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Enzymatic Synthesis of Chiral Organophosphothioates from Prochiral Precursors

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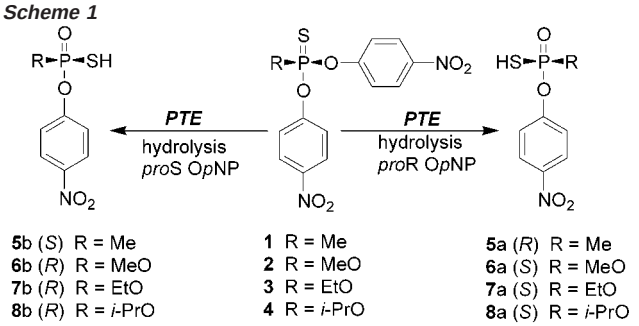
Significant progress has been made in the development of enzyme-based methods for the preparative syntheses of chiral building blocks.[1] As an example, the native bacterial phosphotriesterase (PTE) from *Pseudomonas diminuta* has been shown to be a highly effective catalyst for the kinetic resolution of racemic mixtures of organophosphate triesters.[2] Differential rates of hydrolysis for pairs of chiral enantiomers with the wild-type enzyme have enabled the isolation of pure stereoisomers in very high yield.[1] Moreover, rational reconstruction of the substrate binding site cavity within PTE through site-directed mutagenesis has altered the catalytic properties where the inherent stereoselectivity of the native PTE has been *enhanced*, *relaxed*, or *reversed*.[3] Tsai and co-workers have also shown that the stereoselective properties of adenylate kinase can be manipulated through mutagenesis.[4] The reversal of stereoselectivity for PTE has allowed the isolation of either stereoisomer in a racemic mixture through the judicious choice of a specific mutant from an enzyme library constructed with modified catalytic properties.[2,3]

Scheme 1



In this communication, we describe a novel enzymatic approach toward the efficient generation of chiral enantiomers of alkyl phosphothioates and related alkyl phosphonothioates through the stereoselective hydrolysis of prochiral starting materials. The preferential hydrolysis of a single prochiral *p*-nitrophenolate from bis-*p*-nitrophenyl methyl phosphonothioate (**1**) and bis-*p*-nitrophenyl alkyl phosphothioates (**2**−**4**) will generate the chiral products **5**−**8**. The wild-type PTE is known to catalyze the hydrolysis of phosphorus centers with complete *inversion* of configuration.[5,6] Therefore, cleavage of the *pro-R* substituent from substrates **1**−**4** will generate products **5a**−**8a**, whereas hydrolysis of the *pro-S* substituent will form products **5b**−**8b**. The expected reaction products and stereochemical configurations are summarized in Scheme 1.

The phosphotriesterase from *Pseudomonas diminuta* is a zinc-containing enzyme that catalyzes the hydrolysis of a variety of organophosphorus compounds, including an array of insecticides and chemical nerve agents that are known to inactivate acetyl cholinesterase.[5] The enzymatic reaction is initiated by the direct nucleophilic attack of the hydrolytic water molecule on the phosphorus center.[6] The three-dimensional structure of Zn/Zn−PTE with a bound substrate analogue, diethyl 4-methylbenzylphosphonate, has led to the identification of three distinct binding pockets that are responsible for the orientation and selectivity of substrates within the active site of the enzyme.[7] These binding sub-sites have been designated as the *small*, *large*, and *leaving group* pockets. Prior stereochemical investigations with the native enzyme predict that the wild-type PTE will selectively cleave the *pro-R* substituent from compounds **1**−**4**.[3]

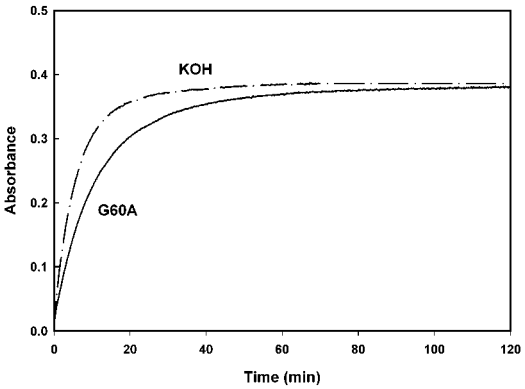


Figure 1. Time course for the hydrolysis of **1** (0.022 mM) by KOH and the mutant G60A at pH 9.0 and 25 °C. The reaction was monitored at 400 nm.

The prochiral substrates **1**−**4** were synthesized by standard methods.[8] A graphical illustration of the reaction time course, monitored at 400 nm, catalyzed by the mutant G60A and KOH for the hydrolysis of substrate **1** is presented in Figure 1. These data demonstrate that a single *p*-nitrophenolate group is cleaved from the prochiral starting material and that the reaction proceeds to the same extent enzymatically or with the addition of KOH.

The relative stereoselectivity for the enzymatic hydrolysis of compounds **1**−**4** was established by ^{31}P NMR spectroscopy, using strychnine as a chiral shift reagent. Illustrated in Figure 2a is the ^{31}P NMR spectrum of the KOH-derived hydrolysis products from **1** in the presence of (−)-strychnine. Resonances for each of the two chiral acids (**5a** and **5b**) are observed at ∼80.3 ppm with a chemical shift difference of 0.05 ppm. Shown in Figure 2b,c are NMR spectra of authentic (S_P)- and (R_P)-stereoisomers of **5**, respectively. The relative downfield resonance for the (S_P)-isomer was confirmed by the addition of a small amount of racemic **5** (Figure 2d). When **1** was hydrolyzed by wild-type PTE there was a preponderance of a single stereoisomeric product (Figure 2e) that was confirmed to be the (R_P)-isomer of **5** after the addition of a small amount of racemic material (Figure 2f). The measured

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Table 1. Kinetic Constants and Enantiomeric Excess (*ee*) for the Enzymatic Hydrolysis of Prochiral Substrates **1**−**4**[a,b]

enzyme	enantiomeric excess (%) 5	6	7	8	1 k_{cat} (min^{-1})	2 k_{cat} (min^{-1})	3 k_{cat} (min^{-1})	4 k_{cat} (min^{-1})
wild-type	86 (R_P)	99 (S_P)	99 (S_P)	99 (S_P)	96	310	420	24
G60A	96 (R_P)	99 (S_P)	99 (S_P)	87 (S_P)	110	290	190	0.1
H254G/H257W/L303T	32 (R_P)	99 (S_P)	99 (S_P)	99 (S_P)	3	5	5	1.0
I106A/H257Y/S308A	14 (S_P)	63 (S_P)	87 (S_P)	96 (S_P)	3	4	1	0.4
I106G/F132G/H257Y	9 (R_P)	46 (S_P)	75 (S_P)	47 (S_P)	55	6	6	0.4
I106A/F132A/H257Y	49 (S_P)	36 (S_P)	99 (S_P)	86 (S_P)	26	9	3	0.2
I106A/F132A/H257W	85 (S_P)	35 (S_P)	99 (S_P)	81 (S_P)	4	0.4	2	0.4
I106G/F132G/H257Y/S308G	23 (S_P)	10 (S_P)	9 (R_P)	88 (R_P)	69	5	2	0.4
I106A/F132A/H254G/H257W	58 (S_P)	24 (R_P)	87 (S_P)	42 (S_P)	96	2	2	0.4

[a] Products **5a**/**5b**, **6a**/**6b**, **7a**/**7b**, and **8a**/**8a**/**8b** were solved by addition of (−)-strychnine in $CDCl_3$. They displayed two distinguishable ^{31}P NMR resonances at 80.34/80.29, 56.05/56.07, 55.07/55.11, and 53.39/53.43 ppm, respectively. The enantiomeric excess was determined from the integral ratios. [b] Reaction conditions: 10% dioxane/CHES (0.5 M, pH = 9.0) with 0.113 mM **1**−**4**.

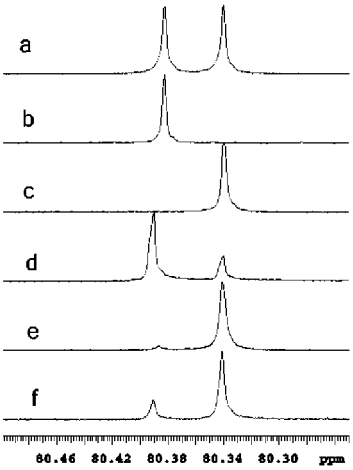

Figure 2. ^{31}P NMR spectra of (−)-strychnine salts of **5a**/**5b**: (a) hydrolysis of **1** by KOH; (b) authentic (S_P)-**5**; (c) authentic (R_P)-**5**; (d) authentic (S_P)-**5** plus racemic **5**; (e) product from hydrolysis of **5** by wild-type PTE; (f) addition of racemic **5** to spectrum 2e.

enantiomeric excess (ee) was 86%. The product ratios for compounds **2**−**4** were determined in an identical manner and the corresponding ee values are presented in Table 1 along with the kinetic constants for the wild-type PTE and selected mutants.

The absolute stereochemistry of the phosphorus centers for the (R_P)- and (S_P)-enantiomers of **5** was confirmed by X-ray diffraction analysis of the isolated (−)-strychnine salts (Figure 3). The racemic methylphosphonothionic acid (**5**) was prepared by hydrolysis of **1** with KOH and converted into the corresponding (−)-strychnine salt. The individual isomers were isolated by successive recrystallizations from MeOH/H_2O (10:1) followed by isopropyl alcohol/H_2O (10:1).

The wild-type PTE cleaves the *pro-R* substituent from compounds **1**−**4** with a stereoselectivity that is greater than previously observed for a related series of racemic organophosphate triesters.[3] The enhancement in the observed stereoselectivity for the wild-type PTE with this series of thiophosphate esters is likely due to a more rate limiting dependence on the specific step for P−O bond cleavage during overall substrate turnover. The utilization of certain variants of the wild-type PTE, where the size and shape of the substrate binding cavity has been specifically modified through site-directed mutagenesis, has altered the stereoselectivity to the point where the *pro-S* substituent is preferentially cleaved from some of the prochiral substrates. A nearly complete reversal in stereoselectivity was observed for substrate **5** with the mutant I106A/F132A/H257W whereas a relaxation in the stereoselectivity was obtained for compounds **6**, **7**, and **8**.

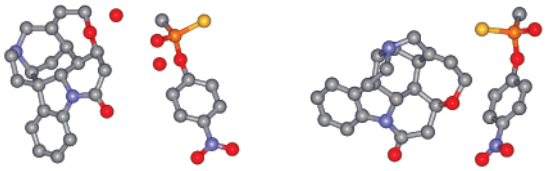

Figure 3. X-ray structures for the strychnine salts of (R_P)-**5** (left) and (S_P)-**5** (right) (−)-strychnine salts.

The chiral thiophosphates generated by these enzymatic methods can be further utilized as chiral synthons for more complex synthetic elaborations. The chiral *p*-nitrophenyl methylphosphonothionic acid (R_P)-**5a**, generated by G60A, was successfully converted into (R_P)-*S*-methyl-*p*-nitrophenyl methylphosphonothiolate by reacting **5a** with excess CH_3I in anhydrous benzene.[9] Products of this type can be utilized as precursors for synthesizing diverse examples of chiral organophosphorus compounds.

In summary, we have demonstrated that formation of chiral organophosphothioic acids can be prepared through the stereoselective hydrolysis of prochiral substrates using engineered mutants of phosphotriesterase. Moreover, the prochiral stereoselectivity inherent to the wild-type PTE can be manipulated by specific modifications to the substrate binding domain within the active site of this protein.

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(8) A new approach was developed to synthesize **2**−**4** through the intermediate, tris(4-nitrophenyl)thiophosphate, which was reacted with the corresponding alcohol in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene at 0−25 °C. The prochiral substrates **2**−**4** were isolated by chromatography or recrystallization (MeOH) in high overall yield (84−96%).
(9) ^1H NMR (300 MHz, $CDCl_3$) δ 2.04 (d, J = 15.6 Hz, 3H), 2.32 (d, J = 13.6 Hz, 3H), 7.44 (dd, J = 8.8, 1.4 Hz, 2H), 8.25 (d, J = 8.8 Hz, 2H).

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