4.33 (m, 1H), 7.34 (dd, $J=1.1$ and 9.4 Hz, 2H, protons at the 2- and 6-positions of the *p*-nitrophenoxy moiety), 8.18 (d, $J=9.4$ Hz, 2H, protons at the 3- and 5-positions of the

p-nitrophenoxy moiety); ^{13}C NMR (75 MHz, CDCl_3) δ 11.8 (d, $J(^{31}\text{P}, ^{13}\text{C})=147.0$ Hz), 16.9, 25.5, 34.9 (d, $J(^{31}\text{P}, ^{13}\text{C})=6.6$ Hz), 82.7 (d, $J(^{31}\text{P}, ^{13}\text{C})=7.5$ Hz), 121.0 (d, $J(^{31}\text{P}, ^{13}\text{C})=4.5$ Hz, C2/C6 position of the *p*-nitrophenoxy moiety), 125.5 (C3/C5 position of the *p*-nitrophenoxy moiety), 144.4, 155.6 (d, $J(^{31}\text{P}, ^{13}\text{C})=7.6$ Hz, C1 position of the *p*-nitrophenoxy moiety). When the I106A/F132A/H257Y mutant was used instead of G60A, the $R_P S_C$ -11 diastereomer was obtained in 61% yield with isomer ratios of $S_P S_C/S_P R_C/R_P R_C/R_P S_C=8:0:0:92$. ($R_P S_C$ -11): ^1H NMR (300 MHz, CDCl_3) δ 0.82 (s, 9H), 1.28 (d, $J=6.4$ Hz, 3H), 1.64 (d, $J=17.6$ Hz, 3H), 4.32 (m, 1H), 7.30 (dd, $J=1.1$ and 9.4 Hz, 2H, protons at the 2- and 6-positions of the *p*-nitrophenoxy moiety), 8.16 (d, $J=9.4$ Hz, 2H, protons at

the 3- and 5-positions of the *p*-nitrophenoxy moiety); ^{13}C NMR (75 MHz, CDCl_3) δ 12.7 (d, $J[^{31}\text{P}, ^{13}\text{C}] = 146.0$ Hz), 16.9, 25.4, 34.8, 83.1 (d, $J[^{31}\text{P}, ^{13}\text{C}] = 8.1$ Hz), 120.8 (d, $J[^{31}\text{P}, ^{13}\text{C}] = 5.0$, C2/C6 position of the *p*-nitrophenoxy moiety), 125.5 (C3/C5 position of the *p*-nitrophenoxy moiety), 144.5, 155.9 (d, $J[^{31}\text{P}, ^{13}\text{C}] = 7.5$ Hz, C1 position of the *p*-nitrophenoxy moiety).

Enzymatic resolution of S_P/R_P -*O*- R_C -pinacolyl *p*-nitrophenyl methylphosphonate (11**).** The diastereomeric mixture of $S_P R_C$ -**11** and $R_P R_C$ -**11** was resolved enzymatically using the G60A and I106A/F132A/H257Y mutants of PTE. The utilization of G60A afforded the $S_P R_C$ -**11** diastereomer with an isolated chemical yield of 84%. Chiral HPLC analysis of the enzymatically prepared $S_P R_C$ -isomer of compound **11** gave isomer ratios of $S_P S_C/S_P R_C/R_P R_C/R_P S_C$ of 8:92:0:0. The utilization of the PTE mutant I106A/F132A/H257Y produced $R_P R_C$ -**11** with an isolated yield of 72% yield and an isomer ratio of $S_P S_C/S_P R_C/R_P R_C/R_P S_C = 4:0:90:6$. Scheme 5 summarizes the synthetic strategy for the chemo-enzymatic synthesis of the four stereoisomers of compound **11**. The toxicity of these compounds is unknown and thus these compounds should be used with caution.

Acknowledgements

The authors thank Dr. Joseph Reibenspies for solving the X-ray structures of compounds S_P -**6** and R_P -**6**. This work was supported in part by the National Institute of Health (GM-33894) and the Office of Naval Research (N0014-99-0235). MAS held a fellowship from Spanish Ministry of Science.

References and Notes

1. Aspelin, A. L.; Grube, A. H. In *Pesticides Industry Sales and Usage 1996 and 1997 Market Estimates*; Environmental Protection Agency: Washington, DC, 1999; pp 9–15.

2. Raushel, F. M.; Holden, H. M. *Adv. Enzymol.* **2000**, *74*, 51.
3. Omburo, G. A.; Kuo, J. M.; Mullins, L. M.; Raushel, F. M. *J. Biol. Chem.* **1992**, *267*, 13278.
4. Omburo, G. A.; Mullins, L. S.; Raushel, F. M. *Biochemistry* **1993**, *32*, 9148.
5. Benning, M. M.; Kuo, J. M.; Raushel, F. M.; Holden, H. M. *Biochemistry* **1995**, *34*, 7973.
6. Vanhooke, J. L.; Benning, M. M.; Raushel, F. M.; Holden, H. M. *Biochemistry* **1996**, *35*, 6020.
7. Jabri, E.; Carr, M. B.; Hausinger, R. P.; Karplus, P. A. *Science* **1995**, *268*, 998.
8. Holm, L.; Sander, C. *Proteins: Struct. Funct. Genet.* **1997**, *28*, 72.
9. Dumas, D. P.; Durst, H. D.; Landis, W. G.; Raushel, F. M.; Wild, J. R. *Arch. Biochem. Biophys.* **1989**, *164*, 1137.
10. diSioudi, B.; Miller, C. E.; Lai, K.; Grimsley, J. K.; Wild, J. R. *Chem-Biol. Interact.* **1999**, *120*, 211.
11. Benschop, H. P.; De Jong, L. P. A. *Acc. Chem. Res.* **1988**, *21*, 368.
12. Benschop, H. P.; Konings, C. A. G.; Genderen, J. V.; De Jong, L. P. A. *Toxicol. Appl. Pharm.* **1984**, *72*, 61.
13. Hong, S. B.; Raushel, F. M. *Biochemistry* **1999**, *38*, 1159.
14. Boter, H. L.; Platenburg, D. H. J. M. *Rec. Trav. Chim. Pays-Bas* **1967**, *86*, 399.
15. Benning, M. M.; Hong, S. B.; Hong Raushel, F. M.; Holden, H. M. *J. Biol. Chem.* **2000**, *275*, 30556.
16. Chen-Goodspeed, M.; Sogorb, M. A.; Wu, F.; Hong, S.-B.; Raushel, F. M. *Biochemistry* **2001**, *40*, 1325.
17. Chen-Goodspeed, M.; Sogorb, M. A.; Wu, F.; Raushel, F. M. *Biochemistry* **2001**, *40*, 1332.
18. Kuo, J. M.; Raushel, F. M. *Biochemistry* **1994**, *33*, 4265.
19. Hoffmann, F. W.; Wadsworth, D. H.; Weiss, H. D. *J. Am. Chem. Soc.* **1958**, *80*, 3945.
20. Mikolajczyk, M.; Omelanczuk, J.; Leitloff, M.; Drabowicz, J.; Ejchart, A.; Jurczak, J. *J. Am. Chem. Soc.* **1978**, *100*, 7003.
21. Michalski, J.; Mikolajczyk, M. *Tetrahedron* **1966**, *22*, 3055.
22. Mikolajczyk, M.; Omelanczuk, J.; Para, M. *Tetrahedron* **1972**, *28*, 3855.
23. Herriott, A. W. *J. Am. Chem. Soc.* **1971**, *93*, 3304.
24. Tawfik, D. S.; Eshhar, Z.; Bentolila, A.; Green, B. S. *Synthesis* **1993**, *19*, 968.
25. Zhu, W.; Li, W.-S.; Raushel, F. M.; Vigh, G. *Electrophoresis* **2000**, *21*, 3249.
26. Pickard, R. H.; Kenyon, J. *J. Chem. Soc.* **1914**, 1115.