Published in final edited form as:

Biochemistry. 2018 October 30; 57(43): 6219–6227. doi:10.1021/acs.biochem.8b00935.

Substrate Profile of the Phosphotriesterase Homology Protein from *Escherichia coli*

Venkatesh V. Nemmara^Ω, Dao Feng Xiang^Ω, A. A. Fedorov^Φ, E. V. Fedorov^Φ, Jeffrey B. Bonanno^Φ, Steven C. Almo^Φ, and Frank M. Raushel^Ω

^ΩDepartment of Chemistry, Texas A&M University, College Station, Texas 77843

^ΦDepartment of Biochemistry, Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Abstract

The phosphotriesterase homology protein (PHP) from *Escherichia coli* is a member of a family of proteins that is related to phosphotriesterase (PTE), a bacterial enzyme from cog1735 with unusual substrate specificity towards the hydrolysis of synthetic organic phosphates and phosphonates. PHP was cloned, purified to homogeneity, and functionally characterized. The three-dimensional structure of PHP was determined at a resolution of 1.84 Å with zinc and phosphate in the active site. The protein folds as a distorted (β/α)₈-barrel and possesses a binuclear metal center in the active site. The catalytic function and substrate profile of PHP was investigated using a structure-guided approach that combined bio-informatics, computational docking, organic synthesis and steady-state enzyme kinetics. PHP was found to catalyze the hydrolysis of phosphorylated glyceryl acetates. The best substrate was 1,2-diacetyl glycerol-3-phosphate with a k_{cat}/K_m of $4.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The presence of a phosphate group in the substrate was essential for enzymatic hydrolysis by the enzyme. It was surprising, however, to find that PHP was unable to hydrolyze any of the lactones tested as potential substrates, unlike most of the other enzymes from cog1735.

Graphical Abstract

Corresponding Authors: raushel@tamu.edu; steve.almo@einstein.yu.edu.

Supporting Information

Details for the chemical synthesis of compounds **2–23** is given in the Supporting Information along with the structures of all compounds tested as substrates for PHP. Also included are the computationally docked possess of high-energy reaction intermediates in the active site of PHP, amino acid sequence alignments, and time courses for enzyme-catalyzed hydrolysis reactions. The Supporting Information is available free of charge on the ACS Publication website at DOI: 10.1021/acs.biochem.8b00935.

The authors declare no competing financial interest.

INTRODUCTION

Advances in genome sequencing technology have enabled the exponential growth in the number of protein sequences in the public databases (1). However, this expansion of available protein sequences presents challenges with regard to systematically determining the function of the associated proteins. A major problem is that the enzymatic functions of many proteins in GenBank are incorrect because of erroneous computational-based annotations of the closest sequenced homologues (2–4). The determination of the catalytic properties for proteins that are biochemically uncharacterized (with unknown or wrongly annotated function) is quite challenging. The challenge of functional discovery has evolved toward the development of comprehensive strategies for annotating enzymes of unknown function through a combination of bioinformatics, crystallography, computational docking, and focused chemical library synthesis for experimental validation of enzyme function. This methodology has been applied to the elucidation of function for many enzymes within the amidohydrolase superfamily (AHS) (5–8).

The amidohydrolase superfamily was first identified by Holm and Sander, on the basis of similarities in the three-dimensional structures of phosphotriesterase, adenosine deaminase and urease (9). Enzymes belonging to this superfamily possess a remarkable functional diversity and have been shown to catalyze the hydrolysis of amide and ester bonds in carbohydrates, peptides and nucleic acids (10). NCBI has subdivided the AHS into 24 clusters of orthologous groups (COG) (11). Enzymes from cog1735 have been previously found to possess an array of catalytic activities that includes the hydrolysis of organophosphate triesters (12), homoserine lactones (13), γ - and δ -lactones (14), phosphorylated sugar lactones (15), and carboxylate esters (8, 16).

Sequence similarity networks (SSN) for cog1735 at BLAST *E*-value cutoffs of 10^{-70} and 10^{-40} are shown in Figures 1a and 1b for approximately 450 proteins (17). At an *E*-value stringency of 10^{-70} , the proteins in cog1735 are partitioned into 18 subgroups that have been arbitrarily color coded and numbered. Proteins from Subgroup 2 have been structurally characterized (PDB id: 3RHG) and catalyze the hydrolysis of methylphosphonate esters (8).

Proteins from Subgroup 3 catalyze the hydrolysis of *N*-acyl homoserine lactones (18, 19) and proteins from Subgroup 4 are esterases with a broad substrate specificity (16). Representative proteins from Subgroup 5, Lmo2620 from *Listeria monocytogenes* (PDB id: 3PNZ) and Bh0225 from *Bacillus halodurans* are phosphorylated sugar lactonases that catalyze the hydrolysis of D-lyxono-1,4-lactone-5-phosphate and L-ribono-1,4-lactone-5-phosphate, respectively (15). Proteins belonging to Subgroup 6, (MS53_0025 (PDB id: 3OVG) from *Mycoplasma synoviae* and MAG_6390 from *Mycoplasma agalactiae* PG2), are also sugar lactonases that catalyze the hydrolysis of D-xylono-1,4-lactone-5-phosphate and L-arabino-1,4-lactone-5-phosphate (20). Subgroup 7 contains proteins that are lactonases, which effectively hydrolyze alkyl substituted γ - and δ -lactones (14). Proteins with phosphotriesterase activity belong to Subgroup 8 with PTE from *Pseudomonas diminuta* as the representative example. PTE can catalyze the hydrolysis of organophosphate and phosphonate esters including the chemical warfare nerve agents GB, GD and VX (21). The enzyme Sso2522 from the hyperthermophilic *Sulfolobus solfataricus* belongs to the Subgroup 9 and has a very weak phosphotriesterase activity, but can efficiently catalyze the hydrolysis of lactone substrates (22).

The present investigation is focused on characterizing an enzyme from Subgroup 1, the phosphotriesterase homology protein (PHP) from *Escherichia coli*. PHP exhibits 28% sequence identity and 66% sequence similarity to PTE from *P. diminuta* (23). Buchbinder *et al.* published the first crystal structure of PHP at a resolution of 1.7 Å resolution (23). Unlike PTE, which functions as a dimer in solution, PHP was found to exist as a monomer. PHP differs from PTE in its catalytic function as this protein could not be demonstrated to possess phosphatase, phosphotriesterase, anhydrase, peptidase or sulfatase activities with a broad array of potential substrates (23). The gene for PHP is located among a cluster of five other genes in *Escherichia coli* K-12 MG1655 (Figure 1c), which, have been predicted to code for a PLP-dependent racemase (*yhfX*), a PLP-dependent lyase (*yhfS*) a sugar isomerase (*yhfW*) and a transport protein (*yhfT*). This gene cluster is conserved in a number of other Gram-negative species, including *Shigella boydii*, *Escherichia albertii*, *Citrobacter koseri*, and *Enterobacter lignolyticus*, among others (www.microbesonline.org).

Overexpression of PHP was found to restore the growth of a strain of *E. coli*, which lacked 4-phosphoerythronate dehydrogenase (PdxB), an essential enzyme involved in the biosynthesis of PLP (24). Kim *et al.* utilized the expression vectors cloned within the ASKA collection of *E. coli* ORFs to identify seven genes, the overexpression of which restored the growth of PdxB knockout strain in minimal media. These genes acted via serendipitous pathways to elevate the metabolic flux leading to the synthesis of PLP (24). Over-production of three proteins, HisB, PHP, or YjbQ complemented the PdxB deletion strain of *E. coli* by producing a novel source of 2-oxo-3-hydroxy-4-phosphobutanoate, which is the product of the reaction catalyzed by PdxB in the PLP biosynthetic pathway. Interestingly, none of the three enzymes have the enzymatic activity catalyzed by PdxB.

Here, we describe the structural and functional characterization of *E. coli* PHP. We utilized a combination of structure-based docking and biochemical screens of a focused chemical library to determine the substrate profile for PHP. We purified PHP to homogeneity and

determined its three-dimensional structure at a resolution of 1.84 Å. The best substrate identified in this investigation was found to be 1,2-diacetyl glycerol-3-phosphate.

MATERIALS and METHODS

Materials.

LB broth was purchased from Research Products International Corp (Mount Prospect, IL). HisTrap HP and gel filtration chromatographic columns were purchased from GE Healthcare (Chicago, IL). *E. coli* BL21 (DE3) competent cells were obtained from Stratagene (San Diego, CA). The standards for inductively coupled plasma emission mass spectrometry (ICP-MS) determination of the metal content of the isolated proteins were purchased from Inorganic Ventures Inc (Christiansburg, VA). The kit for the enzymatic determination of acetate was purchased from Megazyme (UK). Synthetic procedures and structures of all compounds tested for catalytic activity of PHP are shown in the Supporting Information. All other buffers, purification reagents, and chemicals used in this investigation were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Cloning, Expression, and Purification of PHP.

The PHP gene was cloned from the *E. coli* K-12 genome and inserted into a pET30a (+) vector using the *NdeI* and *EcoRI* restriction sites. The plasmid encoding the *php* gene was transformed into *E. coli* BL21 (DE3) competent cells (Invitrogen) and plated on LB agar. A single colony was used to inoculate a 5-mL culture and allowed to grow overnight at 37 °C. The overnight culture was used to inoculate 1 L of LB medium containing 50 µg/mL kanamycin, which was grown with shaking at 30 °C until an OD₆₀₀ of ~0.6 was reached. The cells were supplemented with 1.0 mM ZnCl₂ or 1.0 mM MnCl₂ and induced with 0.5 mM IPTG. After growing for an additional 16 h, the cells were harvested by centrifugation and stored at –80 °C. For purification, the frozen cell pellets were thawed, resuspended in 50 mM HEPES, pH 8.0, and then disrupted by sonication (5 s pulses for 30 min) at 0 °C. After centrifugation, the nucleic acids were removed by addition of 20 mL of 2% (w/v) solution of protamine sulfate in 50 mM HEPES, pH 8.0. The supernatant was further fractionated via 40–60% saturation of ammonium sulfate. The precipitated protein was resuspended in 50 mM HEPES, pH 8.0, and was loaded onto a Hiload 26/60 Superdex-200 gel filtration column (GE Healthcare). Protein was eluted at a flow rate of 1.0 mL/min. The fractions corresponding to the target protein were pooled and loaded onto a RESOURCE™ Q anion exchange column (GE Healthcare). Fractions were eluted with a linear gradient of NaCl in 50 mM HEPES, pH 8.0. The purity of the protein was estimated to be greater than 90% based on SDS-PAGE analysis. The metal content of the purified protein was determined using ICP-MS. For the ICP-MS measurement, the protein sample was digested with concentrated nitric acid by refluxing for 30 min and then diluted with metal-free water to give a final concentration of 1% (v/v) nitric acid.

Kinetic Measurements and Data Analysis.

Activity screening and kinetic measurements were performed using a SpectraMax Plus³⁸⁴ UV-Vis plate reader. Substrate hydrolysis was monitored using three different assay formats. Glycerol-like compounds were assayed using the acetate assay kit as per the manufacturer's

instructions. Reactions were performed in 50 mM HEPES buffer at pH 8.0. Acetate standard curves were made by diluting sodium acetate in 50 mM HEPES, pH 8.0. The final concentrations of the coupling enzymes in the assay were as follows: 12 U/mL L-malate dehydrogenase, 1.4 U/mL citrate synthase, and 3.7 U/mL acetyl-CoA synthetase. The final assay mixture included NAD⁺ (5.0 mM), ATP (1.0 mM), Coenzyme A (2.0 mM), L-malic acid (2.0 mM), and MgCl₂ (2.0 mM). Substrates (0 - 5 mM) were incubated with the cofactors and coupling enzymes for 20 min at 30° C and the reaction was initiated by the addition of PHP (20 nM – 1 μM) to a final volume of 250 μL. Hydrolysis reactions were monitored by following the increase in absorbance at 340 nm.

Lactones were assayed using a pH-sensitive colorimetric assay (14). Protons released from the carboxylate product were measured using the pH indicator, cresol purple. The reactions were performed in 2.5 mM Bicine (pH 8.3) with variable substrate concentrations (0 - 5 mM), 0.1 mM cresol purple and PHP (20 nM - 1 μM). The change in absorbance at 577 nm was monitored. The effective extinction coefficient (ϵ , OD per mole of H⁺) was determined to be $1.76 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ using acetic acid as the standard. PHP was buffer-exchanged with 10 mM Bicine (pH 8.3) before the assay. For compound **28** with 4-nitrophenol as the leaving group, activity screening and kinetic measurements were monitored at 400 nm ($\epsilon = 1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) in 50 mM HEPES, pH 8.0, while for compounds **26** and **27** bearing an acetylphenol group, the reactions were monitored at 294 nm in 50 mM HEPES, pH 8.0 ($\epsilon = 7710 \text{ M}^{-1} \text{ cm}^{-1}$). When indoxyl acetate (16) was used as a substrate, the hydrolysis product was monitored at 678 nm in 50 mM HEPES, pH 8.3, 1% ethanol ($\epsilon = 7.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). For 2-naphthyl acetate (16), the hydrolysis product was monitored at 320 nm in 50 mM HEPES, pH 8.0, 1% methanol ($\epsilon = 600 \text{ M}^{-1} \text{ cm}^{-1}$) (16).

Kinetic parameters were determined by fitting the initial rates to Eqn. 1 using the nonlinear least-squares fitting program in SigmaPlot 9.0, where v is the initial velocity of the reaction, E_t is the enzyme concentration, k_{cat} is the turnover number, $[A]$ is the substrate concentration, and K_m is the Michaelis constant.

$$v/E_t = k_{\text{cat}}A/(K_m + A) \quad (1)$$

Molecular Docking and Computational Modeling.

Computational docking of potential substrates and intermediates to *E. coli* PHP was performed using Autodock Vina (25). The crystal structure of PHP (PDB id: 4LEF) was used in all docking calculations, and the metal ions were retained in the active site. A pdbqt file format of the protein was generated by adding polar hydrogens, and a grid box was centered at the active site of PHP with dimensions $26 \times 26 \times 26 \text{ \AA}$ with grid points spaced every 1 Å. Initial structures of the ligands were generated, and charges were added using MGL tools 1.5.4 version software. Zinc was assigned a net charge of +1.4, as described previously (26). Docking calculations were conducted with an exhaustiveness of 50. Output structures with the best docking poses were considered and analyzed using Discovery Studio Client 3.5 (BIOVIA). Representative images were generated using Chimera (27).

Crystallization, X-ray Data Collection, and Structure Determination.

Phosphotriesterase homology protein (PHP, Uniprot id: P45548) from *E. coli* (24 mg/mL in 20 mM HEPES buffer, pH 8.0) was incubated with 0.1 mM zinc chloride, 5 mM DTT, and 200 mM ammonium phosphate for 6 days on ice. Screening for crystallization conditions was performed using the sitting-drop vapor diffusion method. Equal volumes (0.7 μ L) of the protein solution and precipitant were mixed and allowed to equilibrate at room temperature against the same precipitant solution in clear tape-sealed 96-well INTELLI-plates (Art Robbins Instruments, Sunnyvale, CA). Crystallization setup was performed using a PHOENIX liquid handling robot (Art Robbins Instruments). The final crystallization condition consisted of 1.30 M ammonium phosphate, 10% MPD, 0.1 mM zinc chloride, and 50 mM HEPES, pH 7.5. Prior to data collection, the protein crystals were transferred to cryoprotectant solutions composed of their mother liquors augmented with 40% glucose and flash-cooled in a nitrogen stream at 100 K.

X-ray diffraction data were collected at 100 K on beamline X29A (National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY) at a wavelength of 1.075 Å. Diffraction data were processed and scaled with HKL3000 (28). The crystal structure was determined by molecular replacement with coordinates of the identical PHP determined previously (29) (PDB id: 1BF6) as a starting model using PHASER software as implemented in PHENIX (30, 31). The model was refined using PHENIX and manually adjusted using COOT (32). The structure was deposited to the Protein Data Bank (PDB id: 4LEF). All figures were produced using PYMOL (33). Data collection and refinement statistics are listed in Table 1.

Construction of Sequence Similarity Networks.

Sequence similarity networks were created using Cytoscape (<https://cytoscape.org>). Protein sequences for all members of cog1735 were downloaded from NCBI as a FASTA file. The redundancy of these sequences was checked using the CD-HIT website (34). Protein sequences within this file that shared 90% identity with one another were consolidated. The resulting FASTA file was uploaded to the Enzyme Function Initiative (EFI) enzyme similarity tool website (<http://efi.igb.illinois.edu/efi-est/stepa.php>) (35, 36). The tool-generated data set was analyzed under an organic layout using the Cytoscape software (36).

RESULTS

Cloning, Expression and Purification of *E. coli* PHP.

The gene for PHP was successfully cloned, expressed and purified to homogeneity. The enzyme was grown in the presence of ZnCl₂ or MnCl₂ after induction with 0.5 mM IPTG. The Zn- and Mn-containing proteins were > 90% pure based on SDS-PAGE analysis. The ICP-MS analysis demonstrated that PHP expressed in the presence of Zn contained 1.8 equivalents of Zn, whereas PHP expressed in the presence of added manganese contained 1.6 equivalents of Mn and 0.2 equivalents of Zn.

Three-Dimensional Structure of PHP.

The three-dimensional structure of [Zn/Zn]-PHP was determined to a resolution of 1.84 Å. PHP adopts a distorted (β/α)₈ β -barrel fold with N and C-terminal extensions (Figure 2), which is similar to the previously published structure of this enzyme but with significant differences in the presence and organization of ions in the active site. (23) The eight β -strands of the central barrel include the following chain segments: β -1 (residues 8–13), β -2 (residues 48–53), β -3 (residues 76–81), β -4 (residues 120–127), β -5 (residues 155–158), β -6 (residues 181–184), β -7 (residues 205–208), and β -8 (residues 238–240).

The active site of PHP is located at the C-terminal end of the β -barrel and is open to bulk solvent. The active-site binds two zinc ions with the most deeply buried zinc ion denoted as α and the solvent-exposed zinc ion denoted as β . Zn_α is coordinated by His-12 from strand β -1 and His-14 from the loop succeeding strand β -1, Asp-243 from the loop succeeding strand β -8, and Glu-125 from strand β -4 as illustrated in Figure 3a. Zn_β is coordinated by His-158 from strand β -5, His-186 from the loop following strand β -6, and Glu-125 from strand β -4. In addition to Glu-125 bridging the two zinc ions, a proximal phosphate binds as an additional bridging ligand (Figures 3a and 3b). While the two zinc ions are separated by 3.8 Å, Zn_α and the Zn_β ions interact with O3 of the bridging phosphate at distances of 1.9 and 2.6–2.8 Å respectively. Zn_β is also coordinated by O2 of the bridging phosphate at 2.0–2.1 Å (Figure 3b). A distal phosphate is present at a position near the active site with a P–P distance to the proximal phosphate of 6.2 Å. Two water molecules, Wat198 and Wat214, reside between the proximal and distal phosphates. While Wat198 is equidistant (2.5 Å) from oxygen atoms of both phosphates, Wat214 interacts with the distal phosphate at 2.8 Å and with the proximal phosphate at 3.0 Å distance, respectively. The distal phosphate ion interacts electrostatically with Arg-246 and Lys-213. In addition, this phosphate also makes hydrogen bonds with Tyr-216 and Thr-245 and a water mediated (Wat214) interaction with Lys-23 (Figure 3b).

Computational Docking of High-energy Intermediates in the Active Site of *E. coli* PHP.

The structural basis for the substrate specificity of *E. coli* PHP was investigated using computational docking. To begin, high-energy reaction intermediates (HEI) of potential PHP substrates were generated (Figure 4c). This was accomplished by considering the attack of the hydroxide on the *si*-face of the carbonyl carbon of the substrate (15). Structurally characterized members of amidohydrolase superfamily are known to catalyze the cleavage of esters from the *si*-face of the carbonyl carbon of the ester group (10, 14, 15). Docking conformations were considered productive based on a specific docking pose of the compounds with respect to the enzyme. The specific docking pose involved the interaction of the OH group from the tetrahedral carbon with Zn_α (1.9 – 2.1 Å) and the hydroxide anion with Zn_β metal (2.0 – 2.2 Å) as shown in Figure 4a.

Docked compounds included a series of phosphorylated five-membered and six-membered lactones (Scheme S1a) as well as a series of acetate and butyrate compounds (Scheme S1b). Docking of phosphorylated lactones as well as phosphorylated glycerol acetates resulted in structures with productive conformations. Representative examples of docked structures are shown in as shown in Figures S1a and S1b. Notably, docking of 2-naphthyl acetate in the

active site of PHP revealed a potential hydrophobic pocket lined by residues Phe-161 and Leu-189. This led to the design of phosphorylated glycerol derivatives with a hydrophobic substituent at C2, which were predicted to dock efficiently in the active site of PHP. As expected, compounds **14R**, **17R**, **17S** and **23** docked efficiently with a productive conformation. The phosphate group in these compounds hydrogen bonded with Arg-246, Tyr-216, Lys-23, and Thr-245, similar to the binding determinants of the phosphate ligand in the crystal structure of PHP. A docked pose of compound **23**, is shown in Figure 4b. The 2-butanoyl sidechain in Figure 4b exhibits favorable hydrophobic interactions with the side chains of Phe-161 and Leu-189.

Substrate Specificity and Kinetic Measurements.

The substrate profile of *E. coli* PHP was investigated against a series of peptides, phosphate monoesters, organophosphate esters and carboxylate esters (8, 14–16, 20, 21) that were previously identified as substrates for enzymes from cog1735 (Schemes S2 – S6). None of these compounds were found to be substrates for PHP. However, this enzyme was able to hydrolyze aromatic acetates with structures **26** – **35** as shown in Scheme S1. Of these compounds, 2-naphthyl acetate was efficiently hydrolyzed by PHP with a $k_{\text{cat}}/K_{\text{m}}$ of 1400 M⁻¹ s⁻¹ (Table 2). The Mn-containing enzyme exhibited higher activity (~1.6-fold) than the Zn-containing enzyme with 2-naphthyl acetate as a substrate. The Mn-containing enzyme was used throughout the substrate screening assays and kinetic measurements were supplemented with 1.0 mM MnCl₂.

Perhaps the most important clue for potential substrates of PHP originated from the binding mode of the two phosphates in the active site of this enzyme as observed from the crystal structure (Figure 3b). The phosphate bound to the metal ions was reminiscent of the tetrahedral intermediate generated during substrate hydrolysis. The *distal* phosphate, which is far from the metal center could potentially mimic a phosphate functionality of the substrate. Incorporating this notion, a series of phosphorylated lactones were considered as potential substrates of this enzyme. Docking a subset of phosphorylated lactones in the PHP active site revealed a potential substrate interaction. Pursuing this lead, a library of phosphorylated and non-phosphorylated lactones (Figure S1a) (15, 20) were screened as substrates for this enzyme. Interestingly, no catalytic activity was observed with any of these compounds. This observation suggests that PHP does not possess lactonase activity.

Next, a subset of phosphorylated glycol and glycerol acetates were docked in the PHP active site. Notably, compound **2**, which contains a phosphate group that mimics the *distal* phosphate ion in the crystal structure along with an acetate functionality is hydrolyzed by PHP with a $k_{\text{cat}}/K_{\text{m}}$ of $37 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2 and Figure S6). Compound **4** is comparable to **2** in its reactivity with this enzyme, but not more so. The carboxylate derivative **5**, along with the non-phosphorylated derivatives **1** and **3** were not hydrolyzed by PHP (Scheme S5). Neither the non-phosphorylated *O*-acetyl homoserine derivative **6**, nor the phosphorylated *N*-acetyl derivative **8** are substrates of this enzyme. While the threonic acid derivative **10** is a poor substrate, compounds **9** and **19** are not hydrolyzed by PHP. This suggests that the presence of a chiral substituent within the reaction center is not favorable for catalysis.

A noteworthy result from Table 2 is the reactivity of glycerol derivatives **7**, **12**, and **13**, which are better substrates than **2** with PHP. The monoacetyl derivative **13** is racemic and the progress curve of the time course of enzymatic turnover indicated no preference for a single enantiomer. This was not entirely puzzling as both the *R*- and *S*-isomers of **13** that were docked in the active site of PHP did not reveal any specific binding determinants for the 2-hydroxy group. Progress curve analysis also indicated that both the acetyl groups of **12** are hydrolyzed by PHP at the same rate. On the other hand, the monoacetyl derivative **11** is a considerably poorer substrate of PHP than **13**. This is likely due to the phosphate group, the positioning of which might influence the specificity for optimal catalysis. A more striking result is the reactivity of the 2-acyl derivatives **14**, **15**, and **16**, which are hydrolyzed by PHP at rates much faster than other substrates investigated thus far. The outlier is the 1,2-diacetyl glycerol derivative **16**, which has a $k_{\text{cat}}/K_{\text{m}}$ of $4620 \pm 640 \text{ M}^{-1} \text{ s}^{-1}$ and is 10-fold better than the 1,3-diacetyl derivative **12**. The reactivity of the monoacetyl derivative **17** is similar to that of **11**, whereas **18** is a poor substrate as compared to **14**. In addition to acetate derivatives, PHP can also hydrolyze propionates such as **35** with a $k_{\text{cat}}/K_{\text{m}}$ of $440 \text{ M}^{-1} \text{ s}^{-1}$. Hydrolysis of the dibutyl glycerol derivative **20** by PHP suggests that the enzyme can hydrolyze phosphoglycerol substrates with a butyrate leaving group (Figure S5).

Stereoselectivity of Substrate Catalysis by *E. coli* PHP.

To address the stereoselectivity of PHP for the hydrolysis of chiral acetyl glycerol derivatives, racemic and enantiopure substrates were used. Analysis of the time course for the hydrolysis of **15** suggested that the enzyme prefers the *R*-enantiomer over the *S*-enantiomer (Figure S3). Such a preference however was not reflected in the turnover of **16** by PHP, the hydrolysis of which does not appear to be stereospecific. The progress curve for the hydrolysis of **16** is shown in Figure S4. Enzymatic hydrolysis of **16** with PHP suggests that the enzyme hydrolyzes the 1-acetyl group before acting on the 2-acetyl substituent. The rate of hydrolysis of the 1-acetyl group of **13** by PHP is faster than the 2-acetyl group of **17** and the values from the ^1H NMR experiment matches the kinetic experimental values.

DISCUSSION

Functional Profile for *E. coli* PHP.

E. coli PHP from Subgroup 1 of cog1735 belongs to the amidohydrolase superfamily of proteins (3, 9, 10). Other members of this superfamily from cog1735 have been found to catalyze the hydrolysis of organophosphates, lactones and carboxylate esters (10–20). PHP was initially screened against a small library of compounds that were previously identified as substrates of cog1735 enzymes (8, 14, 15, 16, 20, 21). None of these compounds were substrates of PHP, suggesting that the enzyme is not a broad hydrolase like many other enzymes in cog1735. PHP does not catalyze the hydrolysis of phosphotriesters, diesters, or carboxyesters and lacks phosphatase activity. Nonetheless, this enzyme was able to hydrolyze aromatic acetates such as 2-naphthyl acetate, thereby suggesting a possible role for PHP as a hydrolase.

Computational docking of HEIs of a series of phosphorylated sugar lactones, phosphoalkyl acetates, and phosphoglycerol acetates was conducted with PHP. For the esters and lactones

that were hydrolyzed by PHP, it is highly likely that the *si*-face of the carbonyl carbon will be attacked by the nucleophilic hydroxide (14, 15). This stereochemical preference is reaffirmed by the computational docking, where in the computed poses of **24** and **25** in the active site of PHP highlight a tetrahedral intermediate that would be generated by the *si*-faced attack of the nucleophilic hydroxide ion. In the docked poses, the phosphate functionality of both **24** and **25** interact with Arg-246, Lys-213, and Tyr-216. This interaction is similar to that observed for the phosphate ligands in the crystal structure of this enzyme. The binding determinants of the respective phosphate groups are conserved in all computed poses that rendered a productive conformation.

E. coli PHP was shown to catalyze the hydrolysis of phosphoalkyl and phosphoglycerol acetates, whereas none of the phosphorylated and non-phosphorylated sugar lactones were hydrolyzed at detectable rates. Phosphoglycerol acetates were better substrates than simple phosphoalkyl acetates. The best substrate was found to be the diacetyl phosphoglycerol **16** with a $k_{\text{cat}}/K_{\text{m}}$ of $4620 \text{ M}^{-1} \text{ s}^{-1}$. While the 2-butanoyl derivative **15** is a poorer substrate than **16**, its reactivity may be attributed to its slightly lower K_{m} due to potential binding interaction of the butyryl group with hydrophobic residues Phe-161 and Leu-181 of PHP (Figure 4b). The 2-phenylacetate derivative **14** had an eight-fold lower $k_{\text{cat}}/K_{\text{m}}$ value than **16** and three-fold lower than **15**. This result is perplexing and needs further assessment. Hydrolysis of compound **20** by PHP was interesting due to the fact that PHP was unable to hydrolyze non-phosphorylated carboxyesters with long chain leaving groups. The possibility of longer-chain glycerol phosphates as substrates of PHP cannot be entirely ruled out. It could thus be hypothesized that the active site of PHP might be crafted accordingly to accommodate a longer flexible hydrophobic alkyl chain as compared to a bulkier aromatic group. The substrate profile determined here for PHP does not explain the rescue of the PdxB auxotroph when PHP is overexpressed in *E. coli* (24).

Structural Features of *E. coli* PHP.

The three-dimensional structure of *E. coli* PHP was determined to a resolution of $1.84 \text{ \AA}$ (PDB id: 4LEF) with eight molecules in the asymmetric unit. The distorted $(\beta/\alpha)_8$ -barrel fold of the enzyme is structurally similar those members determined from cog1735 and the amidohydrolase superfamily. A major difference in the structural features of enzymes from cog1735 involves the conformation of the loops that follow the eight β -strands. The active site is located at the C-terminal end of the β -barrel and is lined with polar and non-polar residues. The metal coordination in PHP is quite similar to that of other enzymes and the coordinating amino acids are conserved except the metal ion bridging residue. The metal ions in PHP are bridged by the side chain carboxylate of Glu-125 from β -strand 4, a residue that is replaced by a carboxylated lysine in many enzymes of the amidohydrolase superfamily (37). The metal ions are coordinated to the protein via direct interactions with the sidechains of His-12 and His-14 from β -strand 1 and with His-158 and His-186 from the ends of β -strands 5 and 6 respectively. PHP was crystallized with two phosphates in the active site with one of the phosphates bridging the two metal ions; an interaction that mimics the tetrahedral intermediate, which likely forms during carboxylate ester hydrolysis. Protein sequence alignment of *E. coli* PHP and representative proteins from the relevant groups of cog1735 is shown in Figure S7. The metal binding residues in all these enzymes are

conserved except for the metal bridging ligand, which is a glutamate in PHP, Rsp3690, and Pmi1525, but is replaced by a carboxylated lysine in Lmo2620, MS_0025, and Dr0930.

Structural Comparison of *E. coli* PHP, Lmo2620, and MS53_0025.

The rationale behind comparing the structures of PHP, Lmo2620 and MS53_0025 stems from the fact that all three enzymes recognize phosphorylated substrates. Lmo2620 from *L. monocytogenes* was shown to catalyze the hydrolysis of D-lyxono-1,4-lactone-5-phosphate, (15) whereas MS53_0025 from *Mycoplasma synoviae* efficiently hydrolyzes D-xylono-1,4-lactone-5-phosphate and L-arabino-1,4-lactone-5-phosphate (20). Superimposition of the backbone of all three enzymes is shown in Figure S8. While the central β -barrel, adjoining helices and the metal centers superimpose well, the differences in the loop regions are quite obvious. Loop 2 and Loop 3 in Lmo2620 are conformationally different from PHP and MS53_0025 in the sense that Loop 2 and Loop 3 are longer.

Residues constituting the active sites of PHP, Lmo2620 and MS53_0025 are shown in Figure S9. All three enzymes have a distal phosphate in the three-dimensional structure bound to the phosphate interacting residues. While the position of Lys-213 and Arg-246 in PHP is conserved in the other two enzymes, Tyr-214 and His-249 are replaced by similar residues. A striking difference can be observed from the amino acid residues of Loop 2 and Loop 3 (Figures S8 and S9) in Lmo2620. Lmo2620 has two residues, Asn-96 and Lys-97, instead of Tyr-84 in PHP and His-100 in MS53_0025 that interacts with the substrate in order to facilitate optimal catalysis (15, 20). This difference, however, does not fully explain the difference in substrate specificity.

Another interesting observation is the hydrophobicity of the residues that line the active site entrance of PHP. Figure S10 depicts space-filling renditions of the active site opening of PHP, Lmo2620 and MS53_0025. The active site metal and the position of the *distal* phosphate ligands can be seen in all three enzymes. PHP is lined with hydrophobic residues Phe-22, Ala-87, and Phe-88 in the top and Phe-161, and Tyr-216 in the bottom portion of the opening as seen in Figure S10a, which shows the surface representation of PHP docked with **25**. While interacting specifically with Phe-161 and Tyr-216 by making hydrophobic contacts, the butyryl group is nicely poised to project out of the opening to make potential interactions with the residues lining the entrance of PHP. Active site openings in Lmo2620 (Figure S10b) and MS53_0025 (Figure S10c) are relatively polar with Lys-97, Arg-218, Lys-242, and Lys-244 in Lmo2620 and Glu-34, Glu-37, Lys-103, Lys-217, and Lys-244 in MS53_0025 decorating the active site walls. The preference for substrate **15** by PHP can be explained by the amphipathic nature of the enzyme's active site with the hydrophilic residues making hydrogen bonds with the phosphate group while the hydrophobic residues interact (Figure S11) with the butyryl group thereby rendering effective catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by grants from the Robert A. Welch Foundation (A-840) and the National Institutes of Health (GM 122825 to F.M.R. and U54 GM093342 to S.C.A.)

REFERENCES

1. Benson DA, Karsch-Mizrachi I, Lipman DJ, and Sayers EW (2009) GenBank. *Nucleic Acids Res.* 37, D26–31. [PubMed: 18940867]
2. Juncker AS, Jensen LJ, Pierleoni A, Bernsel A, Tress ML, Bork P, von Heijne G, Valencia A, Ouzounis CA, Casadio R, and Brunak S (2009) Sequence-based feature prediction and annotation of proteins. *Genome Biol.* 10, 2061–2066.
3. Pieper U, Chiang R, Seffernick JJ, Brown SD, Glasner ME, Kelly L, Eswar N, Sauder JM, Bonanno JB, Swaminathan S, Burley SK, Zheng X, Chance MR, Almo SC, Gerlt JA, Raushel FM, Jacobson MP, Babbitt PC, and Sali A (2009) Target selection and annotation for the structural genomics of the amidohydrolyase and enolase superfamily. *J. Struct. Funct. Genomics* 10, 107–125. [PubMed: 19219566]
4. Wu CH, Aweiler R, Bairoch A, Natale DA, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M, Martin MJ, Mazumander R, O'Donova C, Redaschi N, and Suzek B (2006) The universal protein resource (Uniprot): An expanding universe of protein information. *Nucleic Acids Res.* 34, D187–D191. [PubMed: 16381842]
5. Hall RS, Agarwal R, Hitchcock D, Sauder JM, Burley SK, Swaminathan S, and Raushel FM (2010) Discovery and structure determination of the orphan enzyme isoxanthopterin deaminase. *Biochemistry* 49, 4374–4382. [PubMed: 20415463]
6. Hermann JC, Marti-Arbona R, Fedorov AA, Fedorov E, Almo SC, Shoichet BK, and Raushel FM (2007) Structure based activity prediction for an enzyme of unknown function. *Nature* 448, 775–779. [PubMed: 17603473]
7. Irwin JJ, Raushel FM, and Shoichet BK (2005) Virtual screening against metalloenzymes for inhibitors and substrates. *Biochemistry* 44, 12316–12328. [PubMed: 16156645]
8. Xiang DF, Patskovsky Y, Nemmara VV, Toro R, Almo SC, and Raushel FM (2015) Function discovery and structural characterization of a methyl phosphonate esterase. *Biochemistry* 54, 2919–2930. [PubMed: 25873441]
9. Holm L, and Sander C (1997) An evolutionary treasure: unification of a broad set of amidohydrolase related to urease. *Proteins* 28, 72–82. [PubMed: 9144792]
10. Seibert CM, and Raushel FM (2005) Structural and catalytic diversity within the amidohydrolase superfamily. *Biochemistry* 44, 6383–6391. [PubMed: 15850372]
11. Tatusov RL, Galperin MY, Natale DA, and Koonin EV (2000) The COG database: A tool for genome-scale analysis of protein function and evolution. *Nucleic Acids Res.* 28, 33–36. [PubMed: 10592175]
12. Ghanem E, and Raushel FM (2005) Detoxification of organophosphate nerve agents by bacterial phosphotriesterase. *Toxicol. Appl. Pharmacol* 207, 459–470. [PubMed: 15982683]
13. Chow JY, Wu L, and Yew WS (2009) Directed evolution of a quorum-quenching lactonase from *Mycobacterium avium* subsp. *Paratuberculosis* K-10 in the amidohydrolase superfamily. *Biochemistry* 48, 4344–4553. [PubMed: 19374350]
14. Xiang DF, Kolb P, Fedorov AA, Meier MM, Fedorov IV, Nguyen TT, Sterner R, Almo SC, Shoichet BK, and Raushel FM (2009) Functional annotation and three-dimensional structure of Dr0930 from *Deinococcus radiodurans*, a close relative of phosphotriesterase in the amidohydrolase superfamily. *Biochemistry* 48, 2237–2247. [PubMed: 19159332]
15. Xiang DF, Kolb P, Fedorov AA, Xu C, Fedorov EV, Narindoshvili T, Williams HJ, Shoichet BK, Almo SC, and Raushel FM (2012) Structure-based function discovery of an enzyme for the hydrolysis of phosphorylated sugar lactones. *Biochemistry* 51, 1762–1773. [PubMed: 22313111]

16. Xiang DF, Kumaran D, Swaminathan S, and Raushel FM (2014) Structural characterization and function determination of a nonspecific carboxylate esterase from the amidohydrolase superfamily with a promiscuous ability to hydrolyze methylphosphonate esters. *Biochemistry* 53, 3476–3485. [PubMed: 24832101]
17. Atkinson HJ, Morris JH, Ferrin TE, and Babbitt PC (2009) Using sequence similarity network for visualization of relationships across diverse protein superfamilies. *PLoS One* 4, e4345. [PubMed: 19190775]
18. Afriat L, Roodveldt C, Manco G, and Tawfik DS (2006) The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase. *Biochemistry* 45, 13677–13686. [PubMed: 17105187]
19. Uroz S, Oger PM, Chapelle E, Adeline M-T, Faure D, and Dessaux Y (2008) A *Rhodococcus* qsdA-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases. *Appl. Environ. Microbiol* 74, 1357–1366. [PubMed: 18192419]
20. Korczynska M, Xiang DF, Zhang Z, Xu C, Narindoshvili T, Kamat SS, Williams HJ, Chang SS, Kolb P, Hillerich B, J. Sauder M, Burley SK, Almo SC, Swaminathan S, Shoichet BK, and Raushel FM (2014) Functional annotation and structural characterization of a novel lactonase hydrolyzing D-xylono-1,4-lactone- 5-phosphate and L-arabino-1,4-lactone-5-phosphate. *Biochemistry* 53, 4727–4738. [PubMed: 24955762]
21. Gnanem E, and Raushel FM (2005) Detoxification of organophosphate nerve agents by bacterial phosphotriesterase. *Toxicol. Appl. Pharmacol* 207, 459–470. [PubMed: 15982683]
22. Merone L, Mandrich L, Rossi M, and Manco G (2005) A thermostable phosphotriesterase from the archaeon *Sulfolobus solfataricus*: Cloning, overexpression and properties. *Extremophiles* 9, 297–305. [PubMed: 15909078]
23. Buchbinder JL, Stephenson RC, Dresser MJ, Pitera JW, Scanlan TS, and Fletterick RJ (1998) Biochemical characterization and crystallographic structure of an *Escherichia coli* protein from the phosphotriesterase gene family. *Biochemistry* 37, 5096–5106. [PubMed: 9548740]
24. Kim J, Kershner JP, Novikov Y, Shoemaker RK & Copley SD (2010) Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5'-phosphate synthesis. *Mol. Syst. Biol* 6, 436. [PubMed: 21119630]
25. Trott O, and Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J. Comput. Chem* 31, 455–461. [PubMed: 19499576]
26. Irwin JJ, Raushel FM, and Shoichet BK (2005) Virtual screening against metalloenzymes for inhibitors and substrates. *Biochemistry* 44, 12316–12328. [PubMed: 16156645]
27. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem* 25, 1605–1612. [PubMed: 15264254]
28. Otwinowski Z, and Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode, in *Methods in Enzymology* (Carter CWJ, Sweet RM, Abelson JN, and Simon MI, Eds.) pp 307–326, Academic Press, New York.
29. Buchbinder JL, Stephenson RC, Dresser MJ, Pitera JW, Scanlan TS, Fletterick RJ (1998) Biochemical characterization and crystallographic structure of an *Escherichia coli* protein from the phosphotriesterase gene family. *Biochemistry* 37, 5096–5106. [PubMed: 9548740]
30. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser crystallographic software. *J. Appl. Crystallogr* 40 (4):658–674. [PubMed: 19461840]
31. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D. Biol. Crystallogr.* 66 (2): 213–21. [PubMed: 20124702]
32. Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D. Biol. Crystallogr.* 60, 2126–32. [PubMed: 15572765]
33. Delano WL (2002) The PyMOL Molecular Graphics System; Delano Scientific: San Carlos, CA.

34. Huang Y, Niu B, Gao Y, Fu L, and Li W (2010) CD-HIT Suite: a web server for clustering and comparing biological sequences. *Bioinformatics* 26, 680–682. [PubMed: 20053844]
35. Gerlt JA, Bouvier JT, Davidson DB, Imker HJ, Sadkhin B, Slater DR, and Whalen KL (2015) Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST): A web tool for generating protein sequence similarity networks. *Biochim Biophys Acta*. 1854, 1019–1037. [PubMed: 25900361]
36. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, and Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. [PubMed: 14597658]
37. Benning MM, Shim H, Raushel FM, and Holden HM (2001) High resolution X-ray structures of different metal-substituted forms of phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* 40, 2712–2722. [PubMed: 11258882]

Figure 1.

Sequence similarity network representation (<http://www.cytoscape.org>) of cog1735 from the amidohydrolase superfamily obtained at E-value cutoffs of 10^{-70} (a) and 10^{-40} (b). Each node in the network represents a single sequence, and each edge (depicted as a line) represents the pairwise connection between two sequences at the given BLAST E-value. The triangular nodes represent proteins that have been functionally and/or structurally characterized. (c) Organization of the *php* gene cluster in *E. coli*. The adjacent genes encode proteins that are predicted to be a PLP-dependent racemase (*yhfX*), a putative sugar isomerase (*yhfW*), a putative transporter (*yhfT*) and a putative PLP-dependent lyase (*yhfS*).

Figure 2.

Cartoon representation of the *E. coli* PHP (PDB id: 4LEF) with zinc (colored in purple) bound in the active site. The protein is colored based on the secondary structure: α -helices (green), β -strands (maroon) and loops (white). The N-terminus and the C-terminus are labeled as N and C, respectively.

Figure 3.

(a) Coordination geometry of the metal center in the active site of PHP. A phosphate group coordinates with both metal ions. (b) Positioning of the phosphate ligand in the active site of PHP. Metal coordinating histidine residues are not shown for clarity.

Figure 4.

(a) PHP with compound **24** docked in the active site as a high energy intermediate. (b) PHP with **25** (*R*-enantiomer) docked in the active site as a high energy intermediate. (c) Proposed high-energy intermediates of the hydrolysis reaction catalyzed by PHP. Compound **24** from compound **2** and compound **25** from compound **15**.

Scheme 1.
Structures of compounds hydrolyzed by PHP.

Table 1.

Data Collection and Refinement Statistics

PDB identifier	4LEF
Data collection	
Space group	P 1 2 ₁ 1
Unit cell dimension (Å)	
<i>a</i>	83.048
<i>b</i>	100.308
<i>c</i>	168.037
Unit cell angles (°)	
β	104.48
Molecules per ASU	8
Solvent content (%)	52
Beamline	NSLS X29A
Wavelength (Å)	1.075
Structure solution method	molecular replacement
Search model	1BF6
Resolution (Å)	42.57–1.84 (1.92–1.84)
Unique reflections	228414
R_{merge}	0.088 (0.376)
Completeness (%)	99.3 (92.8)
Redundancy	4.1 (4.0)
$I/\sigma(I)$	14.6 (3.8)
Refinement	
Resolution (Å)	42.57–1.84 (1.86–1.84)
R_{work}	0.175 (0.273)
R_{free}	0.208 (0.283)
Number of atoms	
Protein	18468
Water	1729
Ligand	183
Ligand identity	16 Zn ²⁺ , 31 PO ₄ ³⁻ , 1 β -D-glucose
<B-factor>	
Protein	25.5
Water	31.6
Ligand	51.6
RMSD	
Bonds lengths	0.007
Bond angles	1.060

PDB identifier	4LEF
Ramachandran plot statistics (PDB Validation)	
Most favored regions (%)	97.7
Allowed regions (%)	2.3
Outliers (%)	0

Table 2.Kinetic parameters for *E. coli* PHP^a

Compound	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	Compound	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
2	–	–	37 ± 1	21	33 ± 4	10 ± 2	3200 ± 1000
4	–	–	12 ± 2	22	27 ± 2	5.5 ± 0.5	4900 ± 370
7	–	–	100 ± 10	23	6.5 ± 0.1	5.3 ± 1.0	1230 ± 800
10	–	–	11 ± 10	26	–	–	120 ± 3
11	–	–	44 ± 10	27	–	–	270 ± 6
12	0.9 ± 0.1	3.0 ± 0.3	317 ± 10	28	0.40 ± 0.1	2.6 ± 0.3	140 ± 20
13	0.9 ± 0.1	4.2 ± 0.4	217 ± 30	29	–	–	180 ± 10
14	1.6 ± 0.1	2.7 ± 0.2	574 ± 20	30	–	–	56 ± 2
15	5.0 ± 0.2	4.8 ± 0.5	1560 ± 280	31	–	–	52 ± 1
16	28 ± 2	6.0 ± 0.5	4600 ± 650	32	0.6 ± 0.1	2.0 ± 0.2	320 ± 40
17	–	–	48 ± 4	33	2.5 ± 0.1	1.8 ± 0.2	1400 ± 200
18	–	–	100 ± 2	34	0.3 ± 0.1	1.8 ± 0.2	180 ± 20
20	–	–	280 ± 40	35	0.6 ± 0.1	1.3 ± 0.1	440 ± 30

^a Assays for all compounds except **34** were conducted at pH 8.0 and 30 °C; Assays for compound **34** were conducted at pH 8.3 and 30 °C; compounds 11–18 were used as a racemic mixture.