Published in final edited form as:

Curr Drug Discov Technol. 2015 ; 11(4): 279–292.

High Throughput Screen Identifies Natural Product Inhibitor of Phenylalanyl-tRNA Synthetase from *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

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Abstract

Pseudomonas aeruginosa and *Streptococcus pneumoniae* are causative agents in a wide range of infections. Genes encoding proteins corresponding to phenylalanyl-tRNA synthetase (PheRS) were cloned from both bacteria. The two forms of PheRS were kinetically evaluated and the K_m's for *P. aeruginosa* PheRS with its three substrates, phenylalanine, ATP and $tRNA^{Phe}$ were determined to be 48, 200, and 1.2 μM, respectively, while the K_m's for *S. pneumoniae* PheRS with respect to phenylalanine, ATP and $tRNA^{Phe}$ were 21, 225 and 0.94 μM, respectively. *P. aeruginosa* and *S. pneumoniae* PheRS were used to screen a natural compound library and a single compound was identified that inhibited the function of both enzymes. The compound inhibited *P. aeruginosa* and *S. pneumoniae* PheRS with IC_{50}'s of 2.3 and 4.9 μM, respectively. The compound had a K_i of 0.83 and 0.98 μM against *P. aeruginosa* and *S. pneumoniae* PheRS, respectively. The minimum inhibitory concentration (MIC) of the compound was determined against a panel of Gram positive and negative bacteria including efflux pump mutants and hyper-sensitive strains. MICs against wild-type *P. aeruginosa* and *S. pneumoniae* cells in culture were determined to be 16 and 32 μg/ml, respectively. The mechanism of action of the compound was determined to be competitive with the amino acid, phenylalanine, and uncompetitive with AT P. There was no inhibition of cytoplasmic protein synthesis, however, partial inhibition of the human mitochondrial PheRS was observed.

Keywords

Drug discovery; natural product; protein synthesis; *Pseudomonas*; *Streptococcus*

INTRODUCTION

The Gram-negative microorganism *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, is the causative agent in a wide range of infections. *P. aeruginosa* is responsible for over one-seventh of all nosocomial infections, with strains that are multidrug-resistant

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

becoming increasingly common [1,2]. Clinical isolates of antimicrobial resistance strains of *P. aeruginosa* are significant and growing [3] and have become a leading problem in the hospital setting [4]. However, the primary medical problem resulting from *P. aeruginosa* infections are lung colonization associated with cystic fibrosis [5] in which the chronic infections are the main causes of patient morbidity and mortality [6].

Streptococcus pneumoniae, a Gram-positive bacterium, is the most common cause of bacteremia, pneumonia, meningitis, otitis media and other invasive diseases [7] and a primary cause of upper respiratory tract infections in children [8]. The recent explosion of multi-drug resistance among *S. pneumoniae* is increasing worldwide [9]. Resistance to betalactams, macrolides and fluoroquinolone is now common in *S. pneumoniae* [10]. The Assessing Worldwide Antimicrobial Resistance Evaluation (AWARE) surveillance program recently concluded that multi-drug resistance was greater than 30% in *S. pneumoniae* [11,12].

Aminoacyl-tRNA synthetases (aaRS) are potential targets for the development of new antibiotics designed to combat isolates resistant to many antibiotics currently in use. The aaRS are a class of enzymes which catalyze the covalent esterification of an amino acid to its cognate tRNAs during protein biosynthesis. There are two major classes of aaRSs which are based on distinct structural active site regions. The class I aaRS contains a characteristic ATP binding motif, the Rossmann fold, while three structural motifs compose the ATP binding site in class II aaRS [13]. In the class II aaRS, motif 1 appears to be critical for subunit association, and motifs 2 and 3 form parts of the active site during the aminoacylation reaction [14]. The class II aaRSs are further divided into three subgroups: class IIa, class IIb and class IIc. Members of class IIa and class IIc aaRSs are characterized by the lack of a specific C-terminal domain and the absence of a specific N-terminal domain, respectively, that are characteristic of the subgroup class IIb [15]. Phenylalanyl-tRNA synthetase (PheRS) is in the class IIc subgroup aaRSs which also includes AlaRS and GlyRS. PheRS and GlyRS are formed as $\alpha_2\beta_2$ tetramers in most systems and AlaRS is an α_4 tetramer [16]. All of the subunits are required for aminoacylation of the cognate tRNA [17]. The crystal structure of *Thermus thermophilus* PheRS indicates that the tetrameric form of the enzyme is functional as an $(\alpha\beta)_2$ structure, that is a dimer constructed from two heterodimers. Two tRNA molecules are bound by this complex and each of the four subunits in the complex interacts with the three other subunit and with the two bound tRNA substrates [18,19]. Each α-subunit primarily interacts with the CCA acceptor end of the proximal tRNA but recognizes the anticodon stem and loop of the tRNA bound by the distal αβ subunits [18].

Aminoacyl-tRNA synthetases are essential enzymes in protein biosynthesis and individually are attractive targets for the discovery of antibiotics [20]. We describe here the cloning and enzymatic characterization of PheRS from *P. aeruginosa* and *S. pneumoniae* and use of high throughput screening (HTS) systems for the detection of compounds that inhibit the activities of these enzymes. Using these systems, we have screened over 300 natural compounds for inhibitory activity against both of these synthetases. Natural products testing in this area are novel and are under represented in screening in general.

METHODS AND MATERIALS

Materials

Oligonucleotides were purchased from the Integrated DNA Technologies (Coralville, IA). All other materials were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburg, PA). DNA sequencing was carried out at the Howard Hughes Medical Institute (HHMI) laboratory at The University of Texas – Pan American and Functional Bioscience (Madison, WI). Radioactive isotopes were from PerkinElmer (Waltham, MA). *Moraxella catarrhalis* (ATCC 25238), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pneumonia* (ATCC 49619), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Haemophilus influenza* (ATCC 49766), *Pseudomonas aeruginosa* (ATCC 47085) were from the American Type Culture Collection (Manassas, VA). *E. coli tol*C mutant, *P. aeruginosa* PAO200 (efflux pump mutant) and *P. aeruginosa* hypersensitive strain (ATCC 35151) were a kind gift from Urs Ochsner (Crestone Pharma-Boulder CO). Human mitochondrial PheRS (hmPheRS) was prepared as described [21]. Chemical compounds were from Prestwick Chemical (Illkirch, France).

Gel Electrophoresis and Protein Analysis

SDS-PAGE was performed using 4-12% polyacrylamide pre-cast gels (Novex NuPAGE; Invitrogen) with MOPS running buffer (Invitrogen). EZ-Run™ Rec Protein Ladder was from Fisher. Gels were stained with SimplyBlue Safe Stain (Invitrogen). Protein concentrations were determined using protocols from Bradford [22] using bovine serum albumin as the standard. Protein alignments were prepared using Vector NTI Advance ® (Invitrogen).

Cloning and Purification of *P. aeruginosa* and *S. pneumoniae* PheRS

The genes encoding the α and β subunits of *P. aeruginosa* PheRS were amplified by PCR (Bio-Rad MJ Mini Thermo Cycler) from *P. aeruginosa* PAO1 (ATCC 47085) genomic DNA. The genes encoding the α (*phe*S) and β (*phe*T) subunits were cloned as a natural operon using a forward primer (5'-ctgagctagcgaaaacctggatgcgctg-3') designed to add an *Nhe*I restriction site to the 5' end of the operon and a reverse primer (5'-gactaagcttctactatttccttaacgtggcattg-3') which was designed to add a *Hind*III restriction site to the 3' end of the operon. The PCR product was inserted into a pET-28b(+) plasmid (Novagen) digested with *Nhe*I/ *Hind*III. This placed the gene encoding the α-subunit downstream of a sequence encoding six histidine residues. The recombinant plasmid was subsequently transformed into Rosetta 2(DE3) competent cells (Novagen).

The genes encoding the *S. pneumoniae* PheRS α and β subunits of PheRS were amplified individually from *S. pneumoniae* genomic DNA (ATCC 49619). The gene encoding the α subunit was amplified using a forward primer (5'-gactcatatgtcaactattgaagaacaattaaaa-3') designed to add an *Nde*I restriction site to the 5' end of the gene and a reverse primer (5'-agttggatccttatttaaactgttctgagaagcg-3') which was designed to add a *Bam*HI restriction site to the 3' end of the gene. The PCR product was inserted into a pET-28b(+) plasmid (Novagen) digested with *Nde*I/ *BamH*I. The gene encoding the β subunit was amplified using a forward primer (5'-gactcatatgcttgtatcttataaatggttaaaa-3') designed to add an *Nde*I restriction site to

the 5' end of the gene and a reverse primer (5'-gacactcgagacgcacttctgcattgactt-3') which was designed to add an *Xho*I restriction site to the 3' end of the gene. The PCR product was inserted into pET-24b(+) plasmid (Novagen) digested with *Nde*I/*Xho*I. Both recombinant plasmids were transformed individually into Rosetta 2(DE3) competent cells (Novagen).

Bacterial cultures were grown in Terrific Broth containing 25 μg/mL of kanamycin and 50 μg/mL of chloramphenicol. Cultures expressing *P. aeruginosa* PheRS α and β subunits were grown at 37 ºC and the target proteins were expressed at an optical density (A_{600}) of 0.6-0.8 by the addition of isopropyl β3-D-1-thiogalactopyranoside (IPTG) to 0.5 mM. Cultures expressing *S. pneumoniae* α and β subunits were grown to an optical density (A_{600}) of 0.6-0.8 at 37 °C, the temperature was then lowered to 30 °C and target protein expression was induced by the addition of IPTG to 0.25 mM. Growth of all bacterial cultures was continued for 2 h post-induction, and the bacteria were harvested by centrifugation (10000 ×g, 30 min, 4 °C).

To purify the target proteins, bacterial cells were lysed and fraction I lysate was prepared as previously described [23]. *P. aeruginosa* PheRS was precipitated by the addition of ammonium sulfate (AS) to 50% saturation and *S. pneumoniae* α and β subunits were precipitated at 35% and between 45 and 60% AS saturation, respectively. The precipitated proteins were collected by centrifugation (23,000 × *g*, 60 min, 4 °C). The target proteins were further purified to greater than 98% homogeneity using nickel-nitrilotriacetic acid (NTA) affinity chromatography (Perfect Pro, 5 Prime) followed by dialysis (two times) against a buffer containing: 20 mM Hepes-KOH (pH 7.0), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10 % glycerol. *S. pneumoniae* α and β subunits were combined at a molar ratio of 1:1. Finally, all proteins were fast frozen in liquid nitrogen and stored at −80 °C.

ATP:PP_i Exchange Reactions

ATP:PP_i exchange reactions (100 μl) were carried out at 37 °C in 50 mM Tris-HCl (pH 7.5), 10 mM KF, 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2 mM [^{32}P]PP_i (50 cpm/pmol) and contained 0.1 μM of either *S. pneumoniae* or *P. aeruginosa* PheRS. In reactions in which the concentration of ATP was varied, the amino acid phenylalanine (Phe) remained constant at 2 mM; alternatively, when the concentration of Phe was varied, the ATP concentration remained at 2 mM. The exchange of PP_i in these reactions was measured between one and five min. Reactions were stopped by diluting 5 μl of the reaction mix into 45 μl of a buffer containing 20 mM Hepes-KOH (pH 7.0), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and immediately spotting 5 μl of the diluted solution onto PEI cellulose TLC plates (Selecto Scientific). ATP and PP_i were separated using a solution containing 4 M urea and 0.75 M KP_i (pH 3.5) as a mobile phase [24]. Amounts of ATP, PP_i and P_i were quantified using a Typhoon FLA 7000 Laser Scanner (GE Healthcare). Initial velocities for exchange of PP_i were determined and the kinetic parameters (K_M and V_{max}) for the interactions of PheRS with ATP and phenylalanine were determined by plotting the velocities against substrate concentration and fit to the Michaelis-Menten steady-state model using XLfit (IDBS).

tRNA Aminoacylation Assays

All of the aminoacylation reactions were at 37 °C and at time intervals between one and five minutes. Reactions (50 μl) contained 50 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 1.25 mM ATP, 1 mM spermine, 1 mM DTT, 100 μM [^{3}H]Phe (75 cpm/pmol), and 6.25 nM *S. pneumoniae* or *P. aeruginosa* PheRS. The tRNA was from *E. coli* MRE600 (Roche). The $tRNA^{Phe}$ concentrations were varied as indicated. Reactions were stopped by the addition of 2 ml of 5% (v/v) ice-cold trichloroacetic acid (TCA), placed on ice for ten min, then filtered through glass fiber filters (Millipore, type HA 0.45 mm). Filters were washed with 10 ml ice-cold 5% TCA, dried and counted in an LS6500 multi-purpose scintillation counter (Beckman Coulter). Initial velocities for aminoacylation were calculated for all tRNA concentrations. These data were modeled using the Michaelis Menten equation as described above and the K_M and V_{max} values were determined.

Chemical Compound Screening

The natural product compound library (320 compounds) was screened using the tRNA aminoacylation assay adapted to a scintillation proximity assay (SPA) [25]. The chemical compounds were screened using 96-well microtiter plates (Costar). Test compounds dissolved in 100% DMSO (2 μl of compound; 3.3 mM) were equilibrated by the addition of 33 μl of the protein/substrate mix described above for the aminoacylation assay (minus tRNA). Control reactions contained only DMSO with no compound. The concentration of *P. aeruginosa* PheRS was 0.05 μM and *S. pneumoniae* PheRS was 0.1 μM. This mixture was incubated at ambient temperature for 15 min and then reactions were started by the addition of 15 μl *E. coli* tRNA (80 μM total tRNA or 2 μM $tRNA^{Phe}$), followed by incubation for 1 h at 37 °C. Reactions were stopped by the addition of 5 μl of 0.5 M EDTA. 400 μg of yttrium silicate (Ysi) poly-L-lysine coated SPA beads (Perkin-Elmer) in 150 μl of 300 mM citric acid were added and allowed to incubate at room temperature for 1 h. The plates were analyzed using a 1450 Microbeta (Jet) liquid scintillation/luminescent counter (Wallac). Assays to determine IC_{50} values were as described with the test compounds serially diluted from 200 μM to 0.4 μM.

Microbiological Assays

Broth microdilution MIC testing was carried out in 96-well microtiter plates according to Clinical Laboratory Standards Institute guideline M7-A7 [26]. MIC values were determined for *E. coli* (ATCC 25922), *E. coli tol*C mutant, *Enterococcus faecalis* (ATCC 29212), *Haemophilus influenzae* (ATCC 49766), *Moraxella catarrhalis* (ATCC 25238), *P. aeruginosa* (ATCC 47085), *P. aeruginosa* PAO200 (efflux pump mutant), *P. aeruginosa* hypersensitive strain (ATCC 35151), *S. aureus* (ATCC 29213), and *S. pneumonia* (ATCC 49619) from the American Type Culture Collection (Manassas, VA). For quality control (QC) of MIC determination and culture purity, MICs were determined for antibiotics specific for each bacterial strain. The antibiotic and concentration range for each bacteria was: *E. coli* (ampicillin, 0.125-128 μg/ml), *E. faecalis* (vancomycin 0.125-128 μg/ml), *H. influenzae* (ampicillin, 0.03125-32 μg/ml), *M. catarrhalis* (ampicillin, 0.015625-16 μg/ml), *P. aeruginosa* (ampicillin, 0.125-128 μg/ml), *S. aureus* (oxacillin, 0.03125-32 μg/ml), *S. pneumoniae* (penicillin, 0.0625-64 μg/ml).

Eukaryotic Assays

Determination of the effect of inhibitory compound BPP_03B04 on cytoplasmic protein synthesis using wheat germ extracts was as described [27]. To test the effect of BPP_03B04 on the activity of human mitochondrial PheRS (hmPheRS) aminoacylation assays (50 μl) were carried out. The assay mix contained 50 mM Tris-HCl (pH 7.5), 1 mM spermine, 10 mM MgOAc, 2.5 mM ATP, 1 mM DTT, 75 μM Phe, and 0.2 μM hmPheRS. The mix was incubated for 15 min with 132 μM compound, then the reaction was initiated by the addition of $tRNA^{Phe}$ (2 μM). The reaction was stopped by diluting into 2 ml of ice-cold 5% TCA and filtering through glass fiber filters as described above.

Mechanism of Action of Inhibitory Compound

To determine competition with ATP, IC_{50}s were determined in SPA reactions as described above but containing varying ATP concentrations (25, 50, 100, 250, 500, 1000 μM). Final compound concentrations in each IC_{50} reaction ranged from 200 to 0.4 μM. Positive controls contained only DMSO (2 μl) without compound. To determine competition with Phe the same assay was used. However, ATP was held at a constant concentration of 2.5 mM in assays containing the indicated concentrations of Phe (25, 50, 100, 200, 300 μM).

RESULTS

Sequence Analysis

The architecture of the PheRS enzymes has been extensively described, primarily from structural studies of PheRS from *T. thermophilus* [18,28-30] and more recently the structure of PheRS from *E. coli* [31], *Staphylococcus haemolyticus* [32] and *P. aeruginosa* [33] has been solved. The amino acid sequence conservation when comparing that of *T. thermophilus* PheRS with the corresponding enzymes from *E. coli*, *P. aeruginosa*, or *S. pneumoniae* is very similar (Table **1**). Comparison of the α-subunit from *E. coli*, *P. aeruginosa*, *S. pneumoniae* or *S. haemolyticus* with the α-subunit of PheRS from *T. thermophilus* indicates only a small variation in the conservation of the amino acid sequence in which the residues are 53-58% similar and 38-44% identical. There appears to be two regions (α156-170 and α302-310, *T. thermophilus* numbering) that are present in *T. thermophilus* PheRS but are not present in *P. aeruginosa* or *S. pneumoniae* PheRS (Fig. **1**). These are solvent exposed regions on the side of PheRS opposite to that of the bound tRNA and removed from the active site and are unlikely to have an effect on aminoacylation. The amino acid sequence in the β-subunit is only slightly less conserved with a variation of 49-50% similar and 33-36% identical residues. In general, the sequences of functional residues in the subunits of PheRS from *T. thermophilus*, *E. coli*, *P. aeruginosa*, *S. pneumoniae* or *S. haemolyticus* are similar containing 73% and 70% conservation of consensus positions in the α- and β-subunits, respectively.

In the α-subunit, active site residues that have been shown to interact with the amino acid substrate, phenylalanine, are almost entirely conserved with only moderate changes observed (Fig. **1**). In the *T. thermophilus* α-subunit, Trp149, which forms hydrogen bonds with Phe, is replaced with the amino acids, glutamine in *S. pneumoniae* and histidine in *P. aeruginosa*. Trp149 has also been shown to form hydrogen bonds with the terminal 3’

adenosine of the tRNA [18]. Other moderate replacements occur at Val261 (*T. thermophilus* numbering) and Ala283. Amino acids in the α-subunit that interacts with ATP, are also well conserved differing only between alanine, leucine, isoleucine and valine except at position 213, in which a glutamic acid in *T. thermophilus* is replaced by a serine in both *S. pneumoniae* and *P. aeruginosa*. The α-subunit also interacts with the acceptor end of the tRNA during the aminoacylation process. In *T. thermophilus* PheRS, Trp149, Ser180 and Glu220 form hydrogen bonds with the terminal adenosine of the tRNA [18,29]. Of these residues, only Trp149 is not conserved. Phe258 and Phe260 interact with the base of the terminal adenosine in ring-ring interactions and are strictly conserved in the *T. thermophilus*, *P. aeruginosa*, and *S. pneumoniae* α-subunits. In general, functional residues in the active site appear to be well conserved suggesting that the structure of the active site in *P. aeruginosa*, and *S. pneumoniae* α-subunit does not diverge significantly from that of other bacteria in which the structure of PheRS has been solved.

In the β-subunit, amino acids at the N-terminus form salt-bridges with C74 and A73 of the acceptor stem of the tRNA. The amino acid at position two varies from an arginine in *T. thermophilus* PheRS to a lysine and leucine in *P. aeruginosa* and *S. pneumoniae*, respectively. Other amino acids that interact with the acceptor stem in the β-subunit are at positions 160 and 362 (*T. thermophilus* numbering) and only vary from leucine to isoleucine. Amino acids of the β-subunit that act in anticodon recognition are well conserved between the three species and only modest divergences are observed.

Protein Expression and Characterization

The genes encoding the α- and β-subunits of PheRS from *P. aeruginosa* occur as a natural operon and are translationally coupled. Therefore, these genes were cloned together and inserted into a plasmid that allowed the α-subunit to be expressed fused to an N-terminal peptide containing six histidine residues. The α-subunit was purified using affinity chromatography and the β-subunit was co-purified by association with the α-subunit. The genes encoding the α- and β-subunits of PheRS from *S. pneumoniae* occur separated by almost 600 nucleotides, therefore each of these genes was cloned separately. The α-subunit was expressed fused to an N-terminal histidine tag and the β-subunit was expressed fused to a C-terminal histidine tag. The mature from of PheRS contains two copies of both α and β subunits, therefore after being expressed and purified separately the two subunits were mixed at a 1:1 molar ratio (Fig. **2**).

The result of the aminoacylation reaction is the covalent attachment of a specific amino acid to the cognate tRNA and occurs as a two-step process. In the first step, the amino acid and ATP are condensed resulting in the formation of an aminoacyl-adenylate intermediate followed by the release of a pyrophosphate. This reaction is reversible in the absence of a cognate tRNA in most of the aaRS and the parameters governing the kinetic interaction of the aaRS with the amino acid and ATP substrates may be determined. The kinetic parameters K_M, k_{cat} and k_{cat}/K_M for the interaction of both *P. aeruginosa* and *S. pneumoniae* PheRS with the substrates Phe and ATP have been determined by measuring the initial rate of the ATP:PP_i exchange. This data was then fit to the Michaelis-Menten steady-state model. Determination of K_M and k_{cat} values for Phe was determined at a constant

concentration of ATP (2 mM), while the concentration of Phe was varied between 6.25 and 300 μM (Fig. **3A**). The K_M and k_{cat} values for the interaction with Phe calculated from these data were 48 μM and 16 s^{-1} for *P. aeruginosa* PheRS, and 21 μM and 22.5 s^{-1} for *S. pneumoniae* PheRS, all respectively (Table **2**). *E. coli* PheRS has a similar K_M of 40 μM, but the k_{cat} is significantly higher at 138 s^{-1}. A similar analysis was carried out by varying the concentration of ATP between 100 and 750 μM while maintaining the constant concentration of Phe (2 mM) (Fig. **3B**). The K_M and k_{cat} values for ATP calculated from these data are 200 μM and 13 s^{-1} for *P. aeruginosa* PheRS, and 225 μM and 15.5 s^{-1} for *S. pneumoniae* PheRS, all respectively. *E. coli* PheRS has a K_M for ATP (800 μM) that is only 4-fold higher, but the turnover rate (182 s^{-1}) is quite high as compared to that of either *P. aeruginosa* or *S. pneumoniae* PheRS.

The formation of an ester bond between the 2' hydroxyl group (in the case of PheRS) of the 3' terminal adenosine base of the tRNA and the carboxylic acid function of Phe constitutes the second step in the aminoacylation process. This aminoacylation reaction catalyzed by either *P. aeruginosa* or *S. pneumoniae* PheRS was stimulated two-fold by the presence of spermine. The optimal concentration of spermine was observed to be 1-2 mM for both enzymes and the optimal Mg^{++} concentrations was 7.5 and 10 mM, respectively for *P. aeruginosa* and *S. pneumoniae* PheRS. The ability of both *P. aeruginosa* and *S. pneumoniae* PheRS to aminoacylate $tRNA^{Phe}$ was determined at various concentrations of tRNA while maintaining ATP and Phe at 2.5 mM and 75 μM, respectively (Fig. **3C**). The K_M and k_{cat} values were determined to be 1.2 μM and 0.74 s^{-1} for *P. aeruginosa* PheRS and 0.94 μM and 0.93 s^{-1} for *S. pneumoniae* PheRS. The K_M values are 10-fold higher than observed for PheRS from *E. coli* (0.08 μM) but the turnover numbers are similar to those seen with *E. coli* PheRS (1.42 s^{-1}) [34,35].

Screening Natural Compounds Against the Activity of *P. aeruginosa* or *S. pneumoniae* PheRS

Natural products have been a major source of new drugs over the past 30 years and the anti-infective area in particular has been dependent on natural products and derivatives of these products [36,37]. We have screened a phyto-chemical library, a set of 320 natural products derived from plants. This approach capitalizes on recent improvements in the number and quality of natural products available. This compound library was chosen because it is rich in diverse chemotypes therefore, providing a good starting point for hit discovery. Many of the compounds are amendable to modification allowing downstream structure activity relationship (SAR) strategies. The tRNA aminoacylation assay was adapted to a scintillation proximity assay (SPA) in a 96-well microtiter plate format and used to screen the natural compounds as described in the "Methods and Materials" section. In the SPA format, this assay was very robust with a Z factor of 0.50 and a Z' factor of 0.55 across all screening plates. The assay detects the ability of PheRS to aminoacylate $tRNA^{Phe}$ and in the presence of a chemical compound to measure the effect of the compound on the activity of PheRS. Chemical compounds were dissolved in 100% dimethyl sulfoxide (DMSO) resulting in final DMSO concentrations in screening assays of 4%, therefore the ability of PheRS to function in the presence of increasing amounts of DMSO was determined. There was no decrease of activity observed in aminoacylation assays for either *P. aeruginosa* or *S. pneumoniae* PheRS

in assays containing up to 10% DMSO (data not shown). Initial screening assays contained chemical compounds at a concentration of 132 μM and were carried out as single point assays. Compounds observed to inhibit at least 50% of enzymatic activity were re-assayed in triplicate using the filter binding assay as described. These confirmation assays resulted in a single hit compound (BPP_03B04) resulting from the screens of both the *P. aeruginosa* PheRS and *S. pneumoniae* PheRS (Fig. **4**). This compound was assayed using the tRNA aminoacylation assay in an SPA format to determine the concentration at which 50% of the enzymatic activity was inhibited (IC_{50}) (Fig. **5**). The IC_{50} was determined to be 2.3 μM and 4.9 μM for *P. aeruginosa* PheRS and *S. pneumoniae* PheRS, respectively.

Wheat germ extract assays were used to determine whether BPP_03B04 inhibited protein synthesis in a eukaryotic system. Poly-U messenger RNA, yeast $tRNA^{Phe}$, [^{3}H]Phenylalanine and Mg^{++} concentrations were optimized for poly-Phe synthesis in wheat germ extract assays. BPP_03B04 was tested along with cycloheximide, a known inhibitor of protein synthesis [38]. In these assays protein synthesis was inhibited by cycloheximide at 30 μM [27]. In contrast, no inhibition of protein synthesis by BPP_03B04 was observed to 132 μM (Fig. **6A**). PheRS from human mitochondria (hmPheRS) was previously cloned and characterized [21]. To determine if BPP_03B04 affected the activity of hmPheRS, assays containing 132 μM of the compound were carried out. In these assays, the activity of hmPheRS was observed to decrease by approximately 80% (Fig. **6B**).

Microbiological Assays

The compound BPP_03B04 was tested in broth microdilution assays to determine minimum inhibitory concentrations (MIC) against a panel of bacteria (Table **3**). The compound was tested against ten bacteria including efflux pump mutants of *E. coli* and *P. aeruginosa* and a hypersensitive strain of *P. aeruginosa.* BPP_03B04 was observed to inhibit *E. coli* and the efflux pump mutant of *E. coli* at concentrations of 128 μg/ml, all other organisms tested against a panel of bacteria were inhibited at concentrations of 16 or 32 μg/ml. All of the *P. aeruginosa* strains were inhibited with an MIC of 16 μg/ml and *S. pneumoniae* was inhibited with an MIC of 32 μg/ml.

Mechanism of Action

The IC_{50} for BPP_03B04 was determined using the aminoacylation assay in the SPA format at various concentrations of ATP or Phe to determine the mode of action of the inhibitor. The mechanism of inhibition of both *P. aeruginosa* PheRS and *S. pneumoniae* PheRS with respect to ATP was determined at various ATP concentrations (25, 50, 100, 250, 500, 1000 μM) ranging from 8-fold below to 5-fold above the K_M. The IC_{50}s for BPP_03B04 decreased with increasing ATP concentration (Fig. **7**), which is characteristic of an uncompetitive inhibitor [39]. To determine the mechanism of inhibition with respect to the amino acid, the same assay was used, except ATP was held constant at saturating concentrations (2.5 mM) and the IC_{50} was determined at different concentrations of Phe (25, 50, 100, 200, 300 μM). The Phe concentrations ranged from approximately 2-fold below to 10-fold above the K_M. Background amounts of free [^{3}H]Phe in the absence of PheRS were insignificant (less than 3% of positive control). The IC_{50}s for BPP_03B04 increased with increasing Phe concentration (Fig. **8**), which is characteristic of a competitive inhibitor [39].

The shift in IC_{50} with respect to various substrate concentrations is the product of the shift due to each individual substrate in a two-substrate reaction. For the PheRS aminoacylation assay with inhibitors that are phenylalanine competitive and ATP uncompetitive, the relationship is as follows:

$$IC_{50} = \left(1+[\mathrm{Phe}]\,K_M{}^{\mathrm{Phe}}\right)\left(1+K_M{}^{\mathrm{ATP}}/[\mathrm{ATP}]\right)K_i\,[39]$$

This equation was used to calculate K_i for BPP_03B04 with IC_{50}s determined at 75 μM Phe and 2.5 mM ATP. In multiple determinations, BPP_03B04 had a mean K_i of 0.83 μM with respect to *P. aeruginosa* PheRS and 0.98 μM with respect to *S. pneumoniae* PheRS.

DISCUSSION

Aminoacyl tRNA synthetases are essential proteins involved in protein synthesis in all organisms. The bacterial and cytoplasmic eukaryotic forms of the enzymes are sufficiently different as to allow the development of compounds that may inhibit the function of one form without affecting the function of the other form. However, the homolog present in mitochondria carries out the same function yet the structure may favor either the bacterial or cytoplasmic form. Functional regions of PheRS that have been shown to be conserved in *T. thermophilus* PheRS and others, are also conserved in PheRS from *P. aeruginosa* and *S. pneumoniae*. Previous work indicated that the human mitochondrial PheRS (hmPheRS) consists of a single polypeptide chain and is active in a monomeric form which is unlike the $(\alpha\beta)_2$ structure of the prokaryotic and eukaryotic cytoplasmic forms of PheRS which are active in tetrameric forms. The N-terminal 314 amino acids of hmPheRS are analogous to the α-subunit of the prokaryotic form of PheRS, while the C-terminal 100 amino acids correspond to a region of the β-subunit known to interact with the anticodon stem and loop of $tRNA^{Phe}$ [21]. Alignments between the N-terminal 314 amino acids of hmPheRS and the corresponding amino acids in the PheRS α-subunits from *P. aeruginosa* and *S. pneumoniae* were observed to be on average 29% similar and 17% identical. Alignments of the C-terminal 100 amino acids from hmPheRS with the PheRS β-subunits from *P. aeruginosa* and *S. pneumoniae* indicated only a small level of consensus with the C-terminal ends of the prokaryotic proteins. When the α-subunits from *P. aeruginosa* and *S. pneumoniae* PheRS were compared with the human cytoplasmic homolog a similar level of conservation was observed as that seen with hmPheRS. However, when assayed in the presence of the compound BPP_03B04, activity of the wheat germ cytoplasmic form of PheRS was not affected, but the activity of the mitochondrial form of PheRS was reduced. This is similar to results seen by a group from Bayer Healthcare where the PheRS inhibitor, phenyl-thiazoleurea-solfonamide, had no effect on the cytoplasmic counterpart, but the activity of hmPheRS was significantly inhibited [40]. To determine the potential reason for this observation will require an in-depth examination of the structural aspects of the enzyme/compound interaction.

Recently, several attempts have been made to identify inhibitors of bacterial PheRS. As described above, the phenylthiazoleurea-solfonamide compounds were identified by screening *E. coli* PheRS against Bayer's in-house compound library and appears to have

both *in vitro* inhibitory activity against the enzyme and also inhibits growth of bacteria. Another compound series, ethanolamines, was identified by a group at GlaxoSmithKline using crude enzyme preparations from *S. aureus* [41]. These compounds were also observed to have good inhibition of enzymatic activity *in vitro*, however, there was no whole cell antibacterial activity. At about the same time a group from Cubist Pharmaceuticals identified series of spirocyclic furan and pyrrolidine inhibitors of PheRS that exhibited both *in vitro* activity as well as microbiological inhibition [42,43]. Most recently, a group from Pfizer identified a series of benzyl phenyl ethers that exhibited nano-molar IC_{50}s, but had low levels of inhibition against cultures of wild-type *Haemophilus influenza* and *S. pneumoniae* [44]. The compounds identified in all these studies had their origins in synthetic chemistry. Throughout history, a significant role in the treatment of a wide range of medical conditions, including infectious diseases, has been played by medicinal plants. Many of the present clinically used drugs are the product of naturally occurring chemical compounds and with the rise in resistance in pathogenic bacteria, many of them are now being re-assessed as antimicrobial agents [45]. We have screened a set of natural products derived from plants to identify compounds that may inhibit the activity of PheRS from *P. aeruginosa* and *S. pneumoniae*. Many of these compounds have been previously identified as having medicinal properties. From 320 compounds, we have identified one compound that has activity against both forms of PheRS.

Compound BPP_03B04 has been identified as gossypol, a polyphenolic aldehyde extracted from cotton seed. It is thought that gossypol is produced as a natural insecticide by cotton plants [46]. Gossypol has also been studied as a male anti-fertility agent in China for a number of years [47,48]. Many of these studies have had mixed results as to the effectiveness and potential side effects of long term use [49,50]. Gossypol has also been reported to have anti-viral activity [51,52] and a certain level of anti-cancer activity has been observed [53]. Other studies showed, using a disc-diffusion method, that gossypol inhibits the growth of certain bacteria [54,55]. A compound that inhibits a number of biochemical activities may be classified as a “promiscuous” compound [56] and its usefulness as a drug candidate may be limited. To determine if this is the case with gossypol will require additional studies.

We have determined that a possible molecular target for gossypol is PheRS. When assayed against the activities of both *P. aeruginosa* and *S. pneumoniae* PheRS, gossypol inhibited the two enzymes with IC_{50} values in the low micromolar range. We determined that the IC_{50} values increased when determined at increasing concentration of the amino acid substrate indicating that gossypol was competing with Phe for binding both enzymes. Alternatively, the IC_{50} values decreased when determined at increasing concentration of ATP. These results indicate that PheRS inhibition by gossypol was competitive with phenylalanine binding and uncompetitive with ATP binding. It is interesting that the positive cooperativity exhibited between gossypol and ATP parallels the positive cooperativity that has been reported between phenylalanine and ATP [57,58]. The fact that increasing concentrations of ATP enhance amino acid binding fits well with the hypothesis that gossypol binds the amino acid binding site and this binding is enhanced with increasing concentrations of ATP. Thus,

the naturally high physiological concentrations of ATP (2.5 mM) would serve to enhance binding of the inhibitor.

To determine if gossypol would be suitable as a compound that may be developed into an anti-bacterial agent will require more in depth studies. The level of inhibition observed when gossypol was assayed against human mitochondrial PheRS is a concern. Structure activity relationship (SAR) analysis may improve potency against bacterial protein synthesis and reduce the level of inhibition of protein synthesis in mitochondria.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Dr. Linda Spremulli (University of North Carolina – Chapel Hill) for her critical reading of this manuscript. The authors are grateful for the financial support provided by the National Institutes of Health (grant number: 1SC3GM09817301A1). The contents of this article/publication/etc. are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. Funding was also in part from the UT Transform Grant Technology Assessment Committee.

ABBREVIATIONS

aaRS	Aminoacyl-tRNA synthetase
Phe	Phenylalanine
AS	Ammonium sulfate
DTT	Dithiothreitol
TCA	Trichloroacetic acid
MgOAc	Magnesium acetate
hmPheRS	Human mitochondrial PheRS
SPA	Scintillation proximity assay
DMSO	Dimethyl sulfoxide
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
EDTA	Ethylene diamine tetraacetic acid
HTS	High-throughput screening
IPTG	Isopropyl b-D-1-thiogalactopyranoside
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
SAR	Structure-activity relationship
KF	Potassium floride
KPi	Potassium phosphate

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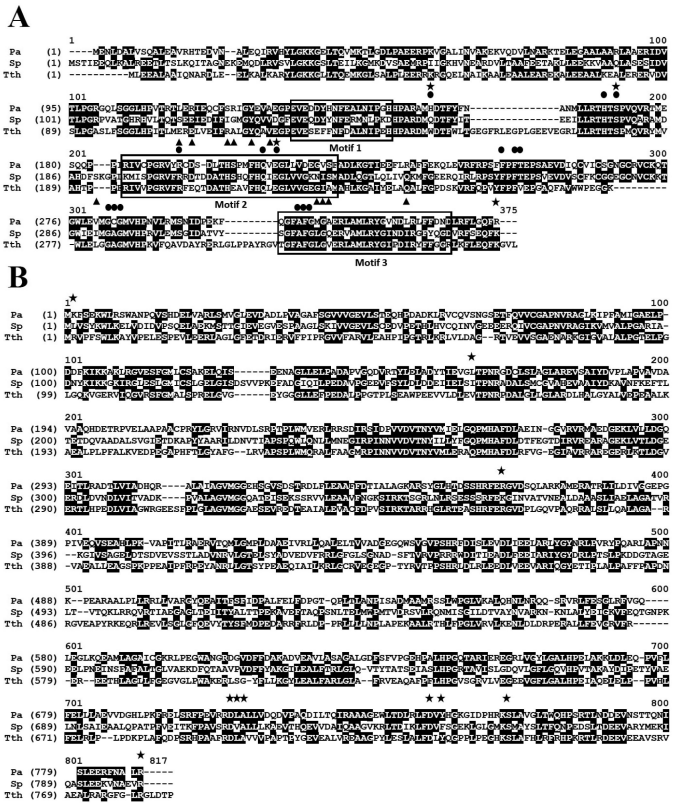

Fig. (1).

An alignment of *P. aeruginosa* and *S. pneumoniae* PheRS with PheRS from *Thermus thermophilus*. The protein sequences were downloaded from the National Center for Biotechnology Information (NCBI). Accession numbers for the a and B subunits of *P. aeruginosa* (Pa), *S. pneumoniae* (Sp) and *T. thermophilus* (Tth) sequences are AAG06128 and AAG06127, ABJ55158 and ACF55813, and WP_011229046 and P27002, respectively. Sequence alignments were performed using Vector NTI Advance (R) 11.5 (Invitrogen). **A.)** Alignment of the amino acid sequences of a subunits. The three structural motifs are enclosed by boxes. Amino acids that interact with Phe (●), ATP (▲) and tRNA (☆) are indicated. **B.)** Alignment of the amino acid sequences of B subunits. Amino acids that interact with tRNA (☆) are indicated.

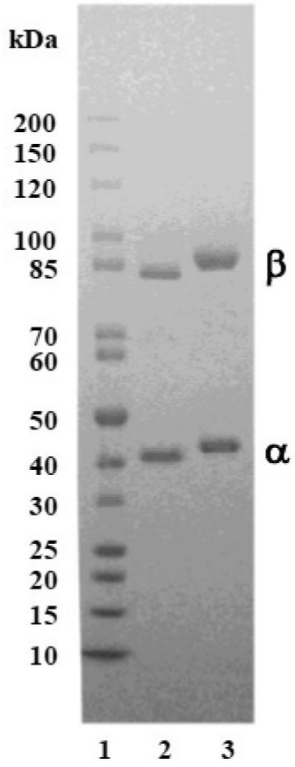

Fig. (2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified *P. aeruginosa* and *S. pneumoniae* PheRS. Samples (1.5 μg) of both forms of PheRS preparations were analyzed on a 4-20% SDS-PAGE gel and the protein bands were visualized by staining with Coomassie blue. Lane #1, contains protein standard; lane #2 contains *P. aeruginosa* PheRS; lane #3 contains *S. pneumoniae* PheRS.

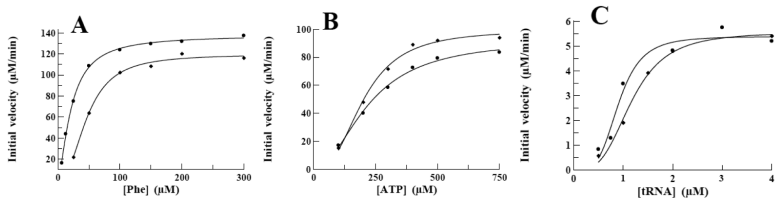

Fig. (3).

Determination of kinetic parameters for interactions of *P. aeruginosa* and *S. pneumoniae* PheRS with the substrates Phe, ATP, and $tRNA^{Phe}$. Initial velocities for the interaction of *P. aeruginosa* and *S. pneumoniae* PheRS with Phe, ATP, and $tRNA^{Phe}$ were determined and the data were fit to a Michaelis-Menten steady-state model using XLfit (IDBS) to determine K_M and V_{max}. Filled diamonds (◆) represent data collected from assays containing *P. aeruginosa* PheRS and filled circles (●) represent data collected from assays containing *S. pneumoniae* PheRS. The graphs contain data from interactions with the substrates **A.)** Phe, **B.)** ATP, and **C.)** $tRNA^{Phe}$.

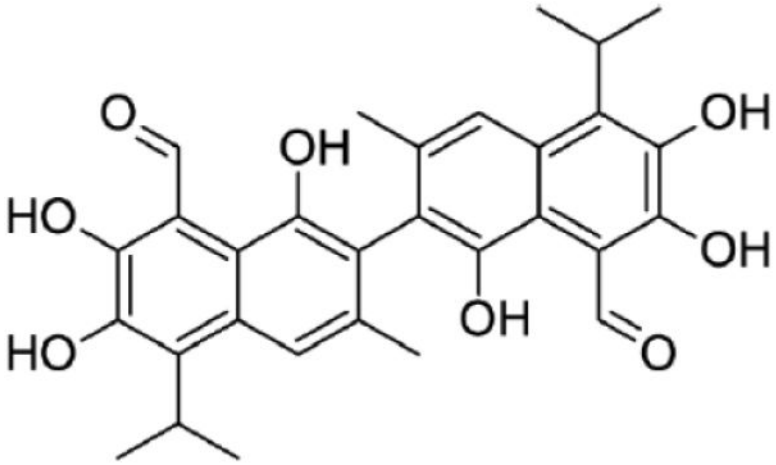

Fig. (4).

The structure of the confirmed hit compound (BPP_03B04). BPP_03B04 was identified as the sole confirmed hit compound that inhibited the activity of both *P. aeruginosa* and *S. pneumoniae* PheRS.

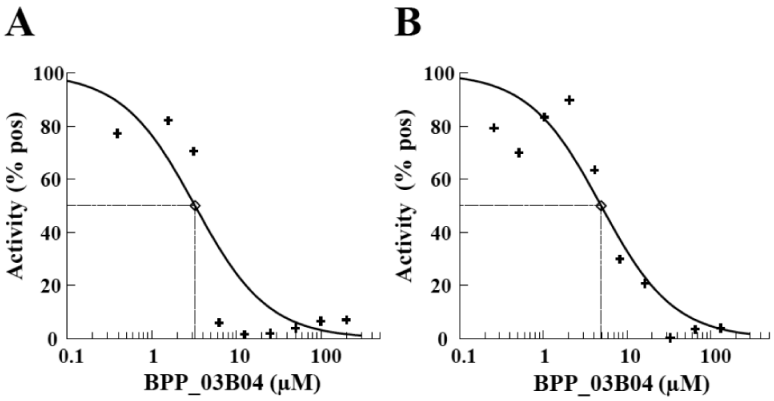

Fig. (5).

Determination of the IC_{50} for BPP_03B04 against the activity of *P. aeruginosa* and *S. pneumoniae* PheRS. IC_{50} values for the inhibitory activity of BPP_03B04 against **A.)** *P. aeruginosa* PheRS or, **B.)** *S. pneumoniae* PheRS were 2.3 μM and 4.9 μM, respectively. IC_{50}s were determined with the test compounds serially diluted from 200 μM to 0.4 μM into aminoacylation assays containing *P. aeruginosa* and *S. pneumoniae* PheRS at 0.05 and 0.1 μM, respectively. The concentrations of ATP and Phe were held constant at 2.5 mM and 75 μM.

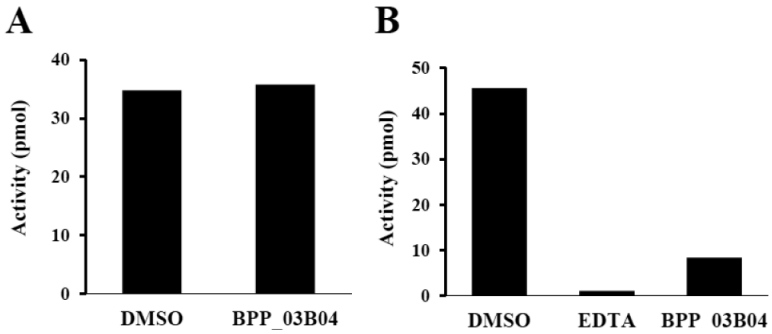

Fig. (6).

The inhibitory activity of BPP_03B04 against eukaryotic protein synthesis. **A.)** Inhibitory activity of BPP_03B04 in wheat germ cell cytoplasmic protein synthesis assays. **B.)** Inhibitory activity of BPP_03B04 against the activity of human mitochondrial PheRS. DMSO represents the positive control where only DMSO was added to the assay in the absence of the compound. EDTA represents the negative control in which reactions contained 20 mM EDTA to inhibit the activity of hmPheRS. Values are the average of three repetitive assays. The concentration of the inhibitory compound BPP_03B04 was 132 μM.

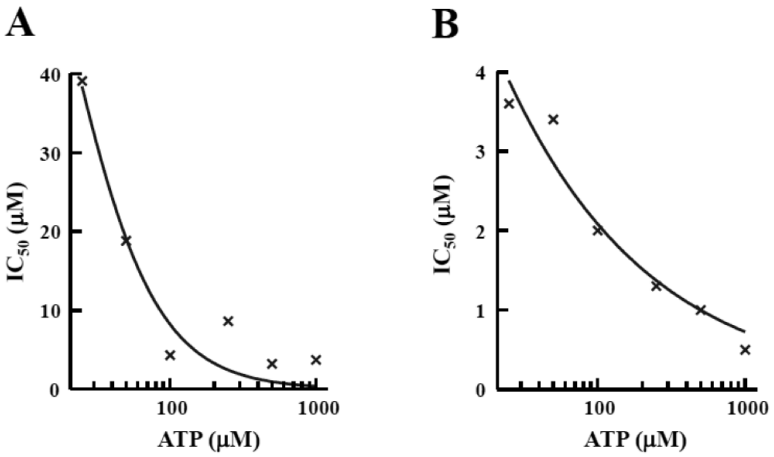

Fig. (7).

Determination of the mode of action of BPP_03B04 relative to ATP. Inhibition by BPP_03B04 is uncompetitive with ATP. BPP_03B04 IC_{50}s were determined using the aminoacylation assay in an SPA format. The phenylalanine concentration was fixed at 100 μM, and IC_{50}s were determined at six different ATP concentrations ranging from 25 to 1000 μM. The data was fit to the sigmoidal dose response model using XLfit (IDBS) using IC_{50}s determined in assays containing **A.)** *P. aeruginosa* PheRS or **B.)** *S. pneumoniae* PheRS.

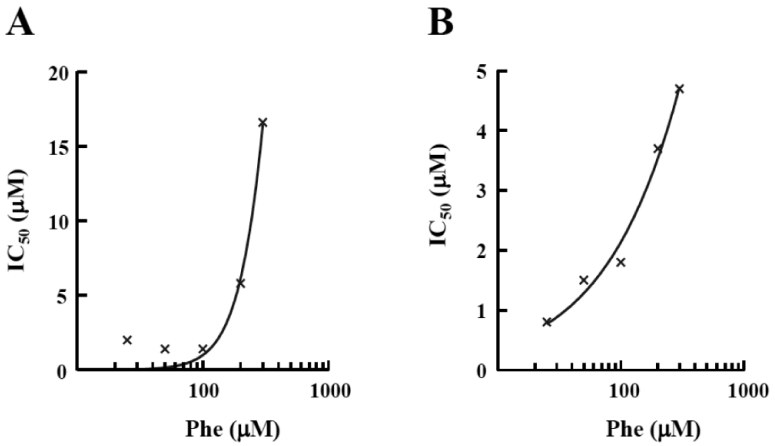

Fig. (8).

Determination of the mode of action of BPP_03B04 relative to Phe. Inhibition by BPP_03B04 is competitive with Phe. BPP_03B04 IC_{50}s were determined using the aminoacylation assay in an SPA format. The ATP concentration was fixed at 2.5 mM, and IC_{50}s were determined at five different Phe concentrations ranging from 25 to 300 μM. The data was fit to the sigmoidal dose response model using XLfit (IDBS) using IC_{50}s determined in assays containing **A.)** *P. aeruginosa* PheRS or **B.)** *S. pneumoniae* PheRS.

Table 1

T. thermophilus PheRS amino acid conservation with homologs.

***T. thermophilus* PheRS**	**α-subunit**	**β-subunit**
E. coli	53.9/43.8[a]	49.3/34.6
P. aeruginosa	54.3/44.3	49.6/36.3
S. pneumoniae	58.0/44.0	50.0/33.6
S. haemolyticus	52.7/37.8	49.3/33.4

[a]% similar amino acids/% conserved amino acids

Table 2

Comparison of the kinetic parameters governing the interaction of PheRS with its substrates.

PheRS		tRNA			Phe			ATP	
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}, μM^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}, μM^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}, μM^{-1})
P. aeruginosa	1.2	0.74	0.64	48	16.2	0.34	200	13.2	0.066
S. pneumoniae	0.94	0.93	1.0	21	22.5	1.1	225	15.5	0.070

Table 3

Minimum inhibitory concentration of the compound against bacterial panel.

Bacteria	MIC (μg/ml)
E. coli (ATCC 25922)	128
E. coli tolC	128
E. faecalis (ATCC 29212)	32
H. influenzae (ATCC 49766)	16
M. catarrhalis (ATCC 25238)	32
P. aeruginosa (ATCC 47085)	16
P. aeruginosa PAO200 (efflux mutant)	16
P. aeruginosa (hypersensitive)	16
S. aureus (ATCC 29213)	16
S. pneumoniae (ATCC 49619)	32