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Review

The bacterial HPr kinase/phosphorylase: A new type of Ser/Thr kinase as antimicrobial target

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Abstract

Protein phosphorylation plays a major role in bacterial cellular regulation as in eukaryotes. The HPr Kinase/Phosphorylase (HprK/P) was the first bacterial serine protein kinase to have had its structure determined, establishing that it is unrelated to the eukaryotic kinases. HprK/P belongs to another large structural family, the P-loop containing proteins. Among them, P-loop containing kinases have been assumed to only phosphorylate small molecules, but the example of HprK/P suggests that some may have proteins as substrates, defining novel cellular signal transduction pathways. Another major result of the studies presented here is that HprK/P also catalyses the phosphorolysis of the phosphoserine, yielding serine and pyrophosphate. The two different catalytic activities are carried out at the same active site. The determination of the structure of the complex with the protein substrates HPr and PserHPr allowed us to propose a catalytic mechanism. Since regulation of HPr phosphorylation has been shown to be involved in the virulence process of pathogenic bacteria, a search for specific inhibitors of HprK/P is of clinical interest and the first hit has already been found.

Keywords: HPr kinase; Crystal; Structure; Complex; Active site; Mechanism

1. Introduction

HPr kinase/phosphorylase (HprK/P), the sensor enzyme for catabolite repression in Gram positive bacteria, phosphorylates Ser46 of HPr, a phosphocarrier protein of a sugar transport and phosphorylation system (PTS) [1]. HprK/P is a bifunctional enzyme. It was first identified as an ATP-dependent serine protein kinase, an activity which is enhanced in the presence of glycolytic intermediates such as fructose-1,6-bisphosphate [1]. However, in the presence of inorganic phosphate (Pi), HprK/P also catalyzes the dephosphorylation of Ser46 [2]. This reaction, for long time assumed to be hydrolytic, has recently been demonstrated to be phosphorolytic and to produce pyrophosphate [3]. X-ray studies played a major role in the identification of this novel reaction [4]. In Gram-positive bacteria, serine-phosphorylated HPr (PserHPr) acts as a co-repressor by binding to CcpA (catabolite control protein A) [5], a LacI/GalR-type repressor [6]. The structure of the CcpA-PserHPr-DNA complex has been solved recently [7]. The interaction allows CcpA to bind to specific operator sites (*cre*) [8], preceding a large number of catabolite-regulated genes [9]. HprK/P is absent in enteric Gram-negative bacteria such as *E. coli*, but homologues are found in pathogens like Neisseriae [10,11]. Phosphorylation of HPr at Ser46 by HprK/P affects the virulence of some pathogenic bacteria. Inactivation of *N. meningitidis* HprK/P leads, for example, to a 10-fold reduced cell adhesion, the first step of the infectious process (Boël et al., 2003). In the Gram-positive *Listeria monocytogenes*, the enhanced formation of PserHPr in the presence of rapidly metabolizable carbon sources was found to be responsible for the inhibition of the transcription activator PrfA, involved in the regulation of virulence gene expression. HprK/P plays, therefore, a regulatory role in the infectious process and a search for specific inhibitors should be of clinical interest. This aspect will be discussed in more detail in Josef Deutscher's accompanying paper.

Sequence analysis revealed that HprK/P showed no similarity with eukaryotic Ser/Thr kinases over its ~300

Abbreviations: HPr, Histidine phosphocarrier protein; PserHPr, seryl-phosphorylated HPr; HprK/P, HPr Kinase/Phosphorylase; P-loop, phosphate binding loop; CcpA, catabolite control protein A; Pi, inorganic phosphate; PEP, phosphoenolpyruvate

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Table 1
X-ray structures of HPrK/P

PDB[a] entry code	Origin	Protein	Ligands	m[b]	Resolution (Å)	Ref.
1JB1	*L. casei*	C-domain	Pi	1	2.8	[14]
1KKL	*L. casei*	C-domain	HPr	3	2.8	[14]
1KKM	*L. casei*	C-domain	P-Ser-HPr,Pi,Ca^{++}	3	2.8	[14]
1KO7	*S. xylosus*	Full-length	Pi, Mg^{++}	2	1.95	[15]
1KNX	*M. pneumoniae*	Full-length	–	6	2.5	[16]

[a] Protein Data Bank http://www.rcsb.org/pdb/.
[b] Number of subunits in the crystal asymmetric unit.

residues. The HprK/P sequences diverge most at the N-terminal domain, which has no defined function yet, and is absent in α-proteobacteria, including *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*. The C-terminal domain carries both kinase and phosphorylase activities. It contains the Walker motif A (G/A)xxxxGK(S/T) [10,12] characteristic of the phosphate binding loop (P-loop) at the nucleotide binding site [13]. Five X-ray structures of HprK/P are available at present (Table 1). After the C-domain of *Lactobacillus casei* HprK/P [14], structures of the full-length protein were obtained for *Staphylococcus xylosus* [15] and *Mycoplasma pneumoniae* [16]. The structures of the complexes between the catalytic domain of *L. casei* HprK/P and either unphosphorylated or phosphorylated HPr were also solved [4]. All are closely related. We review the structure of the free enzyme, and compare it to the complexes. The mode of HPr and inorganic phosphate (Pi) binding observed in the complexes suggest mechanisms for both the phosphorylation and the phosphorolysis reactions [4]. A chemical library has been screened for HprK/P inhibitors, and preliminary results are reported.

2. HprK/P: a completely new structure

2.1. The hexamer

In all X-ray structures, HprK/P is a hexamer with a three-fold axis orthogonal to the three two-fold axes. D3 symmetry is maintained in the complexes with HPr, where the hexamer binds six HPr molecules. It holds by contacts between C-terminal catalytic domains, which form two trimers associated in a hexameric core. Three pairs of N-terminal domains hang outside, making little contact with the core (Fig. 1). In the X-ray structure of the *L. casei* C-domains, the lack of an N-terminal domain has little effect on either the fold or the mode of association of the core (Fig. 4).

The fold of HprK/P N-terminal domain is similar to the MurE/MurF N-terminal domain. At the interface between pairs of N-terminal domains of the *S. xylosus* HprK/P structure, two phosphate ions are bound. They interact with three arginines, which are strictly conserved in the HprK/P sequences of Gram-positive bacteria, but not in Gram negative and in Mycoplasma. This site may be designed to bind phosphorylated metabolites rather than just Pi, and it should play a role in the biological function of the N-terminal domain, whatever that function may be [15].

2.2. The catalytic C-terminal domain

As already suggested by sequence analysis, the catalytic domain of HprK/P shows no similarity with eukaryotic Ser/Thr kinases. The catalytic subunit forms a globular structure, from which the C-terminal helix protrudes (Figs. 1 and 4). An α/β unit consisting of a central five-stranded β-sheet, with two α-helices on one face of it, is characteristic of the P-loop type nucleotide-binding domain [13]. The Walker A motif forms the loop where a Pi ion is found when the protein is crystallized in the presence of phosphate [14,15]. It is the likely binding site for the phosphate moiety of the nucleotide substrate. The HprK/P catalytic domain belongs to the large structural family of P-loop containing proteins. Members include G-proteins, ATPases, and kinases with low molecular weight substrates like nucleoside or nucleotide kinases. HprK/P is the first P-loop protein carrying a protein kinase activity. Thus, HprK/P defines a novel family of protein kinases.

A four-stranded antiparallel β-sheet covers the face of the central β-sheet opposite to the α-helices. An unusual structure corresponding to the conserved HprK/P signature sequence [17], and called the capping motif [14], completes the globular core of the HprK/P subunit.

The closest structural homologue is *E. coli* phophoenolpyruvate (PEP) carboxykinase [18,19] (Fig. 2). The α/β unit of HprK/P and PEP carboxykinase has a different β-sheet topology than adenylate kinase and many other ATP-dependent kinases acting on small metabolites [20]. The similarity of HprK/P with PEP carboxykinase does not extend to the remainder of the C-domain.

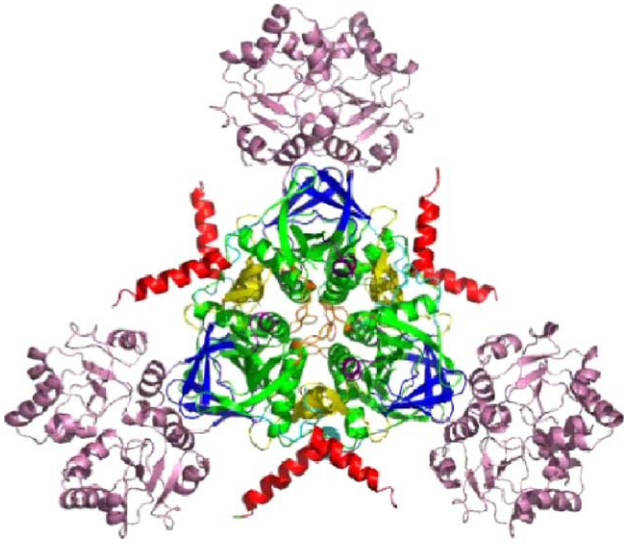

Fig. 1. HprK/P subdomains. N-terminal domain (pink), Conserved nucleotide binding domain (green), P-loop with bound Pi (purple), Capping motif (yellow), Four stranded β-sheet (blue), C-terminal α-helix (red), Central-loop (orange), Flexible loop (cyan).

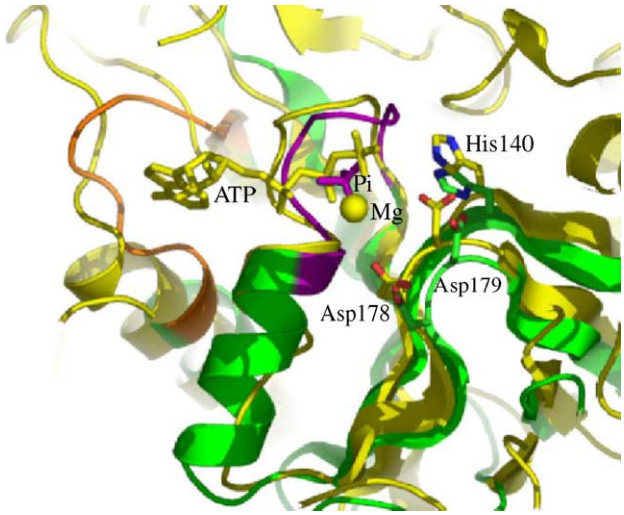

Fig. 2. Superimposition of the nucleotide binding sites of the HprK/P and PEP carboxykinase. *E. coli* PEP carboxykinase (PDB code 1AYL) is shown in yellow with ATP-Mg bound in its P-loop. HprK/P is shown in green with the central loop in orange and the Pi bound P-loop in purple. The nucleotide and the side-chains of the three conserved catalytic residues (*L. casei* HprK/P numbering) are shown in sticks.

2.3. Central loop and ATP binding

In each half of the hexamer, a short loop with a conserved sequence (I/L,P,V/I,xxGR,N/K/SxS/A/P) comes close to the three-fold axis (Fig. 1). Three central loops of a trimer make hydrophobic interactions with each other. The arginine in this sequence also interacts with the P-loop of a neighbouring subunit. In *L. casei* HprK/P, mutating the valine of this conserved sequence to phenylalanine decreases the phosphorylase activity. The kinase activity is unaffected [21].

Although we do not have X-ray evidence yet, a nucleotide can be modeled at the active site of the HprK/P subunit on the basis of a comparison with ATP bound to the PEP carboxykinase (Fig. 2). The phosphate moiety of the nucleotide fits nicely in the HprK/P active site and the β-phosphate superimposes with the Pi ion observed in the *L. casei* structure. However, the nucleotide does not fit in the HprK/P oligomer (Fig. 3), suggesting that the structures we have are in the phosphorylase conformation. Indeed, the base moiety of the modeled nucleotide clashes with the central loop of an adjacent subunit. A conformational change is therefore needed to switch the enzyme for kinase activity. A simple flipping movement of the central loop of each subunit toward the P-loop of the same subunit would be enough to allow ATP binding. Nevertheless, the change may affect subunit contacts within a trimer and, possibly, the quaternary structure of the protein. This is a likely source of the cooperativity seen when *B. subtilis* HprK/P binds ATP analogs and the activator fructose-1, 6-bisphosphate [22].

2.4. The flexible loop and C-terminal helix

A large polypeptide loop (residues 236–260 in *L. casei*) connecting two β-strands of the α/β unit, is located on the surface of the protein above the active site and is disordered in most X-ray structures. A notable exception is the complex with

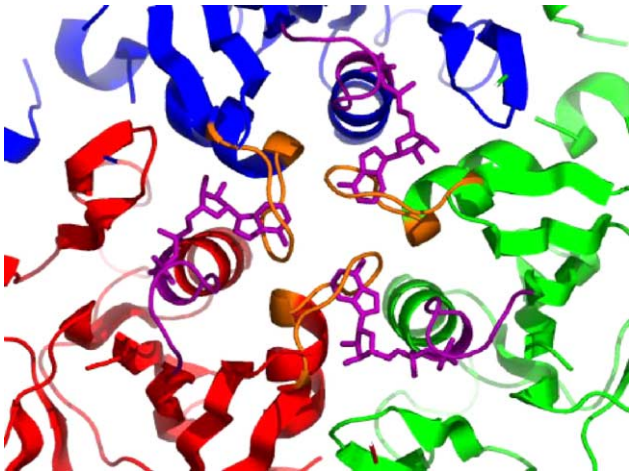

Fig. 3. PEP carboxykinase based model of ATP bound to the HprK/P oligomer. A close view of the top trimer is shown with three HprK/P subunits colored in red, green and blue, respectively. Steric hindrance between the base moiety of the nucleotide (in purple sticks) and the central loop (in orange) of a neighbouring HprK/P subunit is observed in the phosphorylase conformation of the enzyme.

phosphorylated HPr, where a strictly conserved arginine of the loop (Arg245 in *L. casei*) interacts with the phosphoserine of HPr bound to an adjacent subunit (Figs. 4 and 5). In some of the six subunits of the *M. pneumoniae* HprK/P asymmetric unit [16], the loop is also partly ordered in a similar conformation to that in the complex, even though no ligand is present.

Another mobile part of the C-domain is the C-terminal helix of each subunit. It takes different orientations in the various X-ray structures and rotates by about 30° in the C-domains of *L. casei* when HPr binds. The orientation seen in the complex is

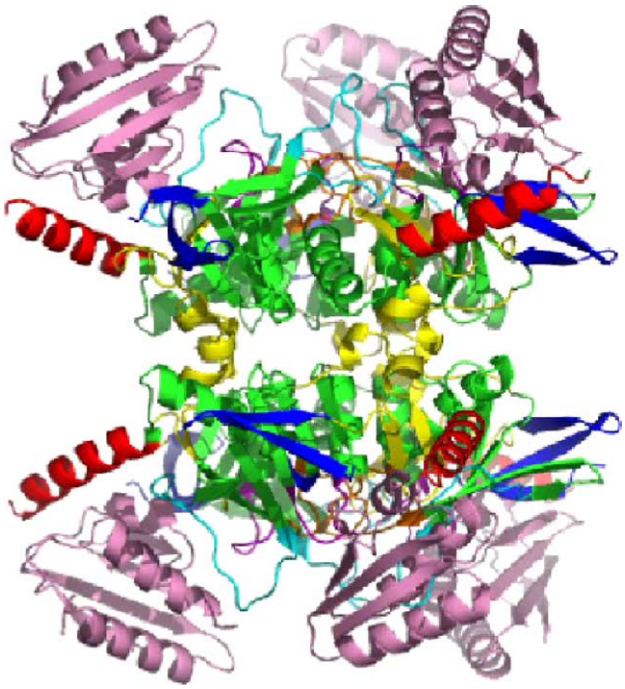

Fig. 4. Side view of the HPr-HprK/P complex. The bound HPr molecules are shown in pink. HprK/P is shown with subdomains of the catalytic core colored as in Fig. 1.

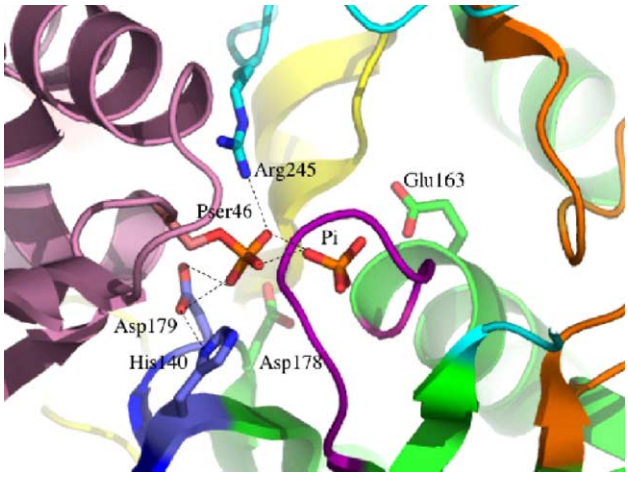

Fig. 5. Close view of the active site in the HPr-HprK/P complex. PserHPr is shown in pink with Pser46 in sticks. HprK/P is colored by subdomains with a Pi ion bound in the P-loop. Catalytic residues (*L. casei* numbering) are shown in sticks.

the same as in the free *S. xylosus* protein, indicating that the helix can move under the influence of crystal contacts as well as HPr binding. The movement is of functional significance, for the C-terminal helix of each HprK/P subunit makes contacts with the HPr molecule bound to an adjacent subunit in the hexamer.

3. Complexes with HPr and PserHPr

The structures of the complexes between the catalytic core of HprK/P and its protein substrates HPr and PserHPr provide a good understanding of protein–protein recognition. It had already been shown that phosphorylation of serine 46 induces no conformational changes in the small HPr protein. Neither does binding to HprK/P. Its binding induces no major change in the enzyme, except for rotation of the flexible C-terminal helix [4]. Three HPr molecules bind on the top of the core, three on the bottom (Fig. 4). Their location makes it unlikely that additional contacts can be made with the N-terminal domains in the full-length protein [23].

Each HPr molecule interacts with two adjacent subunits of the enzyme (Fig. 5). The interface with the first subunit involves the active site of the enzyme and HPr Ser46. A separate interface involves the C-terminal helix of a neighbouring HprK/P subunit (Fig. 4).

4. The catalytic mechanism

4.1. The active site

In HprK/P, the active site comprises the P-loop and surrounding residues (Fig. 5). As in other P-loop proteins [13], the role of the loop must be to first bind the phosphate moiety of the nucleotide substrate. The Pi ion bound to the HprK/P P-loop makes an additional interaction with the side-chain carboxyl of a conserved glutamate just following the Walker motif A (G_{155}xxxxGKS**E$_{163}$**, *L. casei* numbering) (Fig. 5). This interaction is less likely with a nucleotide, where the phosphate moiety is generally unprotonated. It is also missing in the structure with bound PserHPr [4] where the Pi ion interacts with the phosphoserine (Fig. 5).

Outside the P-loop, and in addition to the arginine of the flexible loop mentioned above, a histidine (His140 in *L. casei*) and two consecutive aspartates (Asp178–Asp179) play a major role in catalysis (Fig. 5). Although no homology is detected in the overall sequence, HprK/P shares these active site residues with PEP carboxykinase [18,19] (Fig. 2). His140 and Asp179 side-chains interact with Pser46 and are mobile in the absence of HPr. Asp178 does not directly contact HPr or the phosphates, but it is nevertheless essential to the catalytic activity of the enzyme [18]. It interacts with Ser162, part of the cation coordination sphere, in the complex with HPr and in other P-loop containing proteins.

4.2. The ATP-dependent kinase reaction

Since we still lack a structure with a bound nucleotide, only a model of the ATP-dependent phosphorylation of HPr can be proposed. When a catalytic subunit of HprK/P is superimposed with the corresponding domain of the PEP carboxykinase [18], the phosphate ion in the P-loop of HprK/P superimposes with the β-phosphate of ATP in PEP carboxykinase and the three catalytic residues are conserved. In the complex with unphosphorylated HPr, the carboxylate of Asp179 is in position to receive a hydrogen bond from HPr serine 46, which suggests that it is the catalytic base that removes the serine OH proton upon phosphorylation [4].

However, we have already noted that ATP cannot bind to the form of HprK/P observed in the X-ray structures, in the way it does to PEP carboxykinase. A proper description of the reaction will require additional structures detailing the conformation changes that must accompany ATP binding and may explain the regulation of activity by fructose-1, 6-bisphosphate.

4.3. The phosphorolysis reaction

With PserHPr as substrate, HprK/P catalyzes the dephosphorylation of Ser46 in the presence of Pi [24]. The reaction was initially assumed to be a hydrolysis (and the enzyme a phosphatase), and Pi an activator, until the X-ray structure of the *L. casei* C-domain showed that Pi binds exclusively in the P-loop. This location was compatible with Pi being a competitive inhibitor of the phosphorylation reaction, but not an activator of dephosphorylation or of any other reactions taking place at the same site. This prompted us to analyze the products of the reaction, which proved to be free HPr and pyrophosphate [3]. Thus, dephosphorylation of Ser46 proceeds by phosphorolysis, rather than hydrolysis, of the phosphoester. The reaction is reversible and, in vitro, HprK/P can phosphorylate HPr with pyrophosphate as an alternate phosphate donor. To be efficient in the cell, HPr dephosphorylation actually depends

on coupling phosphorolysis with a third reaction, catalyzed by pyrophosphatases. In *L. casei*, HprK/P is part of an operon that also codes for YvoE, a protein shown to have pyrophosphatase activity [3].

In the complex with phosphorylated HPr, one of the oxygens of the Pi ion bound in the P-loop is at the correct distance (about 3.2 Å) for an in-line nucleophilic attack on the P atom in the phosphoserine, forming the P−O−P bond of pyrophosphate, whilst the Ser−O−P bond is cleaved. Concomitant protonation of the serine may be provided by Asp179, which would act as an acid catalyst in this reaction. The Asp179 side-chain is within hydrogen bonding distance of the phosphoserine and, therefore, likely to be protonated in the presence of its negative charge [4]. Thus, phosphorolysis is likely to involve the same catalytic groups as in the phosphorylation reaction. The metal ion that bridges Pi and the phosphoserine (Ca^{+} in this particular X-ray structure) should assist phosphate transfer by reducing the electrostatic repulsion between the two phosphates. Cations have been shown to play an essential role in the HprK/P mechanism [25], as in many other phosphate transfer enzymes.

5. Conclusions and perspectives: identification of HprK/P inhibitors

The implication of these P-loop containing protein kinases in the virulence process of pathogenic bacteria is a promising starting point for design of specific inhibitors, potentially useful as antimicrobial agents.

A chemical library of 1500 synthetic compounds established by the Laboratoire de Pharmacochimie de la Communication Cellulaire (Strasbourg, CNRS UMR 7081) has been screened in order to identify potential hits acting as *B. subtilis* HprK/P inhibitors [26]. A radioactive kinase assay allowed identification of a symmetrical bis-cationic heterocyclic compound called Do9 (Fig. 6) showing efficient inhibitory effects on both kinase and phosphorylase activities of HprK/P, with respective IC_{50} values of 10 μM and 14 μM. The antimicrobial effect of Do9 was then tested. Preliminary results showed growth inhibition of *B. subtilis* but not of *E. coli*. Structural analysis of the Do9 binding mode should allow hit optimization and design of new antimicrobial agents.

In contrast to most protein kinase inhibitors, competition experiments suggested that Do9 was not competing with the binding of ATP. Such non-ATP mimetic inhibitors may provide interesting challenges for designing potent HprK/P inhibitors with significant increased selectivity profiles. This type of symmetrical heterocyclic amidines is already known to exhibit various pharmacological effects. Antimicrobial activity is well established, e.g., dequalinium, which has also been shown to be an anticarcinoma agent [27]. The biological target of dequalinium appears to be protein kinase C, which is inhibited with an EC_{50} value of about 10 μM. Here again, inhibition is noncompetitive with respect to ATP [28]. It would be interesting to investigate if dequalinium and Do9 may have a similar mode of action with the protein kinase C and HprK/P, respectively.

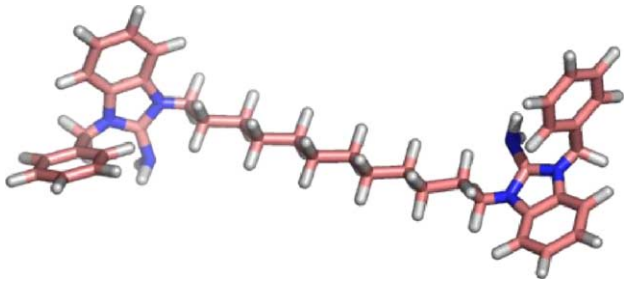

Fig. 6. Structure of Do9, a non-ATP competitive inhibitor of HprK/P.

Acknowledgements

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