Bioorganic & Medicinal Chemistry Letters 19 (2009) 5954–5957

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A fluorescent substrate for carbon–phosphorus lyase: Towards the pathway for organophosphonate metabolism in bacteria

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ARTICLE INFO

Article history:
Received 23 June 2009
Revised 7 August 2009
Accepted 7 August 2009
Available online 13 August 2009

Keywords:
Carbon–phosphorous lyase
phn operon
Phosphonate metabolism
Fluorescent label
Substrate
Escherichia coli

ABSTRACT

Many species of bacteria can use naturally occurring organophosphonates as a source of metabolic phosphate by cleaving the carbon–phosphorus bond with a multi-enzyme pathway collectively called carbon–phosphorus lyase (CP-lyase). Very little is known about the fate of organophosphonates entering this pathway. In order to detect metabolic intermediates we have synthesized a fluorescently labelled organophosphonate and show that this is a viable substrate for the CP-lyase pathway in *Escherichia coli* and that the expected product of CP-bond cleavage is formed. The in vivo competence of one potential metabolic intermediate, 1-ethylphosphonate-α-D-ribofuranose, is also demonstrated.

Appropriately called the 'staff of life',[1] phosphate is an essential component of biological energy (ATP), genetic information (DNA, RNA) and cell signalling (protein phosphorylation, cAMP). Phosphate is also frequently a life-limiting nutrient for microorganisms, particularly in aquatic environments where concentrations can fall to picomolar levels.[2] To make up for this shortfall, many species of bacteria will utilize organophosphonates, which can comprise a significant fraction of total soluble phosphorus.[3] For example, the most abundant naturally occurring organophosphonate, 2-aminoethylphosphonate, is used by phytoplankton as a surrogate for phosphocholine in the biosynthesis of phospholipids.[4] However, for phosphate to be liberated from organophosphonates for other cellular roles, bacteria must cleave the highly stable carbon–phosphorus bond (BDE = 70 kcal mol^{-1}). A multi-enzyme pathway, collectively called carbon phosphorus lyase (CP-lyase), is commonly used by bacteria (primarily gram-negative) to cleave this bond, producing inorganic phosphate and a hydrocarbon.[3,5] Commensurate with its scavenging role, CP-lyase is notably relaxed in its substrate specificity (which varies according to the bacterial species[6]), and is capable of cleaving CP bonds in alkyl-, phenyl-, vinyl-, and alkynylphosphonic acids, as well as doubly cleaving alkylphosphinic acids.[6,7] Such substrate promiscuity makes CP-lyase an excellent candidate for bioremediation applications.

Despite considerable knowledge of the remarkably complex genetic structure of the *phn* operon encoding CP-lyase,[8–10] little is known about the enzymes encoded by the individual *phn* genes (*phnCDEFGHIJKLMNOP*),[11–14] particularly the individual reactions mediated by each and how these reactions might comprise an overall pathway for organophosphonate metabolism. Initial evidence for a metabolic pathway came from the detection of 1-ethylphosphonate-α-D-ribofuranose **1** (Scheme 1) in the medium of an *Escherichia coli* culture subsisting on ethylphosphonate as the sole phosphorus source.[15] This compound bore a striking resemblance to the substrate for PhnN (one of the few CP-lyase enzymes with a demonstrated function), which phosphorylates 5-phospho-α-D-ribofuranosyl phosphate **2** to give the glycosyl donor 5-phospho-α-D-ribofuranosyl diphosphate **3** (Scheme 1).[11] To identify other potential metabolic organophosphonate intermediates it would be useful to directly track the fate of an alkylphosphonate in the cell. Although it is possible to synthesize ^{32}P labelled alkylphosphonates,[15] we opted to avoid radiolabelled substrates.

Inspired by the tolerance of CP-lyase for a diverse range of sizes and structures in the organic moiety of organophosphonates, we hypothesized that a fluorescently labelled alkylphosphonate would also be a viable substrate. To this end we synthesized dansyl labelled 3-aminopropylphosphonic acid **7** in a simple three step procedure (Scheme 2). The commercially available nitrile **4** was reduced with sodium borohydride to obtain 3-aminopropyl phosphonate **5**. Reaction of the amine with dansyl chloride afforded fluorescently labelled **6**, which was deprotected with bromotrimethylsilane to afford **7**. We also synthesized the predicted product **8** of the CP-lyase mediated cleavage of **7** through a direct reaction of dansyl chloride with *n*-propylamine. Details of the

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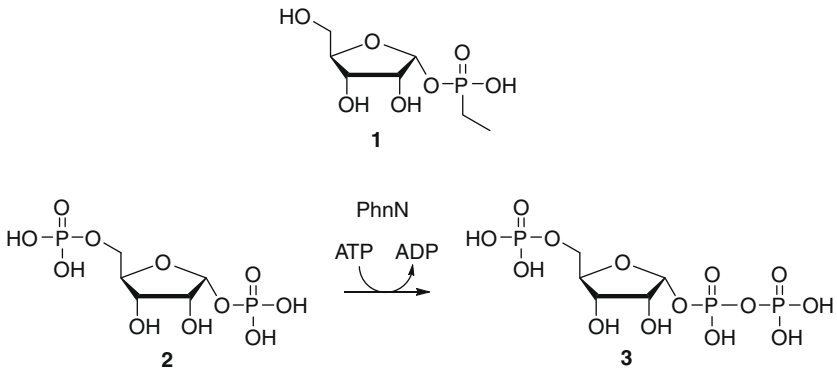
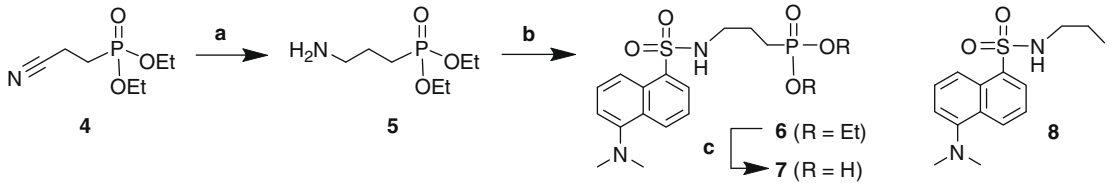
doi:10.1016/j.bmcl.2009.08.035



Scheme 1. The putative metabolic intermediate **1** identified in *E. coli* cultures subsisting on ethylphosphonic acid. The reaction for PhnN, one of 14 enzymes and proteins in the CP-lyase pathway, is shown below. **1** is a potential precursor to the substrate **2** for PhnN.



Scheme 2. Reagents and conditions: (a) $NaBH_4$, $CoCl_2$, THF/H_2O (2:1), 0 °C, 3 h (40%); (b) dansyl chloride, Et_3N, CH_2Cl_2, rt, 32 h (53%); (c) TMSBr, ACN, rt, 24 h then $MeOH/H_2O$ (9:1), rt, 2 h (77%).

synthesis and characterization of **7** and **8** are given in the Supplementary data following this Letter.

We then tested the ability of the *E. coli phn+* strain HO1429 to grow on MOPS minimal media agar plates[16] where **7** (0.5 mM) was the sole source of phosphorus (see Supplementary data for strain preparation and growth conditions). For comparison strain HO1429 was also grown on MOPS-agar media containing 0.5 mM methylphosphonic acid, a known substrate for *E. coli* CP-lyase,[7] and media containing no source of phosphorus. The plates were simultaneously incubated at 37 °C for 3 days (growth of *E. coli* is typically slow under these nutrient deprived conditions). Gratifyingly, *E. coli* HO1429 grew as well on minimal media containing **7** as media containing an equivalent concentration of methylphosphonic acid (Fig. 1). Plates containing no source of phosphorus did

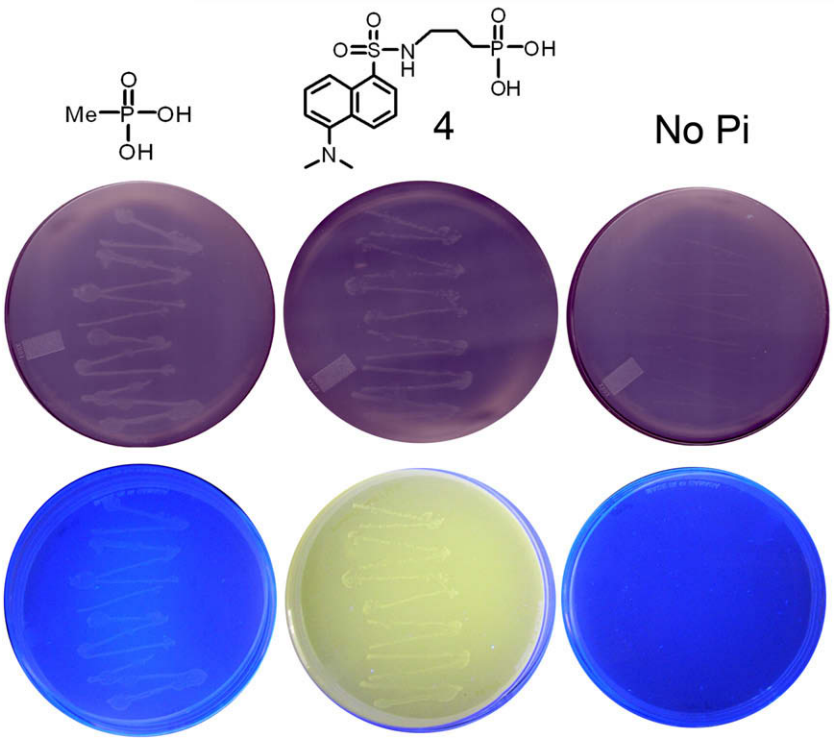


Figure 1. Growth of the *E. coli phn+* strain HO1429 on MOPS minimal media agar containing (l to *r*) methyl phosphonate (0.5 mM), **7** (0.5 mM), and no phosphorus source. The plates were illuminated with ambient light (top row) or 360 nm light (bottom row).

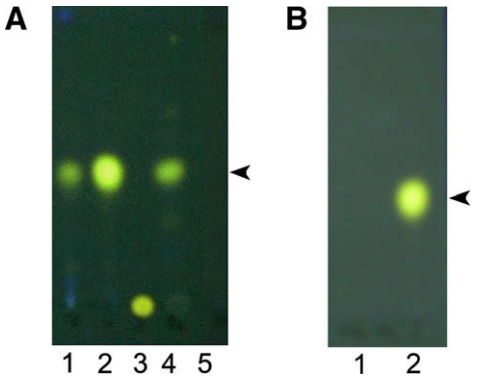


Figure 2. (A) TLC analysis of the metabolism of **7** by *E. coli* in MOPS minimal media. The plate was developed with 96:4 CH_2Cl_2/MeOH and illuminated with 366 nm light. The major product **8** is indicated with an arrow. Lane 1, CH_2Cl_2 extract of the used growth medium of HO1429 grown in liquid culture with **7**; lane 2, synthetic standard **8**, the predicted CP-lyase product; lane 3, substrate **7**, lane 4; major product of *E. coli* culture purified by preparative TLC for EI-MS analysis; lane 5, CH_2Cl_2 extract of minimal media without **7**. (B) Lane 1, CH_2Cl_2 extract of MOPS minimal media incubated with **7** in the absence of HO1429. Lane 2, synthetic standard **8**.

not show bacterial growth. Growth of HO1429 on **7** is not likely to have arisen from contaminating phosphate as no phosphate was present during synthesis and purification of **7**, and glassware was made phosphate-free by liberal washing with dilute nitric acid before use.

The growth of HO1429 on **7** in the absence of contaminating phosphate should lead to the production of the CP-bond cleavage product **8** (Scheme 2). To perform a product analysis HO1429 was cultured in liquid MOPS minimal media containing 0.5 mM **7** until the bacterial culture reached an optical density at 600 nm of ~1 (37 °C, 220 rpm in an air shaker, ~2 days). After removing the cells by centrifugation, the remaining medium was extracted with CH_2Cl_2 and the solvent removed in vacuo. TLC analysis of the concentrated extract on silica gel plates developed with 96:4 CH_2Cl_2/MeOH indicated the presence of a product with an R_f = 0.44 (Fig. 2A, lane 1) which exhibited the characteristic yellow–green fluorescence of a dansyl fluorophore (λ emission = 525 nm) when viewed under long UV light (~360 nm). This compound matched the R_f of synthetically derived **8**, the expected product of the CP-lyase reaction with **7** (Fig. 2A, lane 2). The substrate **7** remained on the baseline (Fig. 2A, lane 3). Incubation of **7** in minimal media in the absence of HO1429 did not result in the formation of **8** (Fig. 2B, lane 1). Likewise, disruption of the *phn* operon in *E. coli* resulted in a strain that was unable to cleave **7** to form **8** (unpublished data). Purification of this primary product by preparative TLC (Fig. 2A, lane 4) and analysis by EI-MS (positive ion mode) revealed a molecular ion peaks at m/z = 292.12 and m/z = 171.11, which correspond to the expected values for the molecular ion of **8** and a dimethylamino naphthalene fragment ion, respectively (Fig. 3). It is clear from these analyses that *E. coli* CP-lyase accepts **7** as a substrate and cleaves the CP bond to form inorganic phosphate (which sustains bacterial growth) and the expected labelled product **8**.

Encouraged by these results, we subsequently tested synthetically derived **1**,[17] a putative metabolic intermediate in the *E. coli* CP-lyase pathway. The *E. coli* strain HO1429 was once again grown on MOPS-agar minimal media supplemented with 0.5 mM methylphosphonic acid, 0.5 mM **1**, or media lacking any phosphorus source. Likewise, glassware was washed with dilute nitric acid to eliminate contaminating phosphate. After 2–3 days incubation at 37 °C, equivalent growth of HO1429 colonies were observed on plates containing methylphosphonic acid or **1**, but not on the medium lacking phosphorus (Fig. 4). This is an intriguing result as **1** is formally a phosphonate ester, and it was previously shown that ethyl ethylphosphonic acid could not sustain growth of *E. coli*, suggesting that this was not a substrate for CP-lyase.[7] Although this is good preliminary evidence that **1** is a competent intermediate in the CP-lyase pathway, it is possible that **1** is hydrolyzed in vivo to release ethylphosphonic acid, which is a viable substrate for *E. coli* CP-lyase.[7] Having demonstrated that fluorophore labelled alkyl phosphonates are acceptable substrates for CP-lyase, this

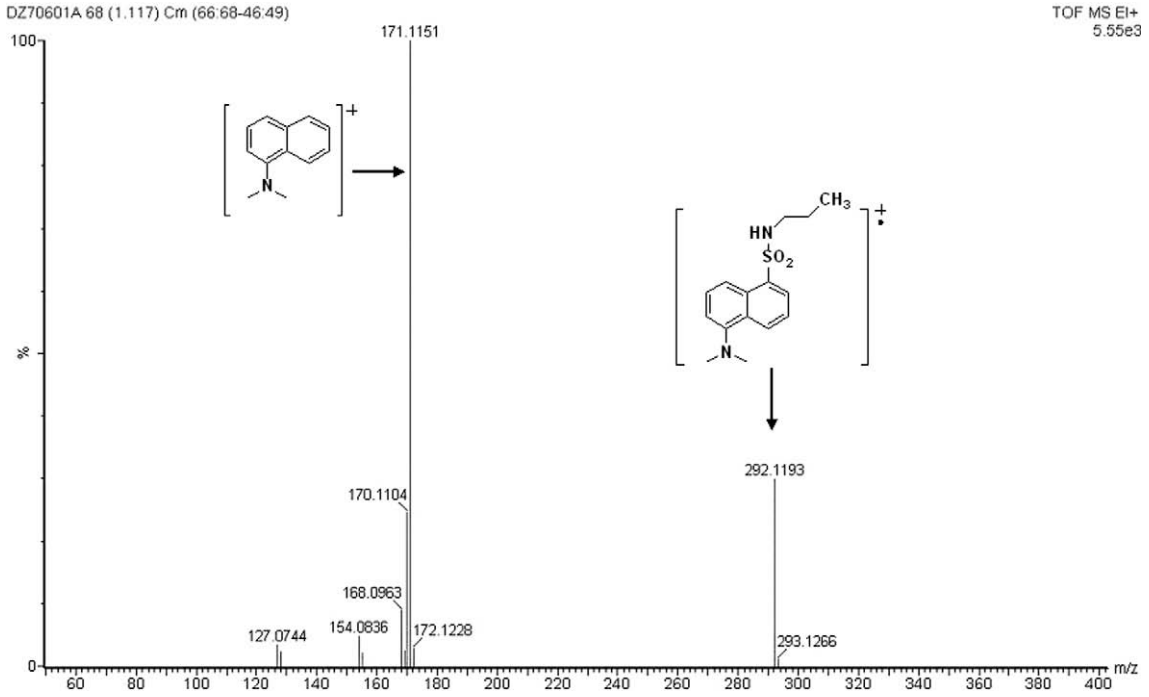


Figure 3. EI-MS spectrum (positive ion mode) of the major product isolated from HO1429 subsisting on **7**. Molecular (m/z = 292.1) and fragment ions (m/z = 170.1) are indicated.

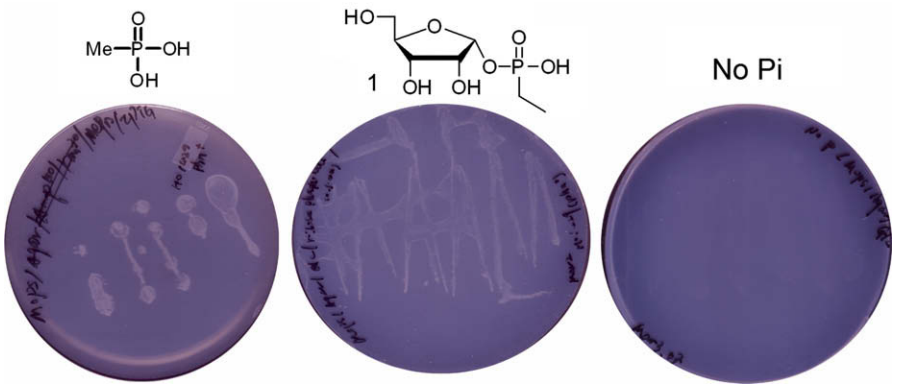


Figure 4. Growth of *E. coli phn+* strain HO1429 on MOPS-agar minimal media containing (l to *r*) methyl phosphonate (0.5 mM), **1** (0.5 mM), and no phosphorus source. The plates were illuminated with ambient light.

possibility can be addressed by synthesizing the corresponding dansyl substituted **1** and tracking its fate in vivo by TLC or HPLC analysis of cell extracts.

The viability of **7** as a substrate for *E. coli* CP-lyase provides a powerful tool for the dissection of the metabolic pathway of organophosphonate degradation. Strains of *E. coli* can be created where the *phn* operon is constitutively expressed so that the strains will degrade organophosphonates even in the presence of low concentrations of inorganic phosphate.[18] Subsequently, individual *phn* genes can be 'knocked out' in this strain and the resulting mutants cultured with **7**. When **7** is incorporated into metabolic intermediates such as **1**, the absence of a downstream CP-lyase enzyme should result in accumulation of the intermediate, which can be easily detected due to the attached fluorophore. Purification of such intermediates for identification by MS and NMR will also be facilitated by the fluorophore. Ultimately the use of **7** in this strategy will allow the assignment of specific reactions and substrates to individual CP-lyase enzymes.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.035.

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