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Atropselective Hydrolysis of Chiral Binol-Phosphate Esters Catalyzed by the Phosphotriesterase from *Sphingobium* sp. TCM1

Dao Feng Xiang, Tamari Narindoshvili, and Frank M. Raushel*

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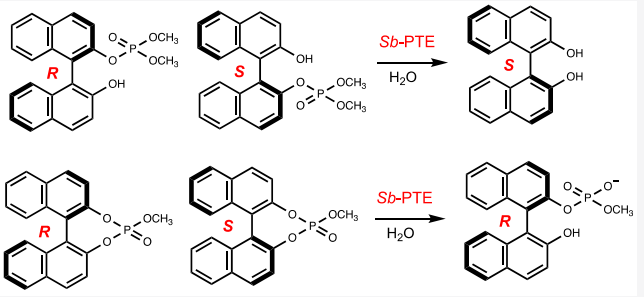
ABSTRACT: The phosphotriesterase from *Sphingobium* sp. TCM1 (*Sb*-PTE) is notable for its ability to hydrolyze a broad spectrum of organophosphate triesters, including organophosphorus flame retardants and plasticizers such as triphenyl phosphate and tris(2-chloroethyl) phosphate that are not substrates for other enzymes. This enzyme is also capable of hydrolyzing any one of the three ester groups attached to the central phosphorus core. The enantiomeric isomers of 1,1′-bi-2-naphthol (BINOL) have become among the most widely used chiral auxiliaries for the chemical synthesis of chiral carbon centers. PTE was tested for its ability to hydrolyze a series of biaryl phosphate esters, including mono- and bis-phosphorylated BINOL derivatives and cyclic phosphate triesters. *Sb*-PTE was shown to be able to catalyze the hydrolysis of the chiral phosphate triesters with significant stereoselectivity. The catalytic efficiency, k_{cat}/K_m, of *Sb*-PTE toward the test phosphate triesters ranged from ∼10 to 10^5 M^{-1} s^{-1}. The product ratios and stereoselectivities were determined for four pairs of phosphorylated BINOL derivatives.



Phosphotriesterases (PTEs) have been shown to be capable of catalyzing the hydrolysis of organophosphates with an unusual degree of regio- and stereoselectivity. For example, the PTE first isolated from *Pseudomonas dininuta* (*Pd*-PTE) catalyzes the hydrolysis of the S_P enantiomer of compound **1** ∼2 orders of magnitude faster than the hydrolysis of the R_P enantiomer with exclusive formation of *p*-acetylphenol as one of the products.[1] This observation has been interpreted to mean that the active site of this enzyme binds (S_P)-**1** in a manner that is more conducive to substrate turnover than it is for the binding of (R_P)-**1**. Consistent with this observation is the fact that mutation of a single residue in the active site, Gly-60, to an alanine enhances the stereoselective preference for the hydrolysis of (S_P)-**1** by 5 orders of magnitude.[2] Alternatively, mutation of His-257 in the active site to tyrosine effectively eliminates any stereoselectivity, whereas construction of the I106G/F132G/H257Y mutant results in a mutant enzyme that preferentially hydrolyzes the R_P enantiomer of compound **1** by 3 orders of magnitude. Thus, the identity of only four residues (G60, I106, F132, and H257) in the active site of this PTE can modulate the stereoselectivity by >8 orders of magnitude.[1]

The PTE from *Sphingobium* sp. TCM1 (*Sb*-PTE) also can hydrolyze a broad spectrum of organophosphate triesters. However, this enzyme is uniquely capable of hydrolyzing any one of the three ester groups attached to the central phosphorus core.[3,4] Thus, with (S_P)-**2**, 79% of the reaction products occur via cleavage of methanol and 21% with cyclohexanol. There is no detectable (<1%) formation of phenol.[4] Remarkably, with the R_P enantiomer, phenol is the predominant reaction product (96%) with cyclohexanol at 4% and no detectable formation of methanol. With *Sb*-PTE, the product ratios are dictated by an earlier transition state, effective protonation of the leaving group, and an active site that accommodates any one of three possible substrate binding orientations.[4,5]

Selective hydrolysis of structurally similar pairs of substrates can also arise from compounds that are sterically restricted from rotating about a carbon−carbon single bond.[6] The most common type of compound within this select group is 1,1′-bi-2-naphthol (BINOL) as illustrated with (S_P)-**3** and (R_P)-**3** in Scheme 1. These compounds are rotationally restricted with a calculated $t_{1/2}$ of 60 min at 220 °C in diphenyl ether solvent.[7] Chemical syntheses of the *R* and *S* enantiomers of BINOL have been reported, and individual enantiomers are available commercially. Such compounds are widely used as chiral auxiliaries in the chemical synthesis of chiral carbon centers.[8]

Enzymatic resolution of racemic BINOL esters has been reported.[9−14] Perhaps the most dramatic demonstration of this achievement was obtained by Kazlauskas[9] using cholesterol

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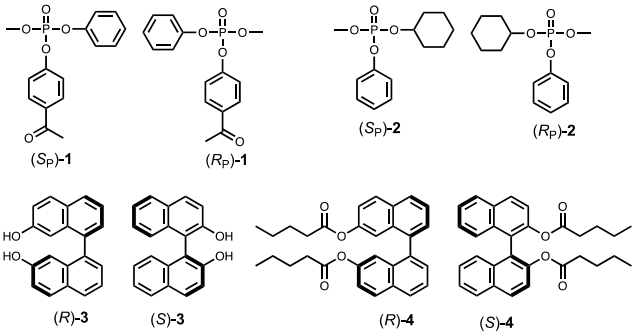
Scheme 1. Chiral Substrates for Phosphotriesterases (compounds 1 and 2), Axially Restricted BINOL (compound 3), and the Dipentanoyl Ester of Compound 3 (compound 4)



esterase to selectively hydrolyze the *S* enantiomer of compound **4** leaving the *R* enantiomer intact. From 200 g of racemic **4**, nearly 70 g each of (*S*)-**4** and (*R*)-**4** were isolated with an enantiomeric excess of 99. In this report, we have tested *Sb*-PTE for its ability to hydrolyze a series of biaryl phosphate esters. These substrates include mono- and bis-phosphorylated BINOL derivatives and cyclic phosphate esters.

MATERIALS AND METHODS

General Materials. Tryptone was purchased from Research Products International. General laboratory chemicals and supplies were obtained from Sigma/Aldrich. The NMR shift reagent Fmoc-Trp(Boc)-OH was obtained from Sigma/Aldrich. Vivaspin columns (10 kDa) were purchased from GE Healthcare.

Expression and Purification of *Sb*-PTE. The wild-type histidine-tagged *Sb*-PTE enzyme was expressed and purified from *Escherichia coli* strain BL21(DE3) as previously described.[15] After purification, *Sb*-PTE was stored in 20 mM HEPES buffer (pH 8.0), and 100 mM NaCl was added to maintain protein stability. The protein purity was judged to be >95% using Mini-PROTEIN TGX gel electrophoresis (Bio-Rad). The yield of purified *Sb*-PTE was ∼10 mg from a 1.0 L cell culture.

Synthesis of (*R*)-2′-Hydroxy[1,1′-binaphthalen]-2-yl Dimethyl Phosphate (5). To a stirred solution of dimethyl chlorophosphate (0.11 mL, 1.0 mmol, 96%, 1.0 equiv) and (*R*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous dichloromethane (8.0 mL) was added triethylamine (0.14 mL, 1.0 mmol, 1.0 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered, and after concentration, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 3:2) yielding 0.25 g (63%) of product: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 8.9 Hz, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.92 (d, J = 8.9 Hz, 1H), 7.86 (d, J = 7.9 Hz, 1H), 7.62 (d, J = 8.9 Hz, 1H), 7.54−7.48 (m, 1H), 7.41 (d, J = 8.6 Hz, 1H), 7.39−7.32 (m, 2H), 7.30−7.24 (m, 2H), 7.05 (d, J = 8.3 Hz, 1H), 6.08 (s, 1H), 3.60 (d, J = 11.4 Hz, 3H), 3.18 (d, J = 11.4 Hz, 3H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −3.06 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{22}H_{20}O_5P$ 395.1048, found 395.1038.

(*S*)-2′-Hydroxy[1,1′-binaphthalen]-2-yl Dimethyl Phosphate (5). To a stirred solution of dimethyl chlorophosphate (0.11 mL, 1.0 mmol, 96%, 1.0 equiv) and (*S*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous dichloromethane (8.0 mL) was added triethylamine (0.14 mL, 1.0 mmol, 1.0 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered, and after concentration, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 3:2) yielding 0.25 g (63%) of product after concentration: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 8.9 Hz, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.92 (d, J = 8.9 Hz, 1H), 7.86 (d, J = 7.9 Hz, 1H), 7.62 (d, J = 8.9 Hz, 1H), 7.54−7.48 (m, 1H), 7.41 (d, J = 8.6 Hz, 1H), 7.39−7.32 (m, 2H), 7.30−7.24 (m, 2H), 7.05 (d, J = 8.3 Hz, 1H), 6.08 (s, 1H), 3.60 (d, J = 11.4 Hz, 3H), 3.18 (d, J = 11.4 Hz, 3H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −3.06 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{22}H_{20}O_5P$ 395.1048, found 395.1038.

(*R*)-[1,1′-Binaphthalene]-2,2′-diyl Tetramethyl Bis-(phosphate) (6). A stirred solution of (*R*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous diethyl ether (7 mL) was chilled to −78 °C, and 1.0 mL of LDA (2.0 M in THF, 2.0 mmol, 2.0 equiv) was added. The solution was stirred for 30 min and transferred via cannula to another flask at −78 °C that contained dimethyl chlorophosphate (0.23 mL, 2.2 mmol, 96%, 2.2 equiv) in diethyl ether (20 mL). The cooling bath was removed, and the reaction mixture was allowed to warm to ambient temperature (23 °C) and stirred for 18 h. The reaction mixture was filtered and concentrated, and the residue purified by silica gel column chromatography (ethyl acetate) yielding 0.25 g (50%) of product. Synthesis of the bis-phosphorylated binol product (**6**) required a stronger base, relative to the synthesis of the monophosphorylated product (**5**), presumably because of hindered access to the intermediate after the first phosphorylation event: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 9.2 Hz, 2H), 7.82 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 9.2 Hz, 2H), 7.39−7.34 (m, 2H), 7.27−7.18 (m, 4H), 3.34 (d, J = 11.4 Hz, 6H), 3.09 (d, J = 11.4 Hz, 6H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −5.31 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{24}H_{25}O_8P_2$ 503.1025, found 503.1010.

(*S*)-[1,1′-Binaphthalene]-2,2′-diyl Tetramethyl Bis-(phosphate) (6). To a stirred solution of (*S*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous diethyl ether (7 mL) chilled to −78 °C was added 1.0 mL of LDA (2.0 M in THF, 2.0 mmol, 2.0 equiv). The solution was stirred for 30 min and transferred via cannula to another flask at −78 °C that contained dimethyl chlorophosphate (0.23 mL, 2.2 mmol, 96%, 2.2 equiv) in diethyl ether (20 mL). The cooling bath was removed, and the reaction mixture was allowed to warm to ambient temperature (23 °C) and stirred for 18 h. The reaction mixture was filtered and concentrated, and the residue purified by silica gel column chromatography (ethyl acetate) yielding 0.25 g (50%) of product: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 9.2 Hz, 2H), 7.82 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 9.2 Hz, 2H), 7.39−7.34 (m, 2H), 7.27−7.18 (m, 4H), 3.34 (d, J = 11.4 Hz, 6H), 3.09 (d, J = 11.4 Hz, 6H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −5.31 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{24}H_{25}O_8P_2$ 503.1025, found 503.1010.

(*R*)-Methyl 1,1′-Binaphthyl-2,2′-diylphosphate (7). To a stirred solution of methyl dichlorophosphate (0.26 mL, 2.2 mmol, 85%, 1.1 equiv) and (*R*)-BINOL (0.57 g, 2.0 mmol, 1.0 equiv) in anhydrous dichloromethane (10 mL) was added triethylamine (0.62 mL, 4.4 mmol, 2.2 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered, and after concentration, the residue was purified by silica gel column chromatography (hexanes/ethyl

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acetate, 3:2) yielding 0.22 g (30%) of product after concentration: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 7.7 Hz, 2H), 7.79 (d, J = 8.1 Hz, 2H), 7.63 (d, J = 8.8 Hz, 1H), 7.54−7.48 (m, 3H), 7.43−7.30 (m, 4H), 4.01 (d, J = 11.4 Hz, 3H); ^{31}P NMR (160 MHz, $CDCl_3$) δ 3.83 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{21}H_{16}O_4P$ 363.0786, found 363.0780.

(*S*)-Methyl 1,1′-Binaphthyl-2,2′-diylphosphate (7). To a stirred solution of methyl dichlorophosphate (0.26 mL, 2.2 mmol, 85%, 1.1 equiv) and (*S*)-BINOL (0.57 g, 2.0 mmol, 1.0 equiv) in anhydrous dichloromethane (10 mL) was added triethylamine (0.62 mL, 4.4 mmol, 2.2 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered, and after concentration, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 3:2) yielding 0.22 g (30%) of product after concentration: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 7.7 Hz, 2H), 7.79 (d, J = 8.1 Hz, 2H), 7.63 (d, J = 8.8 Hz, 1H), 7.54−7.48 (m, 3H), 7.43−7.30 (m, 4H), 4.01 (d, J = 11.4 Hz, 3H); ^{31}P NMR (160 MHz, $CDCl_3$) δ 3.83 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{21}H_{16}O_4P$ 363.0786, found 363.0781.

(*R*)-Phenyl 1,1′-Binaphthyl-2,2′-diylphosphate (8). To a stirred solution of phenyl dichlorophosphate (0.16 mL, 1.1 mmol, 95%, 1.1 equiv) and (*R*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous dichloromethane (10 mL) was added triethylamine (0.3 mL, 2.2 mmol, 2.2 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered and washed with 0.1 N HCl and a saturated solution of $NaHCO_3$ yielding 0.27 g (63%) of product: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 8.1 Hz, 1H), 8.04 (d, J = 9.6 Hz, 1H), 8.01−7.97 (m, 2H), 7.67(d, J = 8.7 Hz, 1H), 7.56−7.50 (m, 2H), 7.48 (d, J = 8.7 Hz, 1H), 7.46−7.29 (m, 8H), 7.27−7.21 (m, 1H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −3.44 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{26}H_{18}O_4P$ 425.0943, found 425.0931.

(*S*)-Phenyl 1,1′-Binaphthyl-2,2′-diylphosphate (8). To a stirred solution of phenyl dichlorophosphate (0.16 mL, 1.1 mmol, 95%, 1.1 equiv) and (*S*)-BINOL (0.28 g, 1.0 mmol, 1.0 equiv) in anhydrous dichloromethane (10 mL) was added triethylamine (0.3 mL, 2.2 mmol, 2.2 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered and washed with 0.1 N HCl and a saturated solution of $NaHCO_3$ yielding 0.27 g (63%) of product: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 8.1 Hz, 1H), 8.04 (d, J = 9.6 Hz, 1H), 8.01−7.97 (m, 2H), 7.67 (d, J = 8.7 Hz, 1H), 7.56−7.50 (m, 2H), 7.48 (d, J = 8.7 Hz, 1H), 7.46−7.29 (m, 8H), 7.27−7.21 (m, 1H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −3.44 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{26}H_{18}O_4P$ 425.0943, found 425.0931.

Dimethyl Naphthalen-2-yl Phosphate (9). To a stirred solution of dimethyl chlorophosphate (0.36 mL, 3.3 mmol, 96%, 1.1 equiv) and naphthalen-2-ol (0.43 g, 3.0 mmol, 1.0 equiv) in anhydrous dichloromethane (15 mL) was added triethylamine (0.46 mL, 3.3 mmol, 1.1 equiv), and the mixture was stirred for 18 h at room temperature (23 °C). The reaction mixture was filtered and concentrated, and the residue purified by silica gel column chromatography (hexanes/ethyl acetate, 2:1) yielding 0.41 g (54%) of a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88−7.80 (m, 3H), 7.71−7.69 (m, 1H), 7.54−7.44 (m, 2H), 7.38 (dd, J_1 = 2.1 Hz, J_2 = 8.9 Hz, 1H), 3.91 (d, J = 11.4 Hz, 6H); ^{31}P NMR (160 MHz, $CDCl_3$) δ −4.00 (s); HRMS (ESI$^+$) m/z [M + H]$^+$ calcd for $C_{12}H_{14}O_4P$ 253.0630, found 253.0632.

Activity Screening of *Sb*-PTE with Binol Esters. To determine if *Sb*-PTE is capable of hydrolyzing the binol phosphate esters, the hydrolysis products from compounds **5**−**9** after incubation with *Sb*-PTE were analyzed by ^{31}P NMR spectroscopy. The enzymatic reaction for each substrate was conducted in 50 mM HEPES buffer (pH 8.5) containing 100 μM $MnCl_2$. DMSO [20% (v/v)] was included in the reactions to increase the solubility of the binol esters. The reaction mixtures were applied to a Vivaspin 500 column (GE Healthcare) to remove the protein after the reaction mixture was incubated (3−24 h) in glass vials at 30 °C. The ^{31}P NMR spectrum was obtained after the addition of 10 mM EDTA and 10% D_2O. For each enzymatic reaction, a control without the addition of *Sb*-PTE was conducted concurrently. The catalytic activities of *Sb*-PTE toward the hydrolysis of compounds **5**−**9** and the ratios of multiple products from a single substrate were determined.

Determination of the Enantioselective Activity of *Sb*-PTE. To enable the identification of the specific enantiomers of the phosphate esters hydrolyzed by *Sb*-PTE, ^{31}P NMR analysis was employed. The specific enantiomer being preferentially hydrolyzed from a racemic mixture by *Sb*-PTE was identified by isolating the remaining substrate from the reaction mixture and then conducting ^{31}P NMR analysis after the addition of the chiral shift reagent Fmoc-Trp(Boc)-OH to the sample. For compound **5**, the reaction was conducted in a reaction volume of 50 mL with 0.15 mM racemic **5**, 100 μM $MnCl_2$, 20% DMSO, 50 mM HEPES (pH 8.0), and 0.6 μM *Sb*-PTE. The progress of the reaction was monitored by removing small aliquots and measuring the absorbance at 334 nm. At various time points, a 10 mL sample was removed from the reaction mixture and the unreactive substrate in the reaction mixture was extracted using ethyl acetate (3 × 20 mL). The organic phase was washed with water to remove DMSO and dried over anhydrous Na_2SO_4, and the solvent removed by rotary evaporation. The remaining substance was dissolved in 750 μL of $CDCl_3$ with addition of 200 mg of Fmoc-Trp(Boc)-OH, and the ^{31}P NMR spectrum was then recorded. For compound **7**, the enantiomer being preferentially hydrolyzed by *Sb*-PTE was also determined by ^{31}P NMR spectroscopy. Conditions and purifications were as described above for compound **5** except 100 μM racemic compound **7** and 0.3 μM *Sb*-PTE were used.

Determination of Extinction Coefficients. The extinction coefficients used to calculate the catalytic efficiency of *Sb*-PTE toward the hydrolysis of substrates **5**−**8** were determined in 50 mM HEPES at pH 8.5. Each substrate was dissolved in DMSO and then diluted to 20−100 μM in 50 mM HEPES (pH 8.5) containing 100 μM $MnCl_2$ and 20% DMSO. The ultraviolet−visible spectra of the solutions were recorded between 240 and 400 nm. The same concentrations of the substrate were completely hydrolyzed by *Sb*-PTE under the same reaction conditions, and the spectrum was recorded. The two spectra were subtracted from each other, identifying 334 nm as being optimal with a delta extinction coefficient, $\Delta\varepsilon_{334}$, of 3520 M^{-1} cm^{-1} for compound **5**; 334 nm with a delta extinction coefficient, $\Delta\varepsilon_{334}$, of 5240 M^{-1} cm^{-1} for compound **6**; 310 nm with a delta extinction coefficient, $\Delta\varepsilon_{310}$, of 7400 M^{-1} cm^{-1} for compound **7**; and 310 nm with a delta extinction coefficient, $\Delta\varepsilon_{310}$, of 12450 M^{-1} cm^{-1} for compound **8**.

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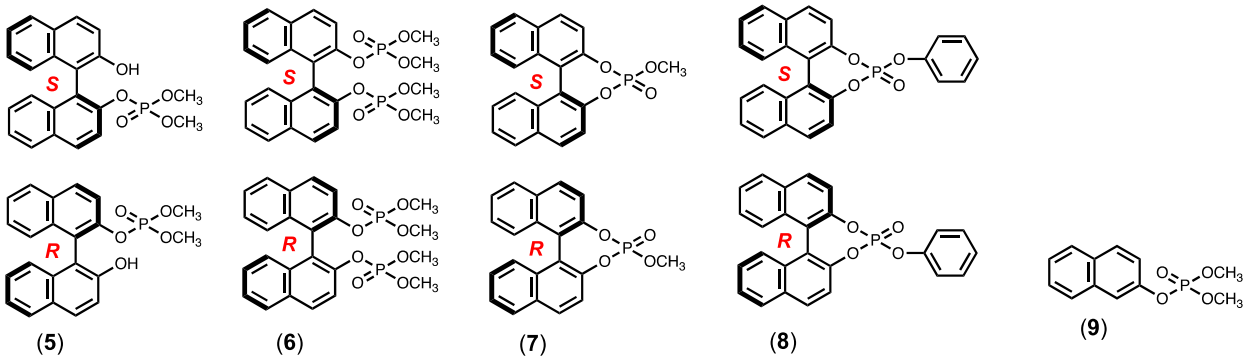
Scheme 2. Biaryl Phosphate Derivatives Tested as Substrates for *Sb*-PTE



Measurement of Kinetic Constants. Due to the limited solubility of compounds **5**−**9**, total hydrolysis with low concentrations of the pure enantiomers of these substrates was employed to determine the catalytic efficiency (k_{cat}/K_m) of *Sb*-PTE for these substrates. In preliminary experiments, we demonstrated that neither dimethyl phosphate (**17**) nor binol (**3**) is inhibitory for the hydrolysis of an equivalent concentration of diethyl *p*-nitrophenyl phosphate (paraoxon). For compounds **5**−**8**, total hydrolysis reactions were conducted in a volume of 1.0 mL containing 50 mM HEPES (pH 8.5), 100 μM $MnCl_2$, and 20% DMSO. Stock solutions of substrates were made in DMSO with final concentrations between 5 and 100 μM in the actual assays. All reactions were followed to completion in 1.0 cm cuvettes using a Molecular Devices Spectramax 364 plus spectrophotometer. The fraction of substrate hydrolyzed was plotted as a function of time. The time courses were fitted to eq 1

$$F = a(1 - e^{-kt}) \quad (1)$$

where F is the fraction hydrolyzed, a is the magnitude of the exponential phase, t is the time, and k is the rate constant. All assays were performed in triplicate at a single fixed substrate concentration (except where noted). The assays were conducted at pH 8.5 because previous experiments have shown that *Sb*-PTE exhibits optimal catalytic activity for the hydrolysis of paraoxon above pH 8.0.[5]

For compound **9**, the catalytic activities of *Sb*-PTE were measured by anion exchange chromatography. The hydrolysis of compound **9** was conducted in a reaction mixture containing 100 μM compound **9** and 1.8 μM *Sb*-PTE in 50 mM HEPES (pH 8.5) containing 100 μM $MnCl_2$ and 20% DMSO. A 150 μL aliquot was injected onto a prepacked 1 mL HiTrap Q HP anion exchange column (GE Healthcare) at different time points to measure the change in product formation at 255 nm. The elution buffer was 20 mM HEPES (pH 8.5) containing 1.0 M NaCl. The reactions were followed to completion, and the fraction of product formed was plotted as a function of time. The value of $^{T}(k_{cat}/K_m)$ was obtained using eq 2

$$^{T}(k_{cat}/K_m) = k/E_t \quad (2)$$

When multiple products are observed, the specific value of k_{cat}/K_m for the formation of each product [$^{X}(k_{cat}/K_m)$ and $^{Y}(k_{cat}/K_m)$] was determined from the experimentally determined product ratios.[15]

RESULTS AND DISCUSSION

Catalytic Properties of *Sb*-PTE with Chiral Binol Phosphate Esters. The Mn^{2+}-dependent phosphotriesterase from *Sphingobium* sp. strain TCM1 (*Sb*-PTE) was previously reported to be capable of hydrolyzing a broad spectrum of substrates, including organophosphate plasticizers, flame retardants, and pesticides.[16] Here we have measured the catalytic properties of *Sb*-PTE for the hydrolysis of four pairs of chiral binol phosphate esters (compounds **5**−**8**) and achiral building block **9** (Scheme 2). The enantiomeric preferences of *Sb*-PTE for the hydrolysis of (*R*/*S*)-**5** and (*R*/*S*)-**7** were determined by ^{31}P NMR spectroscopy. The enantiomers of racemic **5** were differentiated from one another using the chiral shift reagent Fmoc-Trp(Boc)-OH, as illustrated in Figure 1A, where (*S*)-**5** resonates downfield of (*R*)-**5** (Figure 1B). When *Sb*-PTE is added to (*R*/*S*)-**5**, the *S* enantiomer is hydrolyzed significantly faster than is the *R* enantiomer (Figure 1C−E).

The same approach was utilized for the hydrolysis of (*R*/*S*)-**7**. The enantiomers of **7** were also differentiated from one another by ^{31}P NMR spectroscopy after the addition of the chiral shift reagent Fmoc-Trp(Boc)-OH (Figure 2A). (*S*)-**7** resonates at 3.665 ppm, whereas the (*R*)-**7** enantiomer resonates at 3.645 ppm (Figure 2B). The addition of *Sb*-PTE demonstrated that this enzyme hydrolyzes the *R* enantiomer significantly faster than the *S* enantiomer (Figure 1C,D). With racemic mixtures of compounds **6** and **8**, there were no significant differences in the overall rates of hydrolysis of the enantiomers as measured by ^{31}P NMR spectroscopy.

Determination of Product Ratios. Previously, it was shown that *Sb*-PTE can hydrolyze any of the three ester groups contained within a suitably activated organophosphate triester substrate.[3,4] Therefore, it was imperative to determine the product ratios by ^{31}P NMR spectroscopy for each of the targeted substrates **5**−**9**. The structures for the possible products are depicted in Scheme 3. For substrate **5**, the two possible products are **3**/**17** and **10**. For the hydrolysis of (*R*)-**5**, dimethyl phosphate (**17**) is the major product (91%) and product **10** (9%) is the minor product (Figure S1A,B). With the (*S*)-**5** enantiomer, the only product identified was dimethyl phosphate (Figure S1C,D). For substrate **7**, the two possible products are **10** and **13**. With the faster enantiomer, (*R*)-**7**, the only observable product is **10**, formed from the hydrolysis of the naphthyl substituent to the phosphorus (Figure S2A,B). In contrast, with the slower (*S*)-**7** substrate, the two possible products are formed in a 90:10 **10**:**13** ratio (Figure S2C,D). The enantiomers of substrate **8** are hydrolyzed at nearly the same rate, and the two possible products are **14** and **13**. With

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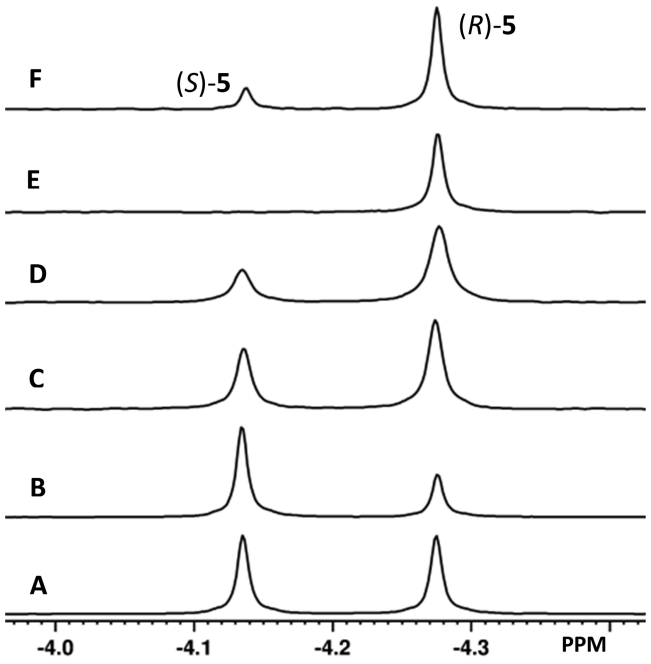

Figure 1. ^{31}P NMR spectra for the hydrolysis of (*R*/*S*)-**5** by *Sb*-PTE. (A) Racemic mixture of compound **5** in the presence of added Fmoc-Trp(Boc)-OH. (B) Mixture of (*S*)-**5** (−4.14 ppm) and (*R*)-**5** (−4.27 ppm) in a molar ratio of 2:1. (C) One hour after the addition of *Sb*-PTE to (*R*/*S*)-**5**. (D) Three hours after the addition of *Sb*-PTE to (*R*/*S*)-**5**. (E) Eight hours after the addition of *Sb*-PTE to (*R*/*S*)-**5**. (F) Spectrum E spiked with (*R*/*S*)-**5**.

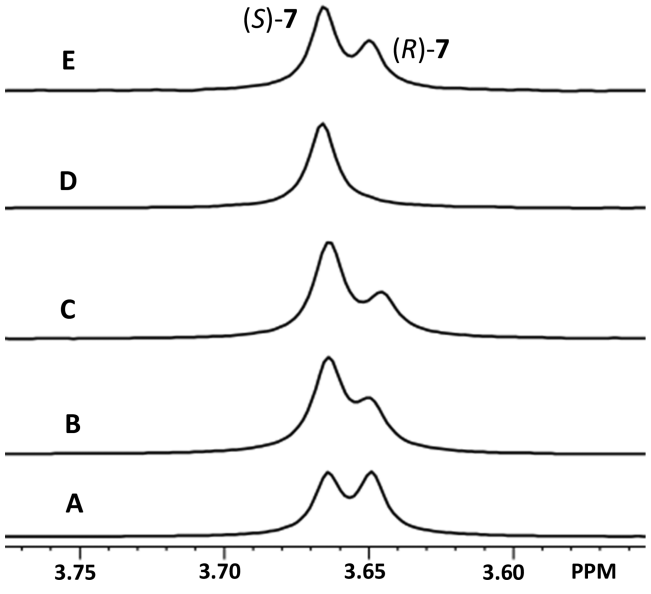

Figure 2. ^{31}P NMR spectra for the hydrolysis of (*R*/*S*)-7 by *Sb*-PTE. (A) Racemic mixture of 7 in the presence of added Fmoc-Trp(Boc)-OH. (B) Mixture of (*S*)-7 (at 3.665 ppm) and (*R*)-7 (at 3.645 ppm) in a molar ratio of 2:1. (C) Ten minutes after the addition of *Sb*-PTE to (*R*/*S*)-7. (D) One hundred ten minutes after the addition of *Sb*-PTE to (*R*/*S*)-7. (E) Spectrum D spiked with racemic 7.

(*R*)-**8**, the only observable product is **14** (Figure S3A,B). However, with the (*S*)-**8** enantiomer, the two products are formed in a 93:7 (*S*)-**14**:(*S*)-**13** ratio (Figure S3C,D).

The hydrolyses of the bisphosphorylated binol esters, (*S*)-**6** and (*R*)-**6**, are a bit more complicated because the two possible initial products (**5** and **11**) can be hydrolyzed a second time, potentially forming a mixture with **3**/**17**, **10**, and **12**. With (*S*)-**6**, the primary reaction product identified in the ^{31}P NMR spectrum of the reaction mixture is dimethyl phosphate (**17**) as illustrated in panels C and D of Figure S4. There are, however, two smaller resonances of equal intensity at −4.28 and −4.54 ppm (6.6% of the total phosphorus integration), which are due to the separate resonances for the two phosphoryl groups of product **11** (Figure 3C,D). These two resonances are also found in the control sample incubated for the same amount of time, but without the addition of enzyme (6.3% of total phosphorus integration) as illustrated in Figure 3C and Figure S4C. Therefore, the initial rate of formation of (*S*)-**11** is <1% of the rate of formation of (*S*)-**5** from the hydrolysis of (*S*)-**6**. For the enzymatic hydrolysis of (*R*)-**6**, the control spectrum (Figure 3A and Figure S4A) also exhibits the non-enzymatic formation of (*R*)-**11** (6.1% of the phosphorus integration). In the ^{31}P NMR spectrum for the enzyme-catalyzed reaction mixture, the relative integration for (*R*)-**11** is 7.6% (Figure 3B and Figure S4B). Because the subsequent enzymatic hydrolysis of the initial product, (*R*)-**5**, is slower than the hydrolysis of (*R*)-**6**, the accumulation of (*R*)-**5** is observed at −4.39 ppm (25.2%). A small amount of (*R*)-**10** (2.4%) is observed at −3.65 ppm. This product is formed from the enzymatic hydrolysis of (*R*)-**5**. Therefore, for the enzymatic hydrolysis of (*R*)-**6** by *Sb*-PTE, the initial product is >98% (*R*)-**5**. There is no evidence to suggest that either (*R*)-**11** or (*S*)-**11** is a substrate for *Sb*-PTE.

For hydrolysis of naphthyl dimethyl phosphate (**9**), two products are observed in the NMR spectrum of the reaction mixture (Figure S5B). The primary product (**16**) results from the hydrolysis of one of the two methyl groups from the substrate, and the other product is dimethyl phosphate (**17**). The two products are formed in an 81:19 ratio.

Measurement of Kinetic Constants. The kinetic constants for the enzymatic hydrolysis of compounds **5**−**9** are listed in Table 1. Due to the limited solubility of compounds **5**−**9** in water, the values of k_{cat}/K_m were determined by measuring the first-order rate constant for the complete hydrolysis of each substrate at low substrate concentrations. Typical time courses for the hydrolysis of compounds (*S*)-7 and (*R*)-7 are presented in Figure S6. The value of k_{cat}/K_m was obtained by dividing the first-order rate constant for hydrolysis by the enzyme concentration (eq 2). The value of k_{cat}/K_m for hydrolysis of the *S* enantiomer of compound **5** (5.3×10^2 M^{-1} s^{-1}) is ∼72 times higher than that for hydrolysis of the *R* enantiomer (7.4 M^{-1} s^{-1}). In contrast, the value of k_{cat}/K_m for hydrolysis of the *R* enantiomer of compound 7 (1.1×10^4 M^{-1} s^{-1}) is ∼58 times higher than that for hydrolysis of the *S* enantiomer (1.9×10^2 M^{-1} s^{-1}). The values of k_{cat}/K_m for hydrolysis of (*R*)-**8** and (*S*)-**8** are 2.9×10^4 and 6.4×10^4 M^{-1} s^{-1}, respectively. The values of k_{cat}/K_m for hydrolysis of (*R*)-**6** and (*S*)-**6** are 53 and 49 M^{-1} s^{-1}, respectively. Compound **9** has a k_{cat}/K_m of 1.1×10^3 M^{-1} s^{-1} when hydrolyzed by *Sb*-PTE.

CONCLUSIONS

The eight chiral binol phosphate esters tested as potential substrates for *Sb*-PTE were shown to be active. The values of k_{cat}/K_m ranged from a low of ∼10 M^{-1} s^{-1} for (*R*)-**5** to a high that approached 10^5 M^{-1} s^{-1} for (*S*)-**8**. The best substrate identified thus far for *Sb*-PTE is triphenyl phosphate with a reported k_{cat}/K_m of 2×10^6 M^{-1} s^{-1}.[16] Stereoselective

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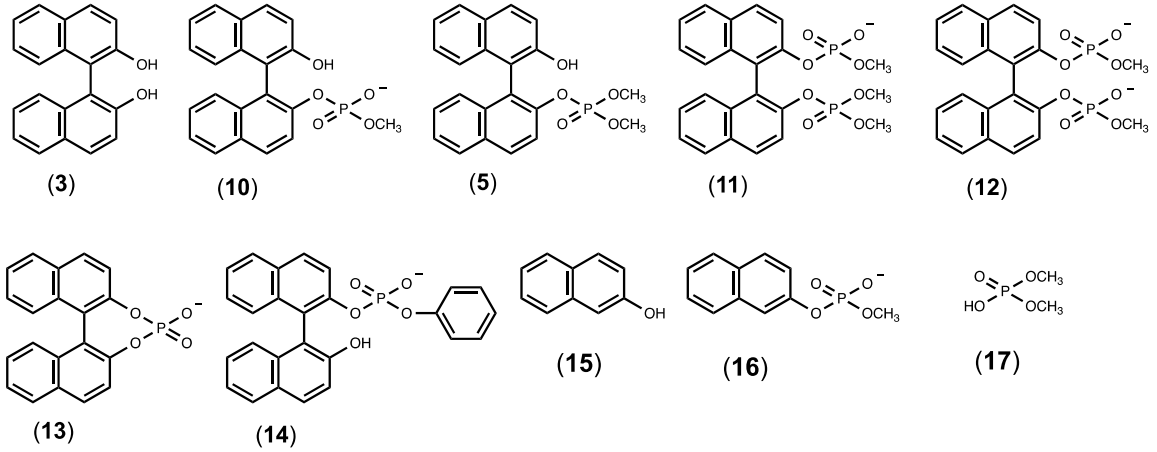
Scheme 3. Structures of Products from the Hydrolysis of Substrates Depicted in Scheme 2



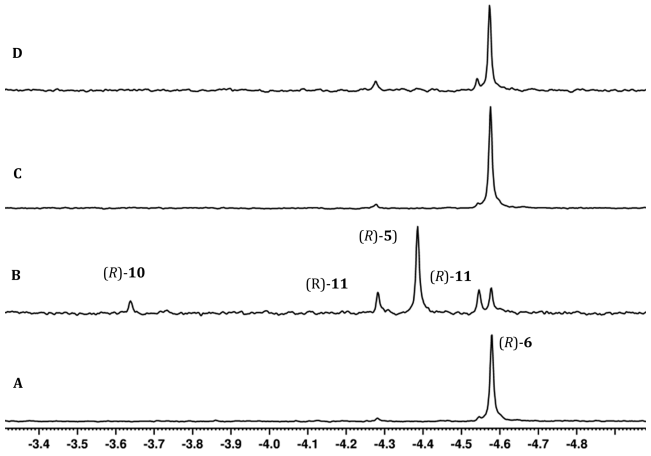


Figure 3. Selected region of the ^{31}P NMR spectra for the product from the *Sb*-PTE hydrolysis of (*R*)-**6** and (*S*)-**6**. (A) Control sample for (*R*)-**6**. (B) Product distribution after the addition of *Sb*-PTE. (C) Control sample for (*S*)-**6**. (D) Product distribution after the addition of *Sb*-PTE. The full ^{31}P NMR spectra are provided in Figure S4. Additional details are provided in the text.

hydrolysis was observed for hydrolysis of the enantiomers of compounds **5** and **7**. The *S* enantiomer of **5** was preferred by a factor of 72, whereas with compound **7**, the *R* enantiomer was preferred by a factor of ∼58. Unfortunately, it is not possible at this stage to rationalize why *Sb*-PTE prefers the *S* enantiomer for hydrolysis of the acyclic phosphate ester (compound **5**) but prefers the hydrolysis of the *R* enantiomer for hydrolysis of the cyclic phosphate ester (compound **7**). The three-dimensional structure of *Sb*-PTE is that of a seven-bladed β-propeller with a binuclear metal center embedded in the central core at the top of the propeller.[17] However, a structure of *Sb*-PTE has not been determined in the presence of a bound ligand. The experiments reported here demonstrate that *Sb*-PTE can be used to kinetically resolve small quantities of racemic phosphate esters from one another. No discrimination of the stereoisomers was observed for the hydrolysis of compound **6** or **8**.

Sb-PTE is known for its ability to hydrolyze any one of three ester groups of an activated organophosphate triester substrate.[3,4] To the best of our knowledge, this is the only known phosphotriesterase with this unique catalytic property. For example, *Sb*-PTE hydrolyzes the R_P enantiomer of cyclohexyl, phenyl, *p*-nitrophenyl phosphate with the following product distribution: phenol (55%), *p*-nitrophenol (38%), and cyclohexanol (7%). With the S_P enantiomer, the product distribution is phenol (8%), *p*-nitrophenol (84%), and cyclohexanol (7%). With the binol phosphate esters employed here, the predominant product is always hydrolysis of the binol ester. With (*R*)-**5**, (*S*)-**7**, and (*S*)-**8**, ≤10% of the alternate product is observed. In contrast, with the naphthyl phosphate ester (**9**), the predominant product is due to the hydrolysis of either of the two methyl groups (81%). This observation must reflect the differential binding of these compounds in the active site of *Sb*-PTE. Given the broad substrate specificity of *Sb*-PTE, it is likely that other axially chiral phenols will be

Table 1. Kinetic Constants for the Hydrolysis of Compounds 5−9[a]

substrate	product ratio X:Y	$^{T}(k_{cat}/K_a)$ (M^{-1} s^{-1})	$^{X}(k_{cat}/K_a)$ (M^{-1} s^{-1})	$^{Y}(k_{cat}/K_a)$ (M^{-1} s^{-1})
(*R*)-**5** (50 μM)	(*R*)-**3**:(*R*)-**10**, 91:9	7.4 ± 0.3	6.7 ± 0.3	0.7 ± 0.1
(*S*)-**5** (50 μM)	(*S*)-**3**:(*S*)-**10**, 100:0	(5.3 ± 0.2) × 10^2	(5.3 ± 0.2) × 10^2	<10
(*R*)-**6** (50 μM)	(*R*)-**5**:(*R*)-**11**, 100:0	(5.3 ± 0.1) × 10	(5.3 ± 0.1) × 10	<1
(*S*)-**6** (50 μM)	(*S*)-**5**:(*S*)-**11**, 100:0	(4.9 ± 0.1) × 10	(4.9 ± 0.1) × 10	<1
(*R*)-**7** (25−100 μM)	(*R*)-**10**:(*R*)-**13**, 100:0	(1.1 ± 0.1) × 10^4	(1.1 ± 0.1) × 10^4	<2 × 10^2
(*S*)-**7** (25−100 μM)	(*S*)-**10**:(*S*)-**13**, 90:10	(1.9 ± 0.1) × 10^2	(1.7 ± 0.2) × 10^2	(1.9 ± 0.2) × 10
(*R*)-**8** (5.0 μM)	(*R*)-**14**:(*R*)-**13**, 100:0	(2.9 ± 0.2) × 10^4	(2.8 ± 0.2) × 10^4	<6 × 10^2
(*S*)-**8** (5.0 μM)	(*S*)-**14**:(*S*)-**13**, 93:7	(6.4 ± 0.2) × 10^4	(6.0 ± 0.2) × 10^4	(4.5 ± 0.5) × 10^3
9 (100 μM)	**15**:**16**, 19:81	(1.1 ± 0.1) × 10^3	(2.1 ± 0.2) × 10^2	(8.9 ± 0.9) × 10^2

[a]All enzymatic reactions were conducted in 50 mM HEPES (pH 8.5) containing 100 μM $MnCl_2$ and 20% (v/v) DMSO at 30 °C. The concentration of the substrate used for the kinetic measurements is provided in the first column.

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differentially recognized as substrates for this enzyme. To the best of our knowledge, this is the only phosphatase that has been demonstrated to differentially hydrolyze chiral binol phosphate esters.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00831.

NMR spectra of reaction products and time courses for the hydrolysis of (*S*)-7 and (*R*)-7 (PDF)

Accession Codes

Sb-PTE, UniProt A0A077JBW9.

■ AUTHOR INFORMATION

Corresponding Author

Frank M. Raushel − *Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States;* orcid.org/0000-0002-5918-3089; Email: raushel@tamu.edu

Authors

Dao Feng Xiang − *Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States*

Tamari Narindoshvili − *Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.biochem.0c00831

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Notes

The authors declare no competing financial interest.

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