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# Analogs of 1-phosphonooxy-2,2-dihydroxy-3-oxo-5-(methylthio)-pentane, an acyclic intermediate in the methionine salvage pathway: a new preparation and characterization of activity with E1 enolase/phosphatase from *Klebsiella oxytoca*

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**Abstract**—The methionine salvage pathway allows the in vivo recovery of the methylthio moiety of methionine upon the formation of methylthioadenosine (MTA) from *S*-adenosylmethionine (SAM). The Fe(II)-containing form of acireductone dioxygenase (ARD) catalyzes the penultimate step in the pathway in *Klebsiella oxytoca*, the oxidative cleavage of the acireductone 1,2-dihydroxy-3-oxo-5-(methylthio)pent-1-ene (**2**) by dioxygen to give formate and 2-oxo-4-(methylthio)butyrate (**3**). The Ni(II)-bound form (Ni–ARD) catalyzes an off-pathway shunt, forming 3-(methylthio)propionate (**4**), carbon monoxide, and formate. Acireductone **2** is formed by the action of another enzyme, E1 enolase/phosphatase, on precursor 1-phosphonooxy-2,2-dihydroxy-3-oxo-5-methylthiopentane (**1**). Simple syntheses of several analogs of **1** are described, and their activity as substrates for E1 enolase/phosphatase characterized. A new bacterial overexpression system and purification procedure for E1, a member of the haloacid dehalogenase (HAD) superfamily, is described, and further characterization of the enzyme presented.


## 1. Introduction

*S*-Adenosylmethionine (SAM, or AdoMet) is a critical source of activated methyl groups in all organisms, and also serves as a source of activated $C_3$ aminopropyl units for polyamine biosynthesis during the cell cycle. The methionine salvage pathway is a ubiquitous biochemical pathway in prokaryotes and eukaryotes that re-incorporates the methylthio group of the proximate metabolite of SAM, methylthioadenosine (MTA) into methionine. Our laboratories are engaged in structural and functional studies of enzymes in the methionine salvage pathway, particularly those enzymes that catalyze the final steps of the pathway, the conversion of 1-phosphonooxy-2,2-dihydroxy-3-oxo-5-methylthiopentane (**1**) to the keto-acid precursor of methionine, 2-oxo-4-(methylthio)butyrate (**3**). Two enzymes have been identified in the bacterium *Klebsiella oxytoca* (American Type Culture Collection (ATCC) strain 8724) that are involved in these transformations, E1 enolase/phosphatase[1,2] and acireductone dioxygenase (ARD).[3–6] E1 catalyzes the dephosphorylation and enolization of **1** to acireductone **2** (Scheme 1). Previous publications have dealt with mechanistic questions regarding this enzyme, and these investigations were aided by the use of desthio analogs **1a**–**c** of E1 substrate **1**.[1,7,8] These compounds are also substrates for E1 (Scheme 2).

However, the reported syntheses of these compounds were laborious, gave low overall yields and required the use of diazomethane.[7] We now report simple syntheses of **1a**–**c**, and provide a comparative analysis of their ability to act as substrates of E1 enolase/phosphatase. We also provide further characterization of the E1 enzyme as obtained in active form upon overexpression in *Escherichia coli*.

*Keywords*: Acireductone dioxygenase; Enolase; Phosphatase; Phosphoramidite.
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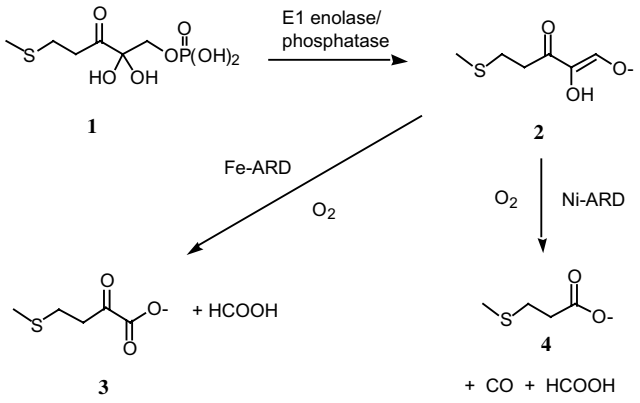
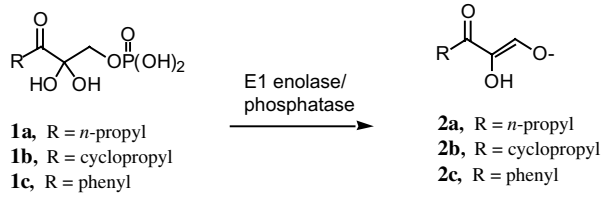



**Scheme 1.** Metabolic disposition of **1** in the methionine salvage pathway of *K. oxytoca*.



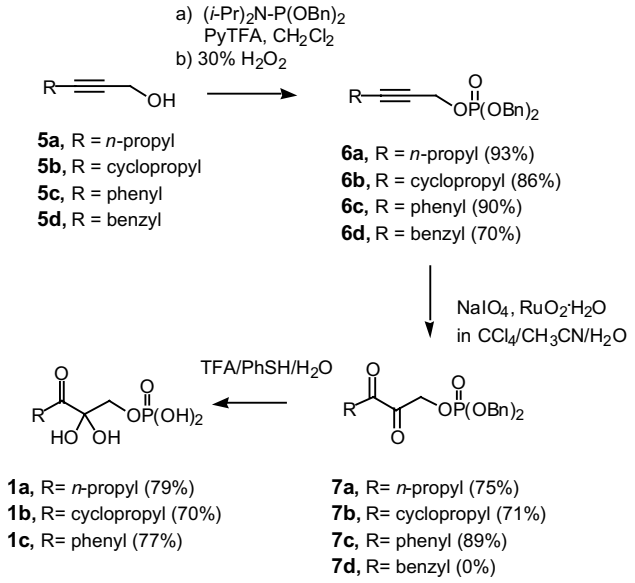

**Scheme 2.** Desthio analogs of **1** that are substrates for E1 enolase/ phosphatase.

## 2. Results and discussion

### 2.1. Synthesis of E1 substrate analogs

The general method for synthesis of E1 substrate analogs **1a–c** is shown in Scheme 3. The starting materials for all three analogs are the 2-alkyn-1-ols **5**. Compounds **5a** and **5c** are commercially available. Alkynols **5b** and **5d** are prepared by deprotonation of the appropriate terminal alkynes with *n*-butyllithium followed by hydroxymethylation with paraformaldehyde at low temperature. Reaction of **5a–d** with dibenzyl *N*,*N*-diisopropylphosphoramide in the presence of pyridinium trifluoroacetate under nitrogen for 25 min provides the intermediate phosphoramidite, which is oxidized with hydrogen peroxide to generate the dibenzyl phosphate ester **6a–d**. This procedure is an adaptation of chemistry commonly used for polynucleotide synthesis.[9]

1*H*-Tetrazole is commonly used as a catalyst for the phosphoramidite chemistry employed for the synthesis of **6a–d**.[10] Presently, solid tetrazole is a restricted compound. However, pyridinium trifluoroacetate (PyTFA) is an effective catalyst for phosphoramidite chemistry.[11] For these reactions, PyTFA proves to be a better catalyst than 1*H*-tetrazole. PyTFA accelerates the reaction considerably (25 min to completion, vs >12 h with 1*H*-tetrazole in THF) and workup is considerably simplified. The use of PyTFA with $CH_2Cl_2$ as the solvent is compatible with hydrogen peroxide oxidation of the phosphite, as opposed to the THF used with 1*H*-tetrazole, which must be replaced with $CH_2Cl_2$ prior to the oxidation step.

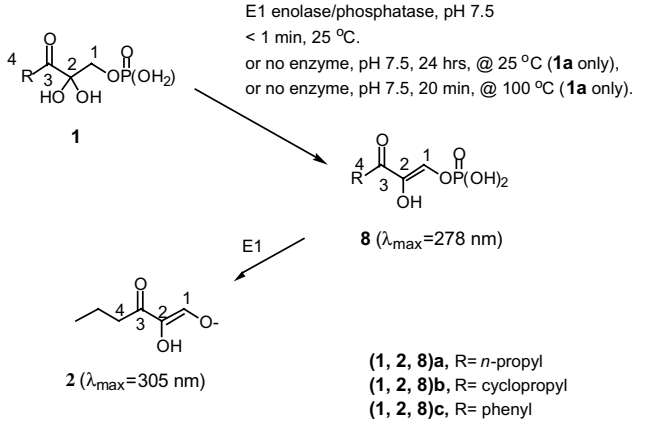



**Scheme 3.** Syntheses of **1a–c**. Percent yields are noted for each compound.

Dibenzyl 2,3-dioxo-1-alkyl phosphates **7a–c** are prepared from compounds **6a–c** by catalytic ruthenium oxide oxidation using sodium periodate as the stoichiometric oxidant.[12] Oxidations are performed in a biphasic $CCl_4/H_2O$ mixture, with acetonitrile added to accelerate the reaction.[13] Although the benzyl-substituted alkynyl phosphate **6d** is readily prepared, it cannot be successfully oxidized to the diketone. It is likely that the enol form of the product diketone is stabilized by the phenyl ring, and that this is susceptible to further oxidation under the reaction conditions. We also note that the oxidation must be closely monitored by TLC to prevent over-oxidation because the benzyl groups of the phosphate esters are slowly oxidized to benzaldehyde.

Compounds **7a–c** are deprotected by stirring with a mixture of trifluoroacetic acid (TFA), thiophenol, and water for 25 min.[14] Sufficient water and methylene chloride are added to form an organic and an aqueous layer; **1a–c** partition into the aqueous layer.

$^{13}C$ NMR spectra of E1 substrate analogs **1a–c** indicate that, as isolated under acidic conditions, the 2-carbonyl is hydrated to form the geminal dihydroxy compound, as indicated by $^{31}P$ coupling to the hydrated carbon at $\delta$ 94–96. Such hydration has been observed for biacetyl in aqueous solution, and is reversible upon transfer of biacetyl into nonpolar solvents.[15,16] The three substrate analogs **1a–c** are stable indefinitely as hydrates if stored as the free phosphoric acid in frozen 0.1 M solutions in $H_2O$. Under slightly basic conditions (pH 7.5 in potassium phosphate buffer), NMR spectra indicate that compound **1a** enolizes almost completely to give enolphosphate **8a** (see Scheme 4). Equilibration occurs within 48 h, with a steady loss of the 1-C$H_2$ signal of the




**Scheme 4.** Proposed sequence of E1 enzymatic activity with substrate **1a**.

hydrate at $\delta$ 3.98 and the appearance of a new signal at $\delta$ 7.47. This signal shows an 8 Hz $^{1}H$–$^{31}P$ coupling, confirming that it is the 1-C*H* enol proton of **8a**. Under the same conditions, the hydrated carbonyl $^{13}C$ signal of **1a** at $\delta$ 95.5 is completely lost, as is the 1-C signal at $\delta$ 68.5. Two new signals are observed for **8a** at 136.5 ($^{3}J_{PC} =$ 9 Hz) and 139.7 ($^{2}J_{PC} = 6$ Hz) corresponding to the 2-C and 1-C enol carbons, respectively. The 3-C carbonyl signal at $\delta$ 212.0 in **1a** shifts upfield to $\delta$ 200.9 in **8a**.

In the course of the enolization experiment in $D_2O$, the 4-C$H_2$ proton signals of **1a** (and therefore **8a**) are rapidly lost (i.e., within several hours) due to exchange with solvent, and the 4-*C*H$_2$ signal at $\delta$ 38 is severely broadened due to $^{2}H$ coupling. The 1-C*H* signal of **8a** is also attenuated by $^{1}H/^{2}H$ exchange, with about half of the expected intensity still observed at $\delta$ 7.47 after 48 h. However, the 1-C signal of **8a** at $\delta$ 139.7 is still clearly observed after 48 h, indicating that $^{1}H/^{2}H$ exchange at this position is considerably slower than at 4-C. Storage of **1a** as a 0.1 M solution at neutral pH for several months, even frozen, also results in considerable isomerization to **8a**, and several minutes of heating in a boiling water bath at neutral pH results in formation of **8a** as well.[7]

## 2.2. Characterization of bacterially overexpressed E1 enolase/phosphatase

E1 enolase/phosphatase was first identified as a component of the methionine salvage pathway in *K. oxytoca* ATCC 8724 (formerly *K. pneumoniae*),[1] and the gene corresponding to E1 was cloned by Balakrishnan and co-workers into pACYC184 to yield a construct pDiox-2, which gives constitutive overexpression of E1 in *E. coli* strain MM294.[2] For our purposes, including NMR characterization of E1, we wished to obtain controllable overexpression of E1 and for that reason chose to use the reliable pET vector expression system. We placed the E1 gene into a pET3a expression vector, and the resulting construct, pTP01, gives good overexpression of the desired enzyme in *E. coli* BL21(DE3)(pLysS). The over-expressed enzyme exhibits electrophoretic and chromatographic mobilities identical to the enzyme described by Balakrishnan et al.,[2] and shows activity with the substrates described here equivalent to the enzyme originally isolated from *K. oxytoca*.[1]

## 2.3. Activity of E1 enolase/phosphatase toward compounds 1a–c

Enzymatic activity assays of E1 enolase/phosphatase with substrates **1a**–**c** were performed anaerobically using 20 mM HEPES (pH 7.4) containing 0.5 mM $MgCl_2$. Typically, substrate concentration for the assay is $\sim$200 μM and the reaction is initiated by the injection of sufficient enzyme to reach a final enzyme concentration of 75 nM. The reactions can be monitored optically, as products **2a** and **2b** have a characteristic optical absorption maximum at $\sim$307 nm. For the phenyl-substituted analog **2c** the maximum is shifted to $\sim$318 nm. In the absence of any enol, there is no significant UV absorbance for any of the substrates under the conditions of the assay with the exception of **1c**, which exhibits the expected phenyl absorbance maxima near 226 and 248 nm. We note that phosphate buffers are not suitable for this assay because we find evidence for significant inhibition of the dephosphorylation reaction by phosphate, which differs from the previous report (Fig. 1).[1] Primary amine buffers such as Tris should be avoided in studies of enzyme activity in this system, as the total amount of product formed is lower than is observed with phosphate or HEPES buffer systems, possibly because primary amines are capable of reacting with the intermediate and/or product that is released from the enzyme (vide infra).

The results of the assays are shown in Figure 2. Product formation is monitored at 320 nm rather than 307 nm, as the intermediate enol-phosphate has an absorption at $\lambda_{max}$ $\sim$278 nm for all three substrates, and total absorbance at 307 nm contains contributions from the intermediate. Extinction coefficients for the acireductone products of substrates **1a**–**c** are similar, $\varepsilon_{320} = 8000\ M^{-1}\ cm^{-1}$. The kinetics of the reaction are complicated by the presence of the intermediate enol–phosphate **8**, which is released from the enzyme active site and builds up transiently in the course of the reaction. It is not yet clear whether the two-step reaction (keto-*gem*-diol-phosphate **1** to enol-phosphate **8** to acireductone **2**) can occur without release of the intermediate enol–phosphate **8** or if release of intermediate **8** is obligate (Scheme 4). A pH profile of enzyme activity as measured by the rate of formation of acireductone **2** shows a maximum near pH 7.8. At pH 5.0, the 307 band is much weaker than the 278 band at all times, suggesting that phosphatase activity is suppressed to a greater extent than enolase activity at lower pH. A more complete study of the mechanism of E1 enolase/phosphatase activity is underway.

Analogs **1a**–**c** are all substrates for E1, suggesting that the nature of the R group is of little importance in determining substrate binding and reactivity with E1. However, the similarity in rates of product formation

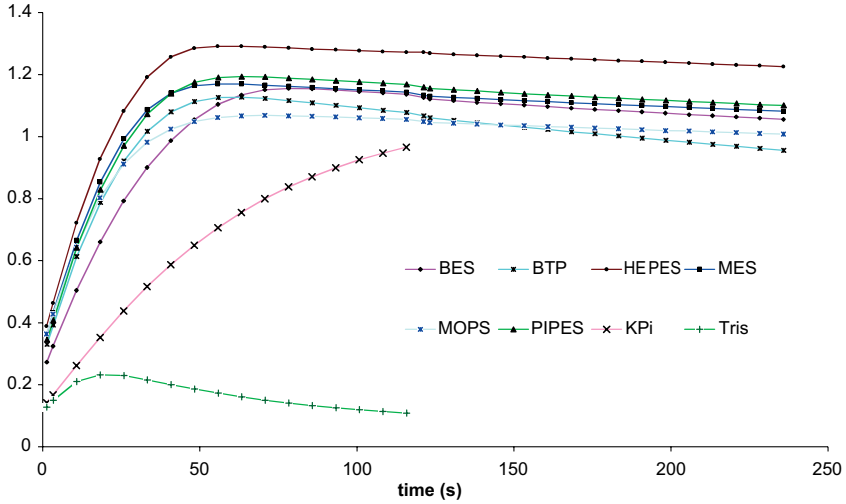



**Figure 1.** Formation of **2a** from **1a** catalyzed by E1 enolase/phosphatase as a function of buffer type. Reaction at 25 °C was monitored by UV absorbance at 305 nm. Concentration of all buffers was 50 mM, pH 7.5, with 0.25 mM $MgCl_2$. Note that the reaction in potassium phosphate buffer ($KP_i$) is inhibited significantly, and total product formed in Tris buffer is less than in other buffers. Enzyme concentration was 120 nM and substrate concentration was 150 μM for all runs. Buffer names are standard abbreviations found in the Sigma-Aldrich catalogue.

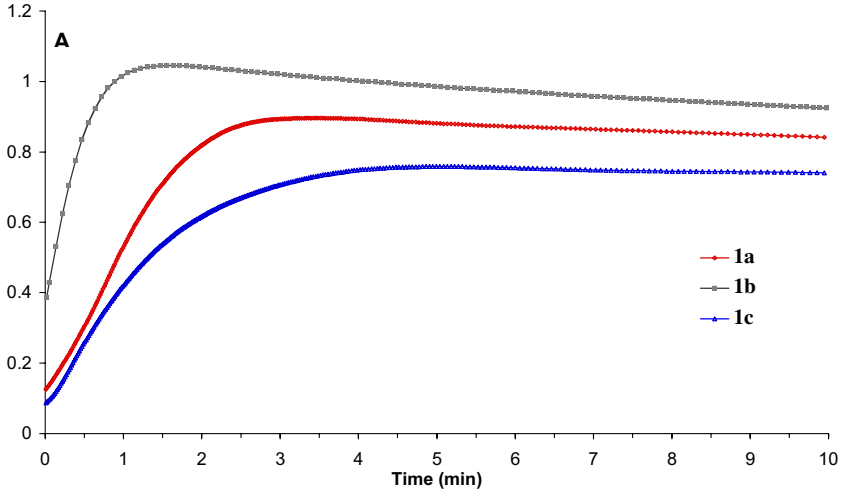



**Figure 2.** Activity of E1 enolase/phosphatase toward substrates **1a**–**c**. All assays were performed at 25 °C in 50 mM HEPES buffer containing 0.5 mM $CaCl_2$. For each assay, substrate was dissolved in buffer in an anaerobic cuvette and degassed by gentle purging with argon gas for 30 min. 2 μL of a 37 μM solution of E1 (80:20 glycerol/water) was added by syringe and mixed rapidly to give a 75 nM final concentration of enzyme. Dead time of the assay was ~10 s. Concentrations of the individual substrates were 184 μM **1a**, 219 μM **1b**, and 152 μM **1c**.

and absence of lag time for the any of the substrates should not be over-interpreted, since the dead time of the current assay is ~10 s. Stopped-flow measurements are underway to characterize the kinetics of E1 activity with these substrates more thoroughly.

## 2.4. Homology modeling of E1 enolase/phosphatase

Sequence homology identifies E1 as a member of the haloacid dehalogenase (HAD) superfamily of enzymes. The HAD superfamily contains L-2-haloacid dehalogenases, epoxide hydrolases, numerous phosphatases and phosphotransferases, and superfamily members have been identified in both prokaryotic and eukaryotic organisms.[17–19] In general phosphatases in this family catalyze reactions initiated by nucleophilic attack on the substrate phosphate group by the side chain carboxylate of a strictly conserved aspartate residue, followed by hydrolysis of the resulting anhydride intermediate. Many members of HAD superfamily require divalent metals (usually $Mg^{2+}$) for their activity, as does E1.[1] Several crystal structures of HAD superfamily members have been solved.[18,20–24] The consensus HAD structure contains two domains, a highly conserved core domain that assumes is α/β hydrolase fold (also known as the Rossmann fold) and a more diverse cap domain, with the active site located between the two domains. A homology model of E1 is shown in Figure 3, a model that is strongly supported by the results of NMR structural analysis currently underway (Milka Kostic, unpublished results).

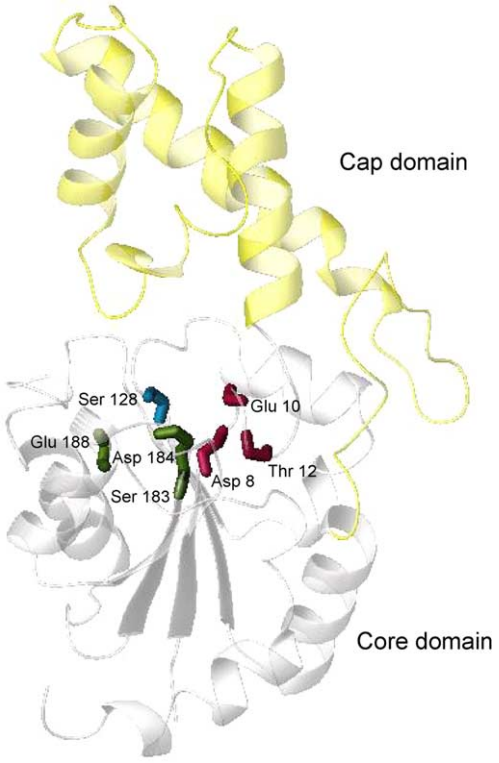



**Figure 3.** Model of E1 enolase/phosphatase based on sequence homology with β-phosphoglucomutase (PDB entry 1LVH).[22] Modeling was accomplished using the nest subroutine of the program Jackal.[28] The positions of proposed active site residues are shown and labeled. These correspond to the strongly conserved residues shown in Figure 4, and are color-coded to correspond to Figure 4. Figure was generated using MOLMOL.[29]

Three conserved sequence motifs are found in the HAD superfamily. The first, motif I, is located close to N-terminus, and contains the consensus sequence hhhhDxDx[T/V][L/V]h, where h is a hydrophobic residue (A, C, F, G, I, L, M, V, W or Y) and x can be any residue (2). The first aspartate residue in this motif plays a critical functional role in HAD enzyme superfamily, acting as the nucleophile in enzyme catalyzed reactions.[25] In E1, motif I starts with Ala 4, with the strongly conserved Asp 8 as a likely nucleophile involved in dephosphorylation and second conserved aspartate conservatively replaced by a glutamate at position 10. Conserved motif II has the consensus sequence hhhhhh[S/T]xx. The conserved serine/threonine (Ser 125 in E1) has been implicated in hydrogen bonding to the substrate.[18] Finally, conserved motif III is located near the C-terminus and is defined as K-$(x)_{18-30}$-(G/S)(D/S)xxx(D/N)hhhh.[18,20] In known structures, this motif forms part of the active site, including residues responsible for divalent metal ligation. The strictly conserved lysine (Lys 159 in E1) forms a salt bridge to the nucleophilic aspartate of motif I.[20–22] Lys 159 in E1 is separated by a 22 amino acid linker from conserved residues Ser 183 and Asp 184 (the conserved G/S and D/S, respectively). The conserved D/N residue of motif III is conservatively replaced by Glu 188 in E1. Details of primary sequence alignment and schematic representations of different motifs organizations are given in Figure 4.

E1 is the only enzyme isolated to date for which both enolase and phosphatase activities have been established. BLAST searching identifies a significant number of putative or predicted enolase/phosphatases from various organisms, ranging from prokaryotes to mammals. Sequence alignment revealed the presence of all three HAD superfamily conserved motifs. It has also revealed two conserved regions in the long linker domain, from which diversity of activity has been shown to arise in other members of HAD superfamily. We are currently attempting to establish the specific residues responsible for substrate specificity and enolase/phosphatase activity of E1.

## 3. Experimental

### 3.1. General

NMR spectra were recorded on a Varian Unity INOVA 400 MHz spectrometer operating at 399.75 MHz ($^1$H) and 100.51 MHz ($^{13}$C). Chemical shifts are reported in $\delta$ with the solvent resonance as the internal standard ($CDCl_3$: $\delta$ 7.27 for $^1$H, $\delta$ 77.0 for $^{13}$C). Spectra obtained in $D_2O$ were referenced to the residual HDO line at $\delta$ 4.8. IR spectra were recorded on a Perkin–Elmer FTIR spectrometer, and all absorption maxima are recorded in $cm^{-1}$. High resolution mass spectra were obtained at the University of California Riverside Mass Spectrometry facility. FAB spectra were obtained on a VG-ZAB instrument with running in negative ion mode using a glycerol matrix. Desorption chemical ionization (DCI) spectra were obtained on a VG 7070 instrument in positive ion mode using ammonia as proton source. Purified yields are reported where appropriate in Scheme 3.

**3.1.1. 3-Cyclopropyl-2-propyn-1-ol (5b).** Under $N_2$, 1.6 M *n*-butyllithium in hexane (9.72 mL, 15.5 mmol) was added dropwise to a solution of cyclopropylacetylene (1.17 mL, 12.9 mmol) in anhydrous ethyl ether (10 mL) at 0 °C. The solution was stirred for 30 min after the addition was completed. To this solution, paraformaldehyde [$(CH_2O)_n$] (0.47 g, 15.5 mmol) was added. The mixture was stirred for 1 h at 0 °C, and allowed to warm to room temperature for another 1.5 h. 20% HCl (5 mL) was added dropwise to the solution in an ice bath. Saturated $NaHCO_3$ solution and $H_2O$ were used to wash the organic layer. The aqueous layer was extracted with diethyl ether, and the organic layers were combined, dried over $Na_2SO_4$, and concentrated. Distillation (53–54 °C at 2 Torr) gave compound **5b** (0.77 g, 62%). The spectral data matched literature values:[26] NMR ($CDCl_3$) $^1$H 0.65–0.73 (m, 2H, cyclopropyl (cp) C$H_2$), 0.75–0.81 (m, 2H, cyclopropyl (cp) C$H_2$), 1.22–1.30 (m, 1H, cp C*H*), 1.55 (t (broad), 1H, $J = 3.6$ Hz,

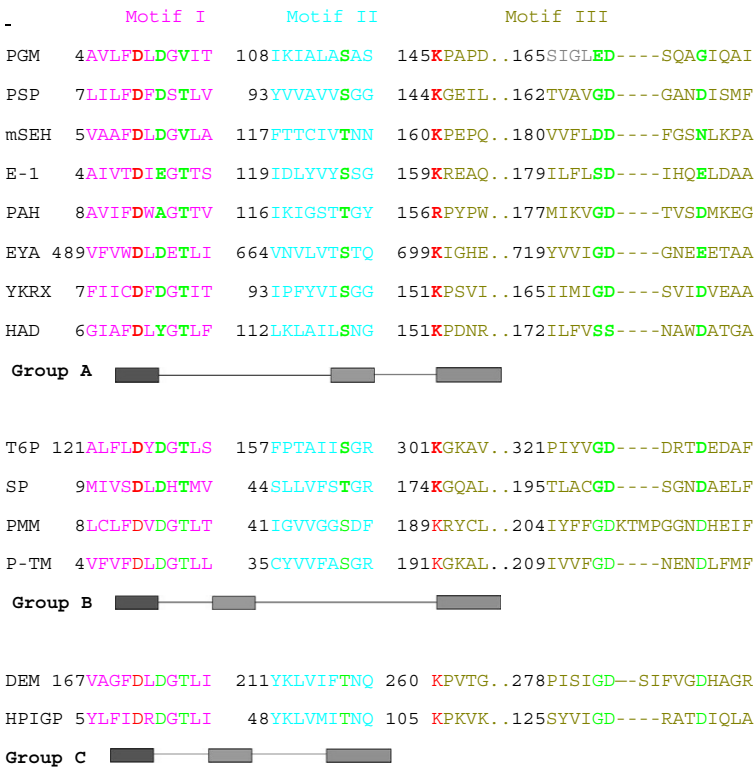



**Figure 4.** Primary sequence alignment of several different members of HAD superfamily. Three conserved motifs of the HAD superfamily are color-coded (purple, domain I, blue, domain II, and green, domain III). The three HAD main domain sequence distribution patterns, groups A, B and C are listed separately (see text). The number of the first residue within a motif indicates the position of motifs within the sequence. Strictly conserved residues are highlighted in red and those that express some variation are highlighted in green. Sequences are as follows: PGM, β-phosphoglucomutase (*Lactoccocus lactis*); PSP, phosphoserine phosphatase (*Methanococcus jannaschi*); mSEH—N-terminal of mammalian soluble epoxide–hydrolase (*Mus musculus*); E1, enolase/phosphatase (*K. oxytoca*); PAH, phosphonacetaldehyde hydrolase (*Bacillus cereus*); EYA, C-terminal of transcription factor (Eya) (*Drosophila melanogaster*); YKRX, YkrX gene product (*B. subtilis*); HAD, L-2-haloacid dehalogenase (*Pseudomonas sp. YL*); T6P, trehalose-6-phosphate phosphatase (*Arabidopsis thaliana*); SP, sucrose phosphatase (*A. thaliana*); PMM, phosphomannomutase (*Homo sapiens*); P-TM, phosphatase (*Thermotoga maritima*); DEM, DNA 5′-kinase-3′-phosphatase (*H. sapiens*); HPIGP, histidinol phosphatase/imidazole–glycerol phosphate phosphatase (*Escherichia coli*).

O*H*), 4.22 (d, 2H, $J = 3.6$ Hz, 1-C$H_2$); $^{13}$C-0.6 (cp *C*H), 8.2 (2C, cp-*C*H$_2$), 51.4 (1-*C*H$_2$), 73.6, 89.7.

**3.1.2. 4-Phenyl-2-butyn-1-ol (5d).** Compound was prepared in the same manner as **5b**, starting with 3-phenyl-1-propyne. Product **5d** (65% yield) was purified by distillation (59–60 °C, 2 Torr). Spectral data matched literature values:[27] NMR ($CDCl_3$) $^1$H 3.64 (t, 2H, $J = 2.0$ Hz, 4-C$H_2$), 4.32 (t, 2H, $J = 2.0$ Hz, 1-C$H_2$), 7.33 (m, 5H, ph C*H*); $^{13}$C 26.1 (4-*C*H$_2$), 52.4 (1-*C*H$_2$), 81.4, 85.0, 127.7 (ph CH), 128.9 (2C, ph–C*H*), 129.5 (2C, ph C*H*), 137.4 (ph C).

**3.1.3. 1-(Dibenzylphosphonooxy)-2-hexyne (6a).** The preparation of compound **6a** is typical. A solution of 1.2 equiv of pyridinium trifluoroacetate (2.3 g, 12.2 mmol), 1.0 equiv (1.0 g, 10.2 mmol) of 2-hexyn-1-ol and 1.2 equiv dibenzyl *N*,*N*-diisopropylphosphoramide (4.2 g, 12.2 mmol) in 10 mL of anhydrous $CH_2Cl_2$ was stirred under $N_2$ at 25 °C for 25 min. The reaction was monitored by TLC on silica gel using a 2% $KMnO_4$ solution spray for visualization. When all the starting alcohol was consumed, 3.5 mL of 30% $H_2O_2$ was added dropwise to the solution with cooling in an ice bath, and the solution was stirred at room temperature for 10 min. The mixture was washed with water, and the aqueous layer extracted with $CH_2Cl_2$. The $CH_2Cl_2$ fractions were combined, dried over $Na_2SO_4$, concentrated, and purified by flash chromatography on silica gel (hexane/ethyl acetate 2:1) to give **6a** (3.40 g, 93% yield) as an oil: NMR ($CDCl_3$) $^1$H, 0.94 (t, 3H, $J = 7.6$ Hz, 6-C$H_3$), 1.48 (tq, 2H, $J = 7.6$, 7.2 Hz, 5-C$H_2$), 2.02 (tt, 2H, $J = 7.2$, 2.4 Hz, 4-C$H_2$), 4.64 (td, 2H, 2.4 Hz, $^3J_{PH} = 9.6$ Hz, 1-C$H_2$), 5.07 (d, 4H, $^3J_{PH} = 7.6$ Hz, benzyl (bnz)–C$H_2$), 7.35 (m, 10H, phenyl (ph)–CH); $^{13}$C 13.4 (6-*C*H$_3$), 20.7, 21.7, 56.1 ($^2J_{PC} = 6$ Hz, 1-*C*H$_2$), 69.2 (2C, $^2J_{PC} = 6$ Hz, bnz–*C*H$_2$), 74.1 ($^3J_{PC} = 8$ Hz, 2-yne-C), 88.8 (3-yne-C), 127.8 (2C), 128.4 (4C), 128.5 (4C), 135.7 (2C, $^3J_{PC} = 7$ Hz); IR (film) 2963, 2237, 1497, 1281, 1017 cm$^{-1}$; $R_f$ (silica gel TLC, 2:1 hexane/EtOAc) 0.40; HRMS (DCI/$NH_3$, pos. ion mode) MH$^+$ calcd. for $C_{20}H_{24}O_4P$, 359.141223, obs. 359.141743.

**3.1.4. 1-(Dibenzylphosphonooxy)-3-cyclopropyl-2-propyne (6b).** NMR ($CDCl_3$) $^1$H 0.64–0.70 (m, 2H, cp–C$H_2$), 0.73–0.80 (m, 2H, cp–C$H_2$), 1.18–1.28 (m, 1H, cp–C*H*), 4.62 (dd, 2H, $^3J_{PH} = 10.4$ Hz, $J = 2$ Hz,

1-$CH_2$), 7.30–7.38 (m, 10H); $^{13}$C-0.6 (cp–*C*H), 8.2 (cp–$CH_2$), 56.1 ($^2J_{PC} = 5$ Hz, 1-*C*$H_2$), 69.3 ($^2J_{PC} = 5$ Hz, bnz–$CH_2$), 72.8 ($^3J_{PC} = 6$ Hz, 2-yne-C), 92.1 (3-yne-C), 127.9 (2C), 128.4 (4C), 128.5 (4C), 135.8 (2C, $^3J_{PC} = 8$ Hz); $R_f$ (silica gel TLC plate, 2:1 hexane/EtOAc) 0.31; IR (film) 2954, 2235, 1497, 1282, 1018 cm$^{-1}$; HRMS (DCI/$NH_3$, pos. ion mode) $MH^+$ calcd. for $C_{20}H_{22}O_4P$, 357.125573, obs. 357.124391.

**3.1.5. 1-(Dibenzylphosphonooxy)-3-phenyl-2-propyne (6c).** NMR ($CDCl_3$) $^1$H 4.85 (d, 2H, $^3J_{PH} = 10$ Hz, 1-C$H_2$), 5.12 (d, 4H, $^3J_{PH} = 8$ Hz, bzl–$CH_2$), 7.25–7.40 (m, 15H); $^{13}$C 55.9 ($^2J_{PC} = 5$ Hz, 1-*C*$H_2$), 69.3 ($^2J_{PC} = 5$ Hz, bnz–*C*$H_2$), 82.7 ($^3J_{PC} = 7$ Hz, 2-yne-C), 87.5 (3-yne-C), 121.7, 127.8 (2C), 128.2 (2C), 128.4 (2C), 128.4 (4C), 128.8 (4C), 131.7, 135.5 (2C, $^3J_{PC} = 7$ Hz); $R_f$ (silica gel TLC plate, 2:1 hexane/EtOAc) 0.36; IR (film): 2955, 2234, 1491, 1280, 1019 cm$^{-1}$; HRMS (DCI/$NH_3$, pos. ion mode: $MH^+$ calcd. for $C_{23}H_{22}O_4P$, 393.125573, obs. 393.127433.

**3.1.6. 1-(Dibenzylphosphonooxy)-4-phenyl-2-butyne (6d).** NMR ($CDCl_3$) $^1$H 3.59 (t, 2H, $J = 2.4$ Hz, 4-C$H_2$), 4.68 (dt, 2H, $^3J_{PH} = 10.4$ Hz, 2.4 Hz, 1-C$H_2$), 5.05 (d, 4H, $^3J_{PH} = 8.0$ Hz, bzl–C$H_2$), 7.25–7.33 (m, 15H, ph–C*H*); $^{13}$C 26.1 (4-*C*$H_2$), 56.9 ($^2J_{PC} = 6$ Hz, 1-*C*$H_2$,), 70.3 ($^2J_{PC} = 5$ Hz, bzl–*C*$H_2$), 84.7 ($^3J_{PC} = 7$ Hz, 2-yne-C), 87.3 (3-yne-C), 127.8, 128.9 (4C), 128.9 (4C), 128.9 (2C), 129.5 (2C), 129.6 (2C), 136.5 (2C, $^3J_{PC} = 8$ Hz), 136.8; IR (film) 2958, 2234, 1493, 1281, 1017 cm$^{-1}$.

**3.1.7. 1-(Dibenzylphosphonooxy)-2,3-dioxohexane (7a).** 4.1 equiv of $NaIO_4$ were added to a solution of 1 equiv of **6a** in $CCl_4/CH_3CN/H_2O$ (15 mL/15 mL/22.5 mL) to form two clear phases. 0.022 equiv of $RuO_2$. $H_2O$ were added, and the solution was stirred vigorously for 20 min at room temperature. The reaction was monitored by TLC and considered complete when no starting material remained, with product showing a clear yellow spot on TLC under ultraviolet light. Additional water (10 mL) was added, and the aqueous layer was extracted with $CH_2Cl_2$. The organic layers were combined, dried over anhydrous $Na_2SO_4$, and concentrated. Flash chromatography on silica gel deactivated with 10% $H_2O$ with gradient elution from pure hexane to 2:1 hexane EtOAc gave pure **7a** as bright yellow oil (2.8 g, 75% yield) after removal of solvent: NMR ($CDCl_3$) $^1$H 0.94 (t, 3H, $J = 7.2$ Hz, 6-C$H_3$), 1.61 (qt, 2H, $J = 7.2$, 7.6 Hz, 5-C$H_2$), 2.74 (t, 2H, $J = 7.6$ Hz, 4-C$H_2$), 5.03 (d, 2H, $^3J_{PH} = 11.2$ Hz, 1-C$H_2$), 5.10 (dd, 2H, $^2J = 12$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$) 5.14 (dd, 2H, $^2J = 12$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$), 7.34–7.37 (m, 10H, bzl–C*H*); $^{13}$C 13.5 (6-*C*$H_3$), 16.1 (5-*C*$H_2$), 38.0 (4-*C*$H_2$), 68.2 ($^2J_{PC} = 6$ Hz, 1-*C*$H_2$), 69.1 (2C, $^2J_{PC} = 6$ Hz, bzl–*C*$H_2$), 128.0 (2C), 128.6 (4C), 128.6 (4C), 135.5 (2C, $^2J_{PC} = 7.6$ Hz), 190.7 ($^3J_{PC} = 6$ Hz, 2-*C*O), 198.3 (3-*C*O); IR (film) 2986, 1743, 1715, 1265, 1045 cm$^{-1}$; HRMS (DCI/$NH_3$, pos. ion mode) calcd. for $C_{20}H_{24}O_6P$, $MH^+$ 391.131052, obs. 391.130697.

**3.1.8. 1-(Dibenzylphosphonooxy)-3-cyclopropyl-2,3-dioxopropane (7b).** NMR ($CDCl_3$) $^1$H 1.14–1.20 (m, 4H, cp–C$H_2$), 2.71–2.78 (m, 1H, cp–C*H*), 5.05 (d, 2H, $^3J_{PH} = 11.2$ Hz, 1-C$H_2$), 5.11 (dd, 2H, $^2J = 11$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$), 5.15 (dd, 2H, $^2J = 11$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$), 7.32–7.38 (m, 10 H, ph–C*H*); $^{13}$C 14.2 (cp–*C*$H_2$), 15.2 (cp–*C*H), 68.3 ($^2J_{PC} = 5$ Hz, 1-*C*$H_2$), 69.7 (2C, $^2J_{PC} = 5$ Hz, bzl–*C*$H_2$,), 128.0 (2C), 128.6 (4C), 128.6 (4C), 135.5 (2C, $^2J_{PC} = 7$ Hz), 190.1 ($^3J_{PC} = 10$ Hz, 2-*C*O), 197.5 (3-*C*O); IR 2958, 1743, 1698, 1498, 1265, 1012 cm$^{-1}$; HRMS (DCI/$NH_3$, pos. ion mode), calcd. for $C_{20}H_{22}O_6P$, $MH^+$ 389.115402, obs. 389.115100.

**3.1.9. 1-(Dibenzylphosphonooxy)-3-phenyl-2,3-dioxopropane (7c).** NMR ($CDCl_3$) $^1$H 5.10 (dd, 2H, $^2J = 11.6$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$), 5.12 (d, 2H, $^3J_{PH} = 11.2$ Hz, 1-C$H_2$), 5.14 (dd, 2H, $^2J = 11.6$ Hz, $^3J_{PH} = 8$ Hz, bzl–C$H_2$), 7.32–7.37 (m, 10 H, bzl–C*H*), 7.50 (dd, 2H, $J = 9$, 8 Hz, *m*-ph–C*H*), 7.67 (tt, 1H, $J = 9$ Hz, 1.2 Hz, *p*-ph–C*H*), 8.04 (dd, 2 H, $J = 8.0$ Hz, 1.2 Hz, *o*-ph–C*H*); $^{13}$C 68.9 ($^2J_{PC} = 5.3$ Hz, 1-*C*$H_2$), 69.8 (2C, $^2J_{PC} = 5.3$ Hz, bzl–*C*$H_2$), 128.1 (4C), 128.6 (4C), 128.7 (2C), 128.9 (2C), 130.4 (2C), 131.6, 135.1, 135.4 (2C, $^2J_{PC} = 7$ Hz), 189.5 (3-*C*O), 194.1 ($^3J_{PC} = 10$ Hz, 2-*C*O); IR (film) 2957, 1732, 1686, 1497, 1216, 1022 cm$^{-1}$; HRMS (DCI/$NH_3$, pos. ion mode), calcd. for $C_{23}H_{23}O_6P$, $MH^+$ 425.115402, obs. 425.115695.

**3.1.10. 1-Phosphonooxy-2,2-dihydroxy-3-oxohexane (1a).** 1.33 g (3.4 mmol) of **7a** was added to a mixture of trifluoroacetic acid/thiophenol/$H_2O$ (65 mL/1.7 mL/1.7 mL) at 25 °C. The mixture was stirred for 25 min and concentrated. $H_2O$ and $CH_2Cl_2$ were added to the residue. The two phases were separated and the organic phase was extracted with water five times. The combined aqueous phases containing compound **1a** were extracted with $CH_2Cl_2$, filtered, passed over decolorizing carbon, and concentrated by rotary evaporation, yielding 0.57 g (79% yield) of **1a**. Purity was established by $^1$H NMR to be >95%. Sufficient distilled water was then added to prepare a 0.1 M solution of **1a**, which was divided into 0.5 mL samples that were stored at −20 °C: NMR ($D_2O$) $^1$H 0.86 (t, 3H, $J = 7.6$ Hz, 6-C$H_3$), 1.55 (tq, 2H, $J = 7.2$, 7.6 Hz, 2-C$H_2$), 2.73 (t, 2H, $J = 7.2$ Hz, 4-C$H_2$), 3.98 (d, 2H, $^3J_{PH} = 4.8$ Hz, 1-C$H_2$), $^{13}$C 12.9 (6-*C*$H_3$), 16.4 (5-*C*$H_2$), 38.6 (4-*C*$H_2$), 68.0 ($^2J_{PC} = 6$ Hz, 1-*C*$H_2$), 94.9 ($^3J_{PC} = 13$ Hz, 2-*C*(OH)$_2$), 212.0 (3-*C*O); IR (film) 3415, 2971,1719, 1654, 1460, 1022 cm$^{-1}$; HRMS (FAB, neg. ion mode), $(M-H)^{-1}$ calcd. for anhydrous $C_6H_{10}O_6P$ (diketone), 209.021502, obs. 209.022200.

**3.1.11. 1-Phosphonooxy-2,2-dihydroxy-3-oxo-3-cyclopropylpropane (1b).** Synthesis was accomplished as for **1a**, except that activated charcoal was replaced by filtration through reverse-phase $C_{18}$-modified 40 μ silica using 7:3 acetonitrile/$H_2O$. Purity was established by $^1$H NMR to be >95%. NMR ($D_2O$) $^1$H 1.12–1.15 (m, 2H, cp–C$H_2$), 1.18–1.22 (m, 2H, cp–C$H_2$), 2.51–2.57 (m, 1H, cp–C*H*), 4.10 (d, 2H, $^3J_{PH} = 4.8$ Hz, 1-C$H_2$); $^{13}$C 14.4

(cp–$CH_2$), 17.6 (cp–CH), 68.9 ($^2J_{PC} = 6$ Hz, 1-$CH_2$), 96.3 ($^3J_{PC} = 10$ Hz, 2-C(OH)$_2$), 212.0 (3-CO); IR (neat) 3412, 2947, 17044, 1668, 1453, 1247 cm$^{-1}$; HRMS (FAB, neg. ion mode), $(M-H)^{-1}$ cald. for anhydrous $C_6H_8O_6P$ (diketone), 207.005852, obs. 207.005200.

**3.1.12. 1-Phosphonooxy-2,2-dihydroxy-3-oxo-3-phenylpropane (1c).** Purity was established by $^1$H NMR to be >95%. NMR ($D_2O$) $^1$H 4.13 (d, 2H, $^3J_{PH} = 5.6$ Hz, 1-C$H_2$), 7.4 (t, 2H, $J = 8.0$ Hz, *m*-ph–C*H*), 7.58 (t, 1H, $J = 8$ Hz, *p*-ph–C*H*), 8.07 (d, 2H, $J = 7.6$ Hz, *o*-ph–C*H*); $^{13}$C 70.0 ($^3J_{PC} = 5$ Hz, 1-$CH_2$), 96.6 ($^3J_{PC} = 11$ Hz, 2-$C$(OH)$_2$), 131.1, 134.3, 135.2, 200.2 (3-CO); IR (film) 3416, 2942,1711, 1659, 1454, 1185 cm$^{-1}$; HRMS (FAB, neg. ion mode), $(M-H)^{-1}$ cald. for anhydrous $C_6H_{10}O_6P$ (diketone), 243.005852, obs. 243.00530.

**3.1.13. 1-Phosphonooxy-2-hydroxy-3-oxohex-1-ene (8a).** 10 mg of **1a** was added to 0.4 mL of buffered (0.05 M potassium phosphate, pH 7.5) $D_2O$ in a standard 5 mm NMR tube. The pH was not corrected for isotope effects. Formation of the enol **8a** was followed by NMR, with enolization complete within 48 h: ($D_2O$) $^1$H 0.92 (t, 3H, $J = 7.6$ Hz, 6-C$H_3$), 1.48–1.60 (m, 2H, 2-C$H_2$), 2.49–2.59 (m, mostly exchanged with $D_2O$, 4-C$H_2$), 7.47 (d, $^3J_{PH} = 8$ Hz, 1-C*H*); $^{13}$C 13.5 (6-$CH_3$), 19.7 (5-$CH_2$), 38.6 (4-$CH_2$), 136.5 ($^3J_{PC} = 9$ Hz, 2-$C$(OH)), 139.7 ($^2J_{PC} = 6$ Hz, 1-*C*H), 200.9 (3-*C*O).

**3.1.14. Subcloning, overexpression, purification, and further characterization of E1 enolase/phosphatase from *K. oxytoca*.** The E1 coding region was PCR amplified from the pDiox2 plasmid with the forward primer 5′-CACTCTGGAGAACATATGATCCGCGCT-3′ and reverse primer 5′-GCGCGCGGATCCTTATGCTGGGATCTGCTCCGGATGAATA-3′. The PCR product was double-digested with BamHI and NdeI (New England Biolabs), and subcloned into pET3a plasmid (Novagen) to generate a new construct named pTP01. The subcloned vector sequences were confirmed by standard methods. Single colonies of BL21(DE3)pLysS *E. coli* transformed with pTP01 were selected from LB-agar plates supplemented with appropriate antibiotics and placed in antibiotic-supplemented LB media. Protein expression was induced by the addition of isopropyl β-D-thioglucopuranoside to 0.5 mM upon the cell density reaching 0.6 at 600 nm. Cells were harvested by centrifugation, and protein expression was confirmed by denaturing gel electrophoresis. If overexpression was observed, cell-free extracts were assayed for enolase/phosphatase activity using the procedures described below.

**3.1.15. Enzyme purification.** A modification of the procedure of Myers et al. was used to purify E1 enolase/phosphatase.[1] Cells overexpressing E1 were harvested by centrifugation and lysed by sonication in 20 mM Tris buffer pH 8.0 with 0.1 % tosyl chloride added to inhibit protease activity. The lysate was cleared by centrifugation (10,000 × *g*, 15 min) and the pellets discarded. An ammonium sulfate precipitation was performed, with the 30–70% ammonium sulfate precipitate containing the majority of the E1. After centrifugation (10,000 × *g*, 30 min) the supernatant was discarded and the 30–70% ammonium sulfate precipitate was dissolved in a minimum amount of 20 mM Tris buffer pH 7.5 and dialyzed overnight against 20 mM Tris buffer pH 7.5 containing 0.5 mM $MgSO_4$ (Buffer A). The resulting dialysate was cleared by centrifugation (14,000 × g, 5 min), and passed through a 5 μ syringe filter onto a MonoQ 10/10 (Pharmacia) column mounted on an AKFA FPLC system. The following chromatographic program was used to elute the E1 with a flow rate of 4 mL/min: 2 column volumes (CV) of buffer A following sample injection (1 CV = 7.854 mL), 10 CV gradient from 0–15% buffer B (Buffer A+1 M NaCl), 50 CV gradient from 15–30% buffer B. E1 elutes between 20 CV (160 mL)and 24 CV (190 mL) (~17% buffer B). These fractions were pooled, and were mixed 1:1 with 2.4 M $(NH_4)_2SO_4$ in buffer A. After centrifugation and filtration, the soluble fraction was applied to a 16/10 Phenyl-superose column (Pharmacia) equilibrated with buffer C (Buffer A + 1.2 M $(NH_4)_2SO_4$). The E1 was eluted from the phenyl-superose column using the following gradient (flow rate of 2 mL/min): 2 CV of buffer C (1 CV = 20.106 mL) following injection, followed by a gradient (6 CV) from 0–40% buffer A, and a second gradient (20 CV) from 40% to 100% buffer A. The E1 eluted after 17 CV (340 mL) (~67% buffer A). Fractions of pure E1, as judged by Coomassie-stained SDS–PAGE, were pooled and concentrated, then dialyzed against 50 mM HEPES buffer (pH 7.5) containing 0.5 mM $MgCl_2$. The enzyme was then either reconcentrated to ~1 mM and stored in liquid nitrogen or mixed with glycerol (80/20 v/v glycerol/protein stock solution) and stored in −20 °C freezer for enzyme assays (2 mg/mL enzyme). Activity of the enzyme was compared to that of the enzyme isolated from *K. pneumoniae* and found to be equivalent, as measured by the rate of formation of acireductone in the absence of oxygen. Overexpression typically gives a yield of purified enzyme of ~50 mg/L of growth medium.

The calculated extinction coefficient of E1 enolase/phosphatase based on the sequence of 21.62 mM$^{-1}$ cm$^{-1}$ at 280 nm, corresponding to an absorbance of 0.844 at 1 mg/mL. Mass spectra of the purified enzyme were obtained on an Applied Biosystems Voyager 2102 MALDI/TOF spectrometer operating in positive ion mode. The calculated molecular mass of E1 is 25482.6 amu, and MALDI/TOF mass spectral analysis yields a molecular mass of 25469+/−3 $m/z$.

Molecular modeling of E1 enolase/phosphatase was performed using the nest subroutine of Jackal, a molecular modeling program available from Columbia University.[28] The structure was modeled using a single molecule from the deposited coordinates of β-phosphoglucomutase (1LVH).[22] The nest subroutine allows the modeled structure to be refined by energy minimization within loop and regular secondary structure regions.

**3.1.16. Enzyme assays.** Enzyme assays were performed in 50 mM HEPES buffer (pH 7.4) containing 0.5 mM $MgCl_2$, using anaerobic cuvettes with 1 cm path length. 1 mL of buffer containing ~200 μM substrate was placed in the cuvette. The solution was then degassed for at least 15 min by bubbling a gentle stream of argon gas introduced through the septum with a syringe needle, with a smaller needle for exhaust. A drop of water was placed on the top of the septum to ensure the seal, and the needles removed. 2 μL of a 40 μM solution of E1 enolase/phosphatase in 80/20 glycerol/water was introduced by syringe, the solution mixed rapidly, and the course of the reaction monitored by UV–visible spectrometry on a HP diode-array spectrophotometer equipped with temperature control. Temperature was maintained at 25 °C. Based on the mass of dehydrated purified samples of **1a**, and assuming 100% conversion of substrate to acireductone product, an extinction coefficient was calculated at 320 nm for the acireductone derivative of **1a** of 8000 $M^{-1}$ $cm^{-1}$.

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## References and notes

1. Myers, R. W.; Wray, J. W.; Fish, S.; Abeles, R. H. *J. Biol. Chem.* **1993**, *268*, 24785–24791.
2. Balakrishnan, R.; Frohlich, M.; Rahaim, P. T.; Backman, K.; Yocum, R. R. *J. Biol. Chem.* **1993**, *268*, 24792–24795.
3. Wray, J. W.; Abeles, R. H. *J. Biol. Chem.* **1993**, *268*, 21466–21469.
4. Wray, J. W.; Abeles, R. H. *J. Biol. Chem.* **1995**, *270*, 3147–3153.
5. Dai, Y.; Wensink, P. C.; Abeles, R. H. *J. Biol. Chem.* **1999**, *274*, 1193–1195.
6. Pochapsky, T. C.; Pochapsky, S. S.; Ju, T. T.; Mo, H. P.; Al-Mjeni, F.; Maroney, M. J. *Nat. Struct. Biol.* **2002**, *9*, 966–972.
7. Dai, Y.; Pochapsky, T. C.; Abeles, R. H. *Biochemistry* **2001**, *40*, 6379–6387.
8. Al-Mjeni, F.; Ju, T.; Pochapsky, T. C.; Maroney, M. J. *Biochemistry* **2002**, *41*, 6761–6769.
9. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859–1862.
10. Wakamiya, T.; Saruta, K.; Yasuoka, J.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 2699–2703.
11. Sanghvi, Y. S.; Guo, Z. Q.; Pfundheller, H. M.; Converso, A. *Org. Process Res. Dev.* **2000**, *4*, 175–181.
12. Zibuck, R.; Seebach, D. *Helv. Chim. Acta* **1988**, *71*, 237–240.
13. Schuda, P. F.; Cichowicz, M. B.; Heimann, M. R. *Tetrahedron Lett.* **1983**, *24*, 3829–3830.
14. Kitas, E. A.; Knorr, R.; Trzeciak, A.; Bannwarth, W. *Helv. Chim. Acta* **1991**, *74*, 1314–1328.
15. Miyata, K.; Nakashima, K.; Koyanagi, M. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 367–371.
16. Lee, Y. J.; Burr, J. G. *Chem. Phys. Lett.* **1976**, *43*, 146–148.
17. Collet, J. F.; Stroobant, V.; Pirard, M.; Delpierre, G.; Van Schaftingen, E. *J. Biol. Chem.* **1998**, *273*, 14107–14112.
18. Wang, W. R.; Kim, R.; Jancarik, J.; Yokota, H.; Kim, S. H. *Structure* **2001**, *9*, 65–71.
19. Selengut, J. D.; Levine, R. L. *Biochemistry* **2000**, *39*, 8315–8324.
20. Morais, M. C.; Zhang, W. H.; Baker, A. S.; Zhang, G. F.; Dunaway-Mariano, D.; Allen, K. N. *Biochemistry* **2000**, *39*, 10385–10396.
21. Hisano, T.; Hata, Y.; Fujii, T.; Liu, J. Q.; Kurihara, T.; Esaki, N.; Soda, K. *J. Biol. Chem.* **1996**, *271*, 20322–20330.
22. Lahiri, S. D.; Zhang, G. F.; Dunaway-Mariano, D.; Allen, K. N. *Science* **2003**, *299*, 2067–2071.
23. Shin, D. H.; Roberts, A.; Jancarik, J.; Yokota, H.; Kim, R.; Wemmer, D. E.; Kim, S.-H. *Protein Sci.* **2003**, *12*, 1464.
24. Argiriadi, M. A.; Morisseau, C.; Hammock, B. D.; Christianson, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10637–10642.
25. Selengut, J. D. *Biochemistry* **2001**, *40*, 12704–12711.
26. Morgan, T. K.; Wohl, R. A.; Lumma, W. C.; Wan, C. N.; Davey, D. D.; Gomez, R. P.; Marisca, A. J.; Briggs, M.; Sullivan, M. E.; Wong, S. S. *J. Med. Chem.* **1986**, *29*, 1398–1405.
27. Claesson, A.; Sahlberg, C. *Tetrahedron* **1982**, *38*, 363–368.
28. Xiang, J. Z. 'Jackal: A protein structure modeling package', URL: http://trantor.bioc.columbia.edu/programs/jackal.
29. Koradi, R.; Billeter, M.; Wuthrich, K. *J. Mol. Graphics* **1996**, *14*, 51.