# Enzymatic Resolution of Chiral Phosphinate Esters

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Phosphotriesterase (PTE) catalyzes the hydrolysis of a wide spectrum of organophosphorus compounds including agricultural insecticides and military nerve agents.[1] High-resolution X-ray structures of PTE have shown that the active site consists of two Zn^{2+} ions which are bridged by hydroxide.[2] The asymmetry of the active site has been exploited for the kinetic resolution of racemic phosphate (**A**) and phosphonate (**B**) esters in high enantiomeric and chemical yield where *L* represents a leaving-group phenol.[3] Manipulation of the active-site structure has enabled mutants of the wild-type enzyme to be prepared with altered stereoselective properties.[4] With organophosphate triesters, variants of PTE were prepared that exhibited *enhancement*, *relaxation*, or *reversal* of the inherent stereoselectivity of the wild-type enzyme.[5] With as few as five amino acid changes the stereoselectivity was altered by nearly 7 orders of magnitude.[5] In this communication we report the synthesis and stereoselective hydrolysis of a series of phosphinate (**C**) esters by PTE. To the best of our knowledge this represents the first time that phosphinate esters have been demonstrated to be substrates for any enzyme. Chiral phosphinate esters can serve as the entry point for the chemoenzymatic preparation of P-chiral phosphines and phosphine oxides.[6]
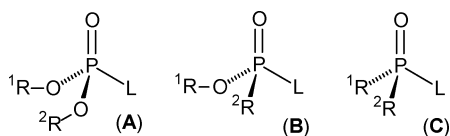
A series of racemic phosphinate esters was synthesized as potential substrates for PTE. Methyl and phenyl substituents were attached directly to the phosphorus center, while the leaving group consisted of a para-substituted phenol. The specific set of compounds is presented in Scheme 1. These compounds are excellent substrates for PTE, comparable to the turnover rates observed for the analogous phosphate and phosphonate derivatives.[5,7] The time courses for the enzymatic hydrolysis (pH 8.0) at low substrate concentrations for each pair of enantiomers were biphasic, a direct indication that one enantiomer was preferentially hydrolyzed relative to its mirror image. The values of k_{cat}/K_m for the preferred enantiomers and five of the more slowly hydrolyzed enantiomers were measured and plotted vs the pK_a of the leaving-group phenol (Figure 1). For the more rapidly hydrolyzed enantiomers a nonlinear Brønsted plot was obtained, indicative of a change in the rate-limiting step that breaks at a pK_a of approximately 8.5. There was a linear dependence of k_{cat}/K_m with the pK_a of the leaving group for the slower enantiomers with a β-value of −0.75.

The experimental results of these exploratory studies demonstrate that there is a substantial difference in the rate of enzymatic hydrolysis between pairs of phosphinate ester enantiomers and that in the most favorable cases, the differential exceeds 3 orders of magnitude. Therefore, it should be practical to use PTE to kinetically resolve phosphinate esters on a preparative scale. Wild-type PTE
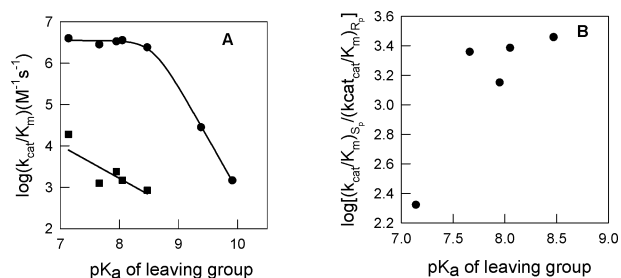
Figure 1. (a) Brønsted plots for the hydrolysis of S_P-(●) and R_P-(■) enantiomers of compounds *1−8* with wild-type PTE. (b) Stereoselectivity for the enzymatic hydrolysis of compounds *1−5* plotted as the ratio of k_{cat}/K_m as a function of the pK_a of the leaving group phenol.

Scheme 1
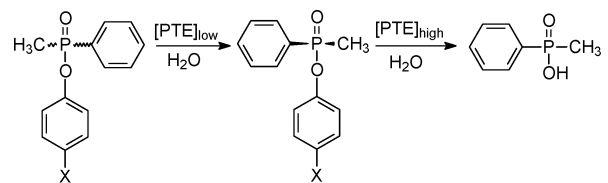
Cpd	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
X	NO_2	CHO	CN	$COCH_3$	$COOCH_3$	Cl	F	H
pK_a	7.1	7.7	8.0	8.1	8.5	9.4	9.9	10.0

(0.65 mg) was added to a 3 L solution of racemic *5* (1.00 g) in aqueous MeOH (20%) buffered with 50 mM HEPES, pH 8.0. The hydrolysis reaction was monitored by following the change in absorbance at 297 nm until the first phase of the reaction was complete after 20 min. The reaction was quenched, and the unreacted enantiomer of *5* was extracted from the reaction mixture by the addition of 200 mL of CH_2Cl_2. A general workup followed by silica gel chromatography gave a crystalline solid (0.38 g). The enantiomeric excess (ee) was quantified by chiral electrophoresis and found to be greater than 99.8%.

The chiral preference for the PTE-catalyzed hydrolysis of the phosphinate esters synthesized for this communication was established through a combination of X-ray crystallography and ^{31}P NMR spectroscopy as summarized in Scheme 2. The diastereomeric mixture of (S_P)-*11* and (R_P)-*11* in a ratio ~1:1 was prepared by the reaction of racemic methyl phenyl phosphinic chloride (*9*) with (S)-2-*tert*-butoxycarbonylamino-3-hydroxypropionic acid *tert*-butyl ester (*10*) in the presence of triethylamine. Chromatography of the crude reaction product on silica gel yielded a crystalline sample of the (R_P)-*11* diastereomer.

The structure and stereochemistry of (R_P)-*11* at the phosphorus center was established unambiguously by X-ray crystallography. The ^{31}P NMR spectrum of the mixture of diastereomers (R_P)-*11* and (S_P)-*11* shows resonances at 45.03 and 45.65 ppm of equal intensity (Figure 2a). The resonance at 45.03 ppm was assigned to

Scheme 2
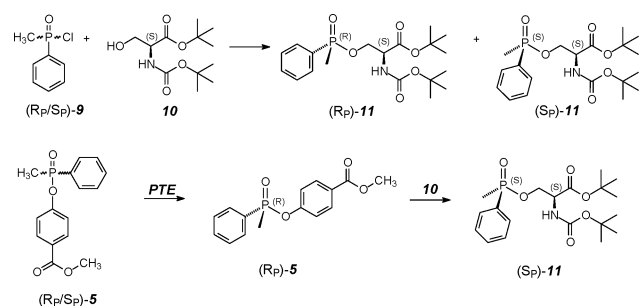
that of (R$_P$)-**11** based on an increase in the intensity of the signal after addition of authentic (R$_P$)-**11** to the diastereomeric mixture of **11** (Figure 2b). Reaction of racemic **5** with **10** catalyzed by triethylamine gives a diastereomeric mixture of (R$_P$)- and (S$_P$)-**11**. Under the same conditions, the chiral product isolated after the enzymatic resolution of racemic **5** reacts with **10** to give initially only (S$_P$)-**11** (Figure 2c). Since the reaction of **5** with **10** is expected to occur with an inversion of configuration at phosphorus,[8] the absolute stereochemistry of the product isolated from the PTE-catalyzed hydrolysis of racemic **5** is that of the (R$_P$)-configuration. Therefore, wild-type PTE preferentially hydrolyzes the series of phosphinate esters prepared for this investigation of the (S$_P$)-configuration. This observation is consistent with the known stereochemical preferences for the hydrolysis of homologous phosphate and phosphonate esters.[3]
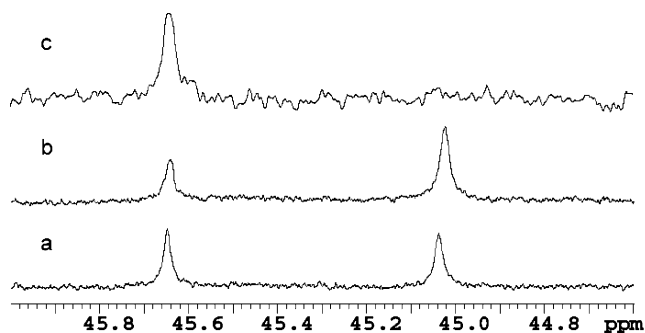
Figure 2. (a) ^{31}P NMR spectrum of (S$_P$)- and (R$_P$)-**11** from reaction of racemic **9** with **10**. (b) NMR spectrum of racemic **11** after additon of authentic (R$_P$)-**11**. (c) NMR spectrum of product isolated from the kinetic resolution of racemic **5** with the wild-type PTE after reaction with **10**.

Prior investigations have demonstrated that alterations in the inherent substrate specificity of wild-type PTE can be obtained by mutation of those amino acids that come in close contact with the substrate in the active site. A series of mutants was screened in an effort to identify a protein catalyst of the opposite stereoselectivity as possessed by the wild-type PTE. For the kinetic resolution of compound **5**, the mutant I106T/F132A/H254G/H257W (TAGW) was the best of the 10 proteins tested. This protein preferentially hydrolyzed the (R$_P$)-enantiomer of **5** over the (S$_P$)-enantiomer by a factor of 17. The catalytic properties of TAGW were quantified for the stereoselective hydrolysis of the phosphinate esters **1**−**8**, and the results are presented in Figure 3. The stereoselectivity of the mutant TAGW is not as dramatic as that of the wild-type enzyme, but this variant of PTE shows a clear preference for the hydrolysis of the (R$_P$)-enantiomer over that for the corresponding mirror image.
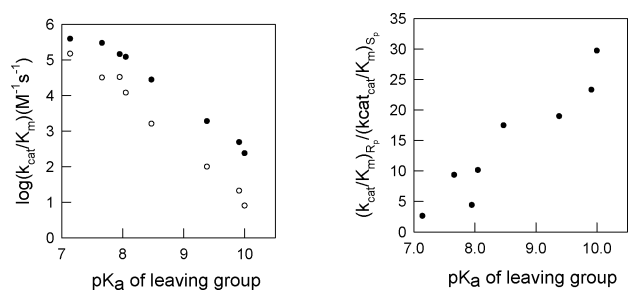
Figure 3. (a) Brønsted plot for the hydrolysis of R$_p$-(●) And S$_p$-(○) enantiomers of compounds **1-8** with mutant TAGW. (b) Stereoselectivity for the enzymatic hydrolysis of compounds **1-8**, plotted as the ratio of k_{cat}/K_m as a function of the pK_a of the leaving group phenol.

The mutant TAGW was used for the isolation of the (S$_P$)-enantiomer of **5**. TAGW (1.2 mg) was added to a solution (600 mL) of racemic **5** (200 mg) in aqueous MeOH (20%) buffered with 50 mM HEPES, pH 8.0. The hydrolysis reaction was monitored by following the change in absorbance at 270 nm. The reaction was quenched, and the unreacted enantiomer of **5** was extracted from the reaction mixture by the addition of 200 mL of CH$_2$Cl$_2$. A general workup followed by silica gel chromatography gave (S$_P$)-**5** as a crystalline solid (44 mg) with an ee > 90%.

PTE catalyzes the hydrolysis of phosphinate esters. For methyl phenyl phosphinates, the (S$_P$)-enantiomers are preferred by the wild-type PTE. Racemic phosphinate esters can be resolved enzymatically on a preparative scale to give a chiral product with high enantiomeric excess. Structural variants of PTE have been identified that possess the opposite stereoselectivity. The chiral phosphinate esters may function in the chemoenzymatic preparation of P-chiral phosphines and phosphine oxides.

Supporting Information Available: Preparation and characterization of the substrates, coordinates for the crystal structure, and ee determinations by CE. X-ray crystallographic data in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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