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Development of phenylthiourea derivatives as allosteric inhibitors of pyoverdine maturation enzyme PvdP tyrosinase

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ABSTRACT

Infections caused by *Pseudomonas aeruginosa* become increasingly difficult to treat because these bacteria have acquired various mechanisms for antibiotic resistance, which creates the need for mechanistically novel antibiotics. Such antibiotics might be developed by targeting enzymes involved in the iron uptake mechanism because iron is essential for bacterial survival. For *P. aeruginosa*, pyoverdine has been described as an important virulence factor that plays a key role in iron uptake. Therefore, inhibition of enzymes involved in the pyoverdine synthesis, such as PvdP tyrosinase, can open a new window for the treatment of *P. aeruginosa* infections. Previously, we reported phenylthiourea as the first allosteric inhibitor of PvdP tyrosinase with high micromolar potency. In this report, we explored structure-activity relationships (SAR) for PvdP tyrosinase inhibition by phenylthiourea derivatives. This enables identification of a phenylthiourea derivative (**3c**) with a potency in the submicromolar range (IC_{50} = 0.57 + 0.05 μM). Binding could be rationalized by molecular docking simulation and **3c** was proved to inhibit the bacterial pyoverdine production and bacterial growth in *P. aeruginosa* PA01 cultures.

Pseudomonas aeruginosa is a Gram-negative pathogen that causes infection in many organs. Although these bacteria barely infect healthy individuals, cystic fibrosis patients and immunocompromised individuals suffer from high morbidity and mortality in *P. aeruginosa* infections.[1] However, the development of antibiotic resistance increasingly limits the treatment options for *P. aeruginosa* infections.[2] *P. aeruginosa* employs intrinsic, acquired and adaptive mechanisms to counter most antibiotics including aminoglycosides, quinolones, and β-lactams. Expulsion of antibiotic molecules by efflux pumps is one of the examples of the intrinsic and adaptive mechanisms. The acquired resistance mechanisms can be obtained through either horizontal transfer of resistance genes or mutational changes. Biofilm formation is one of the examples of adaptive antibiotic resistance.[3] In 2017, the World Health Organization (WHO) has classified carbapenem-resistant *P. aeruginosa* into category 1, which is a category of the most urgent need for developing new antibiotics to treat the infection.[4]

Bacterial transport mechanisms to acquire iron provide attractive targets to develop new antibiotics since iron is an essential element that acts as a cofactor in many biological reactions in bacteria including *P. aeruginosa*.[5] Moreover, the low availability of iron in the human body is one of the growth-limiting factors for *P. aeruginosa*. Since iron III (Fe^{3+}) is hardly soluble and cannot passively diffuse into the bacterial cells, these bacteria secrete siderophore pyoverdine, an iron III (Fe^{3+}) chelating molecule, to transport iron into the cell via the FpvA transporter.[6] Pyoverdine biosynthetic enzymes have been proposed as novel antibiotic targets because small molecule inhibitors would downregulate the production of pyoverdine resulting in limited iron acquisition. In support of this concept, several studies have reported that *P. aeruginosa* pyoverdine-deficient mutants have reduced virulence in animal and plant infection models.[7,8] Therefore, small molecule inhibitors that interfere with the pyoverdine biosynthetic enzymes have potency for the development of new treatments for *P. aeruginosa* infections.

In a low iron condition, the biosynthesis of pyoverdine is induced. Initially, in the absence of complex Fe-ferri uptake regulator (FUR) which otherwise binds to the PvdS promoter, to block the production of the extracytoplasmic function (ECF)-sigma factor PvdS. After translation, PvdS binds to the IS boxes of PVDI genes, starting the transcription and translation of PVDI proteins. The non-ribosomal peptide

Abbreviations: IPTG, isopropyl 1-thio-β-D-galactopyranoside; PTU, phenylthiourea

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synthetases (PvdL, PvdI, PvdJ, and PvdD) together with MbtH, PvdG, PvdH, PvdA and PvdF assemble the precursor siderophore PvdIq in the cytoplasm. Subsequently, PvdE transports PvdIq to the periplasm then it is cleaved by PvdQ to form ferribactin and myristoleic acid. The conversion of ferribactin to pyoverdine involves at least four different enzymes: PvdM, PvdN, PvdO, and PvdP at the final step of the biosynthesis.[9,10] PvdP is a tyrosinase known from its role for the maturation of pyoverdine chromophore.[11] Even though, tyrosinases are found in almost every organism: human, animal, plant, mushroom, and bacteria, the PvdP enzyme is different compared to other tyrosinases. The closest homolog is a tyrosinase of *Bacillus megaterium* (PDB ID = 3NQ5) sharing sequence identity 30,7% (Clustal Omega, http://www.ebi.ac.uk/Tools). Moreover, PvdP also has a unique homodimer structure where each monomer consists of 2 different domains i.e.: beta-barrel domain (BBD) and tyrosinase domain (TYD) are not found in the other tyrosinase structure based on the recent reports. As in other tyrosinases, its active site is formed by a di-copper center of six histidine residues,[12] but in the case of PvdP, this is uniquely covered by a C-terminal lid in the apo state.[13]

In line with the facts above, we have reported that most of the tyrosinase inhibitors, for example, mushroom tyrosinase inhibitors do not inhibit PvdP tyrosinase activity. Surprisingly, phenylthiourea (PTU) was found to inhibit PvdP tyrosinase activity at micromolar concentration. Moreover, a crystal structure demonstrated the binding of PTU to an allosteric binding site instead of the tyrosinase active site.[13] This provides opportunities to develop inhibitors that target the allosteric binding site, which seems to be confined to fluorescent pseudomonads. Therefore, identifying PvdP tyrosinase inhibitors that selectively interfere with the allosteric binding site, might result in a specific antibiotic with limited side effects as it does not bind to other tyrosinases.

To obtain sub-micromolar inhibitors, we designed and synthesized a series of PTU derivatives. Some of the generated derivatives show inhibition at the low micromolar range. Kinetic studies were performed to determine the type of inhibition and, with molecular docking revealing the binding of the compound at the allosteric binding site, the high affinity was being explained.

Design and synthesis of PTU derivatives

In our previous study, PTU was discovered as an allosteric inhibitor that binds at the interface of the beta-barrel domain (BBD) and C-terminal domain (TYD) of PvdP and thereby inhibits the tyrosinase enzymatic activity.[13] A focused compound collection of PTU derivatives was synthesized to improve the PvdP tyrosinase inhibitory potency and to explore structure–activity relationships. The desired PTU derivatives were generated by the synthesis of the required phenylthiocyanates that were reacted with the corresponding amines to provide the desired phenylisothiocyanates as final products (Scheme 1). The phenylisothiocyanates were prepared through a one-pot procedure, in which the phenylamines and carbon disulfide were reacted to provide the dithiocarbamate that was desulfurized with sodium persulfate to give the products in good yields after filtration and wash with water.[14] The phenylisothiocyanates were applied without prior purification for coupling with the corresponding amines to generate the disubstituted thioureas (Table 1). The compounds were subjected to filtration to obtain the pure products as white precipitates, except for **1b** and **3b** for which flash column purification was needed. The final product was obtained in variable but sufficient yields (38–99%) over two steps. Phenylurea (**1a**) was synthesized as an equivalent of phenylthiourea by charging aniline with potassium cyanate in an aqueous 10% AcOH solution. The product **1a** was formed and isolated by extraction and recrystallization to provide **1a** in a yield of 98%.[15]

Structure-activity relationship of PTU derivatives

The structure-activity relationships for PvdP tyrosinase inhibition by the 14 membered compound collection were investigated. First, modification of the thiourea functionality of phenylthiourea provided phenylurea **1a**, which provided less than 50% inhibition of PvdP tyrosinase activity at a concentration up to 100 μM. The crystal structure of PvdP bound to PTU (PDB ID = 6RRP) showed the thioketone group interacting with Trp320 and Ser329. The substitution of this group with a ketone group breaks the interaction between the compound and those residues resulting in the loss of its inhibitory activity on PvdP. This means the thio ketone group is important for the inhibitory activity of PTU on the tyrosinase activity of PvdP.

Next, the substitution of the thiourea functionality of PTU was explored (Table 1) and it was found that methyl (**1b**), vinyl (**2b**) or phenyl (**3b**) substitution decreased the potency. In contrast, ethyl phenyl or benzyl substitution of phenylthiourea improved the inhibitory potency by a factor 10 or more. Structural variation of the benzyl substitution provided compound **3c** with a more than 100-fold improved potency compared to non-substituted PTU with an IC_{50} of 0.57 μM. Other substitution patterns, such as 2-Cl, 2,6-Cl, 4-F, 4-H, 4-Br or 4-OCH_3, provided IC_{50} between 7.6 and 23.5 μM. Further substitutions of the phenyl ring of PTU were investigated based on **3c** as a starting point. However, **1d** and **2d** with a 4-methyl or 4-dimethylamino provided IC_{50} values that indicated reduced potency compared to **3c**.

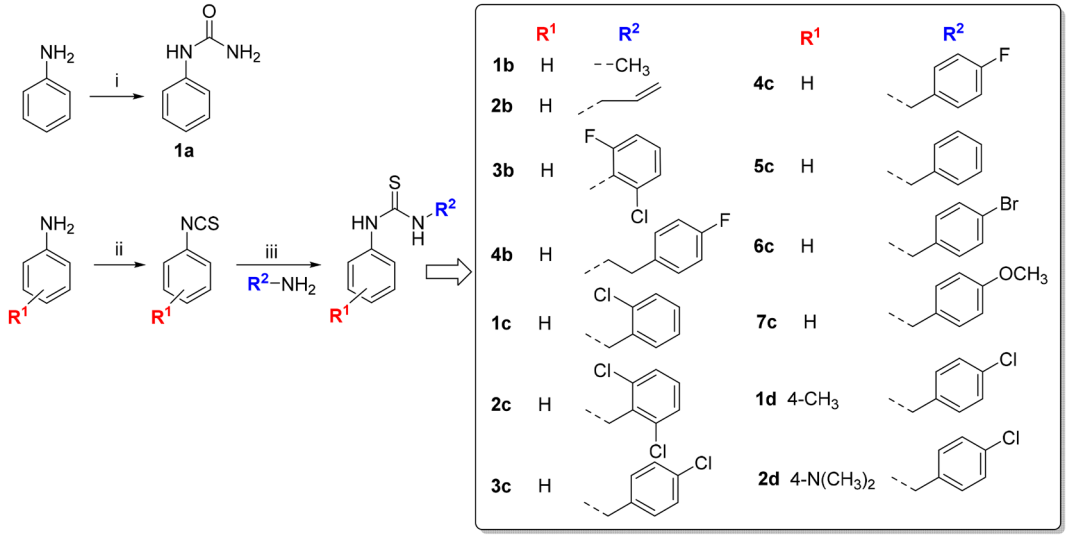
Kinetic study and inhibition activity on PvdP-trunc

To establish the mechanism of PvdP tyrosinase inhibition, Michaelis–Menten enzyme kinetics analysis in the presence of inhibitor **3c** was performed. The Lineweaver–Burk double reciprocal plot shows that inhibitor **3c** causes a decrease in the V_{max} values, whereas the K_m values remain constant (Table 2), indicating a non-competitive inhibition. These results are in line with the data obtained previously for PTU for which we also reported non-competitive inhibition. Obviously, the substitution on the thiourea functionality of PTU with 4-chlorobenzyl improved the inhibition activity but did not change the mechanism of inhibition. Previously, we described that PTU mediated inhibition of PvdP tyrosinase activity involves a C-terminal lid rearrangement and the absence of this lid precludes PTU mediated inhibition of PvdP tyrosinase activity. Inhibitor **3c** was tested for inhibition of wild-type PvdP in comparison to a PvdP variant that is truncated for the C-terminal lid. Similar to the parent compound, this PTU derivative lost its potent inhibition activity on the truncated enzyme, which indicates that inhibitor **3c** has the same mechanism of inhibition as described for PTU that stabilizes the C-terminal lid covering the active site (Fig. 1D).

Mushroom tyrosinase inhibition

In a follow-up experiment, three most potent of the PTU derivatives were tested on mushroom tyrosinase to investigate their effect on other tyrosinases. The IC_{50} values of the compounds increased dramatically, the IC_{50} of **1c** and **2c** were greater than 500 μM and the IC_{50} of the most potent compound (**3c**) increased up to 328 ± 27 μM, an increase of more than 500 times compared to PvdP (Table 3). It means that the derivatives of PTU are not only non-competitive inhibitors, but also very specific inhibitors for PvdP.

As reported, PTU is a competitive inhibitor of mushroom tyrosinase[16] and it binds at the active site of tyrosinase.[17] Knowing that the inhibition of the new derivatives on mushroom tyrosinase is lost, this suggests that its ability to act as a competitive inhibitor is gone. This is most likely attributed to the change in the size and shape of the molecule. The addition of a chlorinated phenyl ring has increased the size of the molecule in such a way that it does not fit into the active site



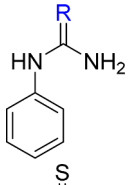
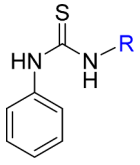
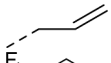
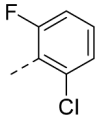
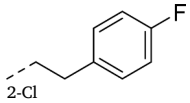
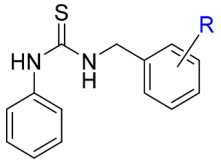
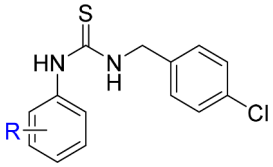
Scheme 1. Synthesis of PTU derivatives. Reagents and conditions: i. KNCO, AcOH, water, r.t., overnight, 90%; ii. 1) K_2CO_3, CS_2, water, r.t., overnight; 2) $Na_2S_2O_8$, K_2CO_3, water, r.t., 1 h, 99%; iii. R_2NH_2, EtOH, r.t., 4 h, 45–97%.

anymore. In conclusion, the PTU derivatives are specific to PvdP due to the nature of their inhibition and their size which prevents them from competitively inhibiting other tyrosinases such as mushroom tyrosinase. This implies that the compounds do also not cross-react with human tyrosinases, which makes them interesting candidates for antibiotics.

Table 2
The kinetic parameters of PvdP tyrosinase activity in the presence of a various concentration of compound **3c**.

Compound	Concentration (μM)	Vmax (A_{475}/min)	Km (mM)
3c	0	0.0155	1.136
	0.2	0.0126	1.131
	0.5	0.0111	1.136

Table 1
Structure and activity of PTU derivatives against PvdP.

Structure	Compound	R	IC_{50} (μM)[a]
	**PTU**	S	174.4 ± 8.9
	1a	O	N.I.
	**1b**	CH_3	N.I.
	2b		N.I.
	3b		N.I.
	4b		22.6 ± 2.6
	**1c**	2-Cl	7.9 ± 0.5
	2c	2,6-Cl	7.6 ± 0.8
	3c	4-Cl	0.57 ± 0.05
	4c	4-F	13.6 ± 3.4
	5c	H	19.4 ± 2.5
	6c	4-Br	23.5 ± 3.1
	7c	4-OCH_3	19.3 ± 2.5
	1d	4- CH_3	22.3 ± 3.3
	2d	4- $N(CH_3)_2$	14.3 ± 3.7
			

N.I. = no inhibition.
[a] = averages derived from three experiments + SD (standard deviation).

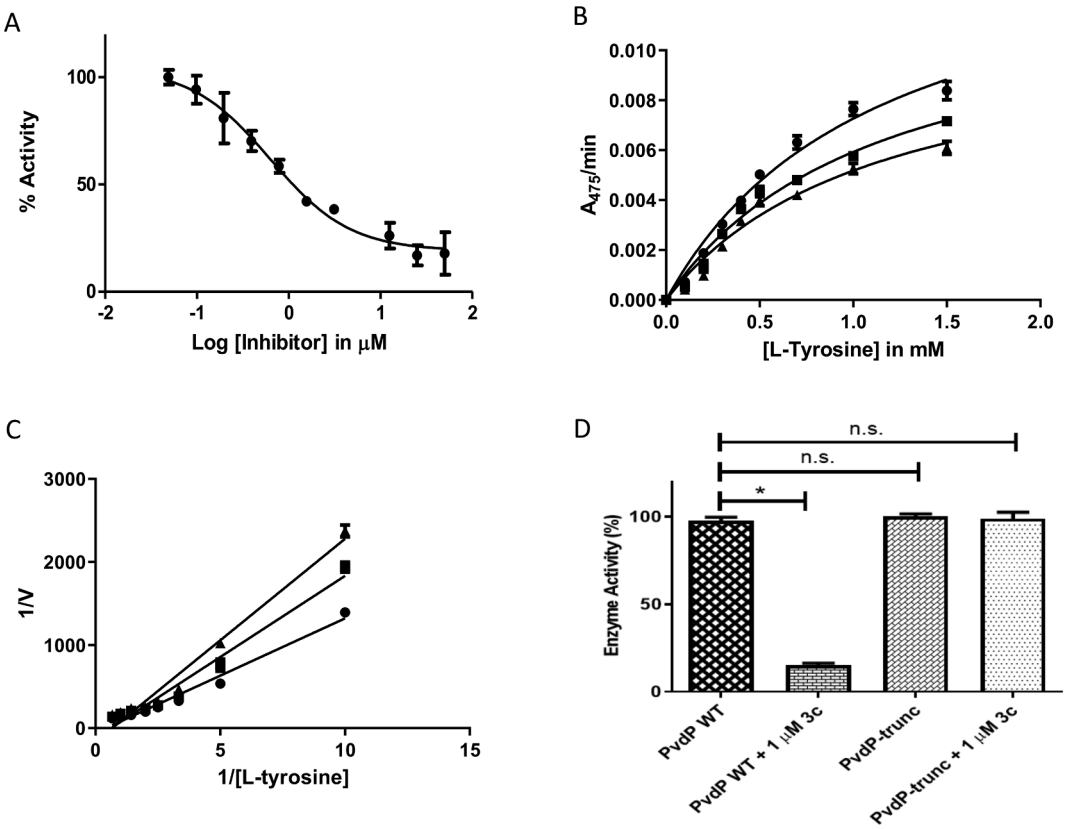


Fig. 1. Inhibition activity of **3c** on PvdP tyrosinase activity. (A) IC_{50} graph of **3c**, (B) Michaelis-Menten graph of **3c**, (C) Lineweaver-Burk plots of **3c**; ● = 0, ■ = 0.2, ▲ = 0.5 μM of compound **3c**, (D) effect of **3c** on PvdP WT (wild type) and PvdP-trunc, an asterisk (*) indicates a significant and n.s. indicates a non-significant difference between treatments ($P < 0.05$, Student *t* test). Data were presented as mean + SD.

Table 3
Inhibitory activity of the potent compounds on PvdP and mushroom tyrosinase.

Compound	IC_{50} + SD (μM)	
	PvdP	Mushroom tyrosinase
1c	7.9 + 0.5	N.I.
2c	7.6 + 0.8	N.I.
3c	0.57 + 0.05	328 + 27

SD = standard deviation, N.I. = no inhibition.

Molecular docking analysis

In order to link the observed SAR to binding information, **3c** was docked at the allosteric binding site of the PvdP enzyme in a similar fashion as with the previously identified non-competitive inhibitor. The docking result showed **3c** provided a binding configuration similar to PTU (Fig. 2A), in which the thioketon moiety forms hydrogen bonds with Trp320 and Ser329. The 4-chlorobenzyl group extended into the available binding site and extra interactions were formed with surrounding residues. The benzyl ring interacts with Asp56 and Ala319 and the chlorine group interacts with Arg57 (Fig. 2B).

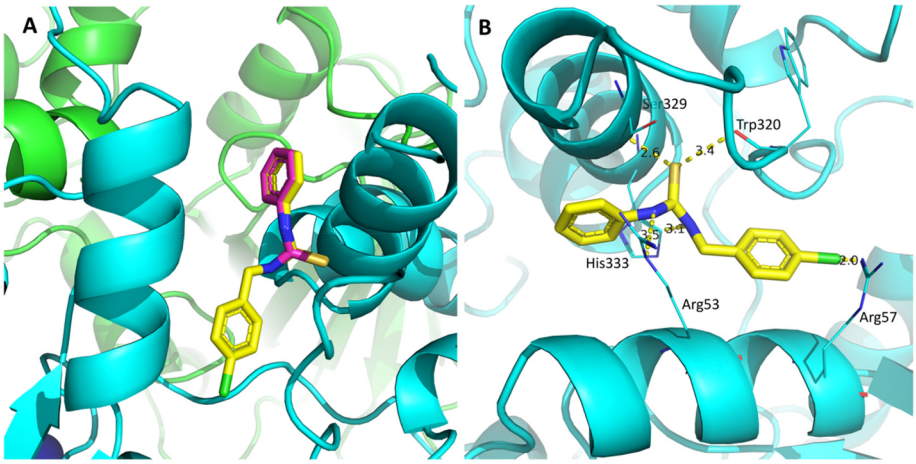


Fig. 2. Result of docking study of **3c** on PvdP. (A) Superimposition of docking result (yellow) and crystal structure of PvdP bound to PTU (PDB ID = 6RRP, magenta), (B) interactions between **3c** and the residues of PvdP.

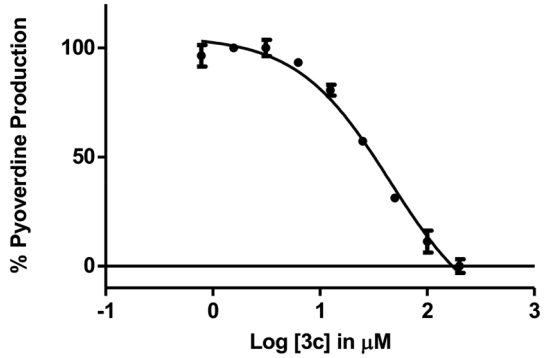


Fig. 3. Bacterial cell assay of **3c**. IC_{50} graph of **3c** on inhibition of the pyoverdine production. Data are presented as average + standard deviation.

Based on the docking result above, it is concluded that substitution with the chlorobenzyl group in the para position gave extra interaction of the compound to PvdP. As reported in our previous study, PTU inhibits the enzymatic activity of PvdP due to the re-arrangement of the C-terminal lid covering the active site.[13] Apparently, these extra interactions provided more anchor points on PvdP resulting in a more stable re-arrangement of the lid. In consequence, compound **3c** has a stronger effect on PvdP than the parent compound.

Inhibition of pyoverdine production

To investigate the inhibition of pyoverdine synthesis by the compound, we tested compound **3c** against living cells of *P. aeruginosa* PA01 for 24 h in a low iron medium. In an iron-depleted condition, the bacteria will start to produce pyoverdine as an iron transporter. A serial dilution of compound **3c** (200 – 0.79 μM, final concentration in DMSO 1%) was prepared and mixed with the bacterial culture in a low iron medium. DMSO 1% was used as a negative control. After 24 h incubation, we measured the production of pyoverdine at 405 nm and the cell-growth at 600 nm. The results showed a dose-dependent inhibitory activity of **3c** on the production of pyoverdine. The production of pyoverdine was inhibited by **3c** with an IC_{50} value of 44.9 ± 5.9 μM (Fig. 3) after being normalized with the cell-growth. The effect observed on bacterial cell assay is in line with the inhibition of PvdP tyrosinase activity. There is however a concentration difference of almost two orders of magnitude between the *in vivo* and the in vitro tests. These differences might be caused by a limited ability of the compound to reach the site of action, i.e. the periplasm. A similar difference between in vitro activity and inhibition inside the periplasm is seen more often.[18,19] This might be improved in the future by optimizing the water solubility, the log P and other physical–chemical properties important for the compound to pass the outer membrane of *P. aeruginosa*.

Several studies have reported targeting pyoverdine production to develop antivirulence agents of *P. aeruginosa*.[18–21] A recent study reported that some pyoverdine inhibitors reduce the pathogenicity of *P. aeruginosa* and improve the survival of *Caenorhabditis elegans* infection model.[22] Our report provided a new alternative to develop antivirulence against *P. aeruginosa*.

In this study, the structure–activity relationships of PTU derivatives against PvdP enzymatic activity were investigated. Several compounds showed low micromolar potency against PvdP tyrosinase activity. Remarkably, the most potent compound **3c** exhibited a strong inhibition against PvdP with an IC_{50} of 0.57 ± 0.05 μM. **3c** was confirmed to be an allosteric inhibitor similar to PTU, but with much higher potency. Binding modes of **3c** with PvdP were studied by molecular docking simulation and found to be consistent with PTU binding mode. In addition, **3c** was active in a bacterial cell assay in inhibiting the production of pyoverdine and bacteria cell growth with an IC_{50} = 44.9 ± 5.9 μM. The discovery and development of PTU derivative as an allosteric inhibitor against the production of iron chelators to suppress bacterial growth could be an effective strategy to treat infections and also to solve the issue of antibiotics resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127409.

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