Chapter 1

General Introduction and Scope of the Thesis

Introduction

Pseudomonas aeruginosa was documented for the first time in the 1850s when a French surgeon frequently observed green-blue color on discharged wound dressings[1],[2]. The green-blue color was later identified in 1860 as pyocyanin. Following the identification of the pigment, the bacterium that was producing pyocyanin was later isolated for the first time in 1882. The species name of the bacterium was standardized in 1951 after previously being known under several different names, e.g., *Bacillus pyocyaneus, Bacteria aeruginosa, Pseudomonas polycolor*, and *Pseudomonas pyocyanea*[3]. This bacterium is a Gram-negative organism that can be found in almost every environment, both aerobic and anaerobic. Therefore, it can infect plants, animals, and also humans[4]. Nowadays, *P. aeruginosa* is known as an opportunistic pathogen with green-blue fluorescent color during the culture, especially in low iron media.

As mentioned above, *P. aeruginosa* is an opportunistic pathogen because it rarely infects healthy individuals. However, immunocompromised patients suffering from diseases, such as serious illness, burn wounds, cystic fibrosis (CF), and Acquired Immuno-deficiency Syndrome (AIDS) are vulnerable to being infected. Moreover, due to its ability to live in very diverse environments, the bacterium can infect almost all of the host's organs, such as lungs, blood, urinary tracts, kidneys, and burn wounds. Although *P. aeruginosa* is not as virulent as other pathogenic bacteria (e.g., *Staphylococcus aureus* and *Streptococcus pyogenes*), its ability to form an extensive colony and biofilm can cause chronic infections[5].

Antibiotic resistance

One of the most challenging problems in treating *P. aeruginosa* infections is the increase in antibiotic resistance. In 2017, the World Health Organization (WHO) released a list of the emergence of research to obtain a new treatment for antibiotic-resistant bacteria. On this list, *P. aeruginosa* is classified into category 1, a group where it is critically needed to find a new therapy against this bacterial's infection[6]. When talking about the impact on the economy, in the US, the Centers for Disease Control and Prevention (CDC) estimated the cost due to antimicrobial resistance up to $55 billion annually: $20 billion in excess of healthcare costs and $35 billion society cost for the loss of productivity[7].

Resistance mechanisms

Recently, the treatment of *P. aeruginosa* infections has become a big challenge in the clinical setting due to the ability of this species to resist a lot of available antibiotics, including aminoglycosides, quinolones, and beta-lactams[8]. In general, the resistance mechanisms of *P. aeruginosa* against antibiotic agents can be categorized into three major mechanisms.

1. Intrinsic antibiotic resistance

   Intrinsic antibiotic resistance is the ability of the bacteria to reduce or even inactivate the efficacy of antibiotics given through functional or structural

characteristics. *P. aeruginosa* possesses intrinsic resistance to most antibiotics by lowering the outer membrane permeability, expressing efflux pumps that "kick" the antibiotics molecules out of the bacterial cell, or by producing antibiotic-inactivating enzymes such as betalactamases[9],[10].

2. Acquired antibiotic resistance
   It is a mechanism of resistance where the bacteria obtain the resistance gene through mutational changes or horizontal transfer from other bacteria. The mutational changes can cause reduced antibiotic uptake, altered antibiotic targets, overexpression of efflux pumps, and production of antibiotic-inactivating enzymes[11]. The resistance genes can be carried by plasmids, transposons, integrons, or prophages; they can be acquired by *P. aeruginosa* from different or the same species[10].
3. Adaptive antibiotic resistance
   The two most elucidated adaptive resistances are biofilm formation and generation of persister cells resulting in persistent infections and poor prognosis in CF patients[12].

Strategies to combat *P. aeruginosa* infections

Following the discovery of penicillin by Alexander Fleming in 1928, many antibiotic agents of various classes were developed and used to treat infections. However, the overuse and misuse of antibiotics for decades have triggered the increase of antibiotic-resistant bacteria. Therefore, developing novel therapeutic strategies to treat infections especially caused by *P. aeruginosa* is urgently needed. In the past decade, several new strategies have been investigated and proposed to be alternative therapies against the infection.

1. Quorum quenching (QQ)
   Quorum sensing (QS) is the ability of *P. aeruginosa* to communicate to sense and coordinate the cell density by releasing communication molecules, such as N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL)[13]. QQ is the interruption of QS. The most studied QQ agent is PvdQ which is naturally produced by *P. aeruginosa.* It has been demonstrated to degrade 3-oxo-C12-HSL *in vitro*[14]. Moreover, it is also reported to be effective in animal infection models i.e. *Caenorhabditis elegans*[15] and mice[16].
2. Lectin inhibition
   Lectins are outer membrane proteins that are found associated with biofilm formation. Lectin inhibitors have a high affinity for lectins[17]. Therefore, the binding of lectins to the host surface can be blocked by lectin inhibitors resulting in the interruption of their function. Recently identified lectin inhibitors are glycocluster, glycopolymer, and glycodendrimer.
3. Iron chelation
   Some iron chelators such as 2,2' dipyridyl (2DP), diethylenepentacetic acid (DPTA), ethylenediaminetetraacetate (EDTA), and gallium were reported to effectively impair *P. aeruginosa* and biofilm formation[18]. Other iron chelators were used alongside

antibiotics. For example, the combination of tobramycin with deferoxamine and deferasirox reduced biofilm biomass *in vitro* and enhanced tobramycin-mediated killing of *P. aeruginosa*[19].

4. Phage therapy
   Phage replication at the infection site, ease to be administered, high specificity to bacterial target, bactericidal activity, and fewer side effects are several advantages of phage therapy[20]. Recently, at least 137 phages have been identified targeting the Pseudomonas genus. For instance, phage PA709 significantly reduced *P. aeruginosa* viability[21], P3-CHA increased the survival rate, and decreased the bacterial burden in the lungs of mice infected with *P. aeruginosa* strain CHA[22], PELP20 killed *P. aeruginosa* strain LESB65[23]. However, clinical trials with phage therapy have been hardly conducted recently. The reasons are the safety problem of the phage clearance after use and the impurity of the phage, poor stability of phage preparations, and limited knowledge of the mechanism of action of the phage[24].
5. Nanoparticles
   The nanoparticles ease the penetration of antibiotic molecules to the bacterial cell membrane, inhibit biofilm formation, show various antibacterial mechanisms, and are good antibiotic carriers[25]. For example, the silver nanoparticles effectively killed and inhibited the growth of *P. aeruginosa in vitro*. In addition, the silver nanoparticles showed low cytotoxicity to mammal cells[26].
6. Antimicrobial peptides
   Several antimicrobial peptides (AMPs) including GL13K, LL-37, T9W, NLF20, cecropin P1, indolicidin, magainin II, nisin, ranalexin, melittin, and defensin, showed bactericidal activity against *P. aeruginosa* or disrupted the biofilm formation[27]–[29]. Their tedious production and isolation hamper their use as antibiotics.
7. Electrochemical scaffolds
   Electrochemical scaffolds generate a low and constant concentration of H_2O_2 to destroy the biofilm formation and improve the penetration of antibiotics. A study demonstrated the efficacy of this method to enhance the susceptibility of *P. aeruginosa* PAO1 to tobramycin[30]. However, a clinical study needs to be conducted before it can be applied to the patients.

Iron uptake mechanism

Iron is an essential compound for all living organisms, including bacteria such as *P. aeruginosa*. In the host, iron is typically in the Fe^{3+} state and characterized by low solubility[31]. Therefore, iron is also a major growth-limiting factor[32]. To obtain a sufficient amount of iron, the bacteria secrete siderophores, iron chelator molecules to capture iron from the environment. The bacteria produce two main siderophores, i.e., pyoverdine, a high-affinity chelator, and pyochelin, a low-affinity chelator[33].

The production of pyoverdine involves a complex biosynthesis mechanism that starts in the cytoplasm and ends up in the periplasm. It is built up by a number of non-ribosomal peptidase synthetases (NRPs) and enzymes (**Figure 1**)[34].

Several studies have reported their success in the exploitation of the iron acquisition mechanism as a target to develop a novel treatment against *P. aeruginosa* infections. PvdQ, an NTN hydrolase that provides the substrate for PvdP, attracts the most attention in this approach. Drake et al. successfully identified and synthesized some novel compounds having inhibition activity on PvdQ[35], moreover another study also reported their finding that some PvdQ inhibitors show their effect in reducing the pyoverdine production in living *P. aeruginosa*[36]. Developing a compound that directly interacts with pyoverdine so it can compete with the iron to bind to also has been investigated in another study[37].

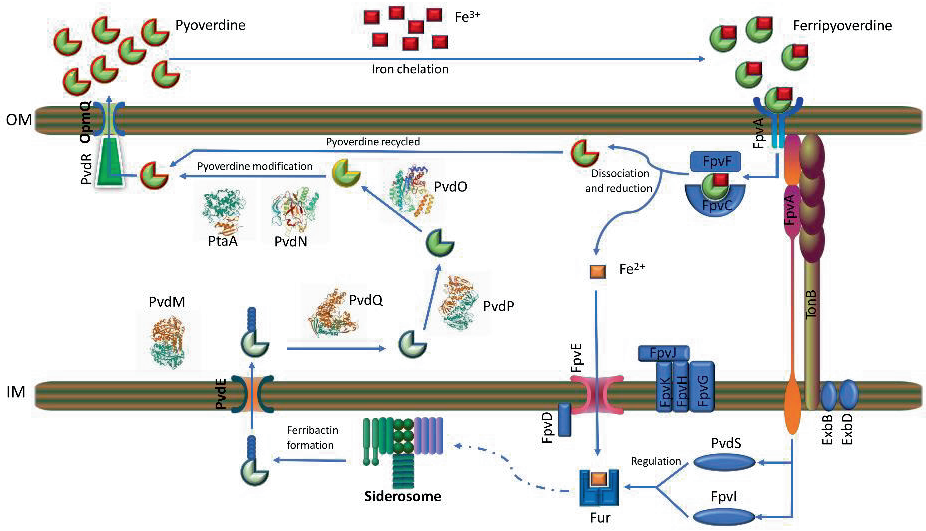

Figure 1. The biosynthesis of pyoverdine involves several non-ribosomal peptidase synthetases (NRPs) and enzymes in the cytoplasm and periplasm. The acylated ferribactin precursor is synthesized in the cytoplasm by NRPs and auxiliary enzymes organized in membrane-associated complexes called siderosomes. Next, it is most likely exported by PvdE to the periplasm, where it is deacylated by the Ntn-type hydrolase PvdQ. PvdP then catalyzes the oxidative cyclization, which leads to dihydropyoverdine. PvdO, possibly in conjunction with other proteins, facilitates the final oxidation, resulting in the characteristic pyoverdine chromophore. Thereafter, side-chain modification pathways transform the original L-glutamic acid side chain to either succinamide catalyzed by PvdN or ketoglutarate catalyzed by PtaA. The modified pyoverdines are then secreted via various transport systems such as PvdRT-OmpQ and bind ferric irons (Fe^{3+}). The complex (ferripyoverdine) binds to FpvA and is taken up in a TonB-dependent manner into the periplasm. FpvF and FpvC dechelate and reduce the Fe^{3+} to be Fe^{2+} then taken up into the cytoplasm by the FpvDE transporter. The apo pyoverdin is recycled.

Considering the encouraging results above, in this thesis, we explore another enzyme in the same pathway: PvdP. Based on the fact that this enzyme is a tyrosinase, it attracts our attention to be investigated and exploited as a novel target of antivirulence

against *P. aeruginosa*. In addition, we also tried to find novel inhibitors of PvdQ through screening a compound library based on certain scaffold.

Scope of the Thesis

The results presented in this thesis mainly focus on the step-by-step strategy to find a novel treatment against *P. aeruginosa* infections through the inhibition of pyoverdine biosynthesis. Two important enzymes (PvdP and PvdQ) that are responsible for the biosynthesis of pyoverdine are targeted to develop a novel treatment against infections.

Part A. Inhibition of PvdP Tyrosinase

Concisely, the strategy includes investigating virulence-related phenotypes of PvdP knockout mutant, crystallization of PvdP, the finding of a novel PvdP inhibitor, and the development of PvdP inhibitors. The application of the most potent PvdP inhibitor in an infection model is also discussed in this thesis.

In **Chapter 2**, we demonstrate the role of PvdP in *P. aeruginosa* virulence. A PvdP knockout mutant of *P. aeruginosa* showed losing its ability to produce pyoverdine and to form biofilm. Furthermore, the mutant showed reduced virulence in the *Galleria mellonella* infection model.

Based on the results of the previous chapter, we started our effort to find the inhibitor for PvdP. In **Chapter 3**, we report our main finding in which phenylthiourea is an allosteric inhibitor of PvdP. In support of this finding, we also successfully co-crystallized phenylthiourea bound to PvdP. Moreover, we also crystallized the apo- and the holo-structure of PvdP.

Since our finding in chapter 3 is new information in terms of PvdP inhibition by phenylthiourea, we did a follow-up study in **Chapter 4**. In this chapter, we designed and synthesized phenylthiourea derivatives by quantitative structure-activity relationship (QSAR) approach. In the end, we were able to synthesize a series of compounds in which the most potent compound showed sub-micromolar potency against PvdP tyrosinase activity.

Furthermore, to know the efficacy of the PvdP inhibitor in an infection scenario, in **Chapter 5**, as a follow-up of our findings in chapter 4, we performed an assay to evaluate the most potent PvdP inhibitor in an infection model. *G. mellonella* larvae were used as an infection model to perform the assay in this chapter.

Part B. Inhibition of PvdQ Acylase

Not only targeting PvdP, but our effort to disrupt the iron uptake mechanism of *P. aeruginosa* also inhibits the acylase activity of PvdQ. PvdQ is an enzyme that provides the substrate for PvdP in the biosynthesis of pyoverdine. In **Chapter 6**, a low throughput screening of a compound library against acylase activity of PvdQ resulted in compound **4d** as the most potent PvdQ inhibitor and acting as a competitive inhibitor according to kinetic studies. The binding mode of the inhibitor to PvdQ was predicted

through a molecular docking experiment. The application of the most potent PvdQ inhibitor in an infection model is also discussed in this chapter.

Finally, **Chapter 7** describes a summary and future perspectives of the findings described in this thesis.

References

[1] J. B. Lyczak, C. L. Cannon, and G. B. Pier, "Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist," *Microbes Infect.*, vol. 2, no. 9, pp. 1051–1060, Jul. 2000, doi: 10.1016/S1286-4579(00)01259-4.

[2] C. Gessard, "On the Blue and Green Coloration that Appears on Bandages," *Rev. Infect. Dis.*, vol. 6, no. Supplement_3, pp. S775–S776, Sep. 1984, doi: 10.1093/CLINIDS/6.SUPPLEMENT_3.S775.

[3] R. T. Villavicencio, "The history of blue pus," *J. Am. Coll. Surg.*, vol. 187, no. 2, pp. 212–216, Aug. 1998, doi: 10.1016/S1072-7515(98)00137-9.

[4] S. P. Diggle and M. Whiteley, "Microbe Profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat," *Microbiology*, vol. 166, pp. 30–33, 2020, doi: 10.1099/mic.0.000860.

[5] N. Høiby, O. Ciofu, and T. Bjarnsholt, "Pseudomonas aeruginosa biofilms in cystic fibrosis," *Future Microbiology*, vol. 5, no. 11. Future Medicine Ltd London, UK , pp. 1663–1674, Nov. 06, 2010, doi: 10.2217/fmb.10.125.

[6] M. A. Fischbach and C. T. Walsh, "Antibiotics for Emerging Pathogens," *Science (80-.).*, vol. 325, no. 5944, pp. 1089–1093, Aug. 2009, doi: 10.1126/science.1176667.

[7] F. Prestinaci, P. Pezzotti, and A. Pantosti, "Antimicrobial resistance: A global multifaceted phenomenon," *Pathogens and Global Health*, vol. 109, no. 7. pp. 309–318, Oct. 01, 2015, doi: 10.1179/2047773215Y.0000000030.

[8] R. E. W. Hancock and D. P. Speert, "Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment," *Drug Resist. Updat.*, vol. 3, no. 4, pp. 247–255, 2000, doi: 10.1054/drup.2000.0152.

[9] J. M. A. Blair, M. A. Webber, A. J. Baylay, D. O. Ogbolu, and L. J. V. Piddock, "Molecular mechanisms of antibiotic resistance," *Nature Reviews Microbiology*, vol. 13, no. 1. pp. 42–51, Jan. 11, 2015, doi: 10.1038/nrmicro3380.

[10] E. B. M. Breidenstein, C. de la Fuente-Núñez, and R. E. W. Hancock, "Pseudomonas aeruginosa: All roads lead to resistance," *Trends in Microbiology*, vol. 19, no. 8. pp. 419–426, Aug. 01, 2011, doi: 10.1016/j.tim.2011.04.005.

[11] J. M. Munita and C. A. Arias, "Mechanisms of antibiotic resistance," *Microbiol. Spectr.*, vol. 4, no. 2, pp. 1–24, 2016, doi: 10.1128/microbiolspec.VMBF-0016-2015.

[12] P. K. Taylor, A. T. Y. Yeung, and R. E. W. Hancock, "Antibiotic resistance in Pseudomonas aeruginosa biofilms: Towards the development of novel anti-biofilm therapies," *J. Biotechnol.*, vol. 191, pp. 121–130, Dec. 2014, doi: 10.1016/j.jbiotec.2014.09.003.

[13] P. Williams, K. Winzer, W. C. Chan, and M. Cá mara, "Look who's talking: communication and quorum sensing in the bacterial world," doi: 10.1098/rstb.2007.2039.

[14] C. F. Sio *et al.*, "Quorum Quenching by an N-Acyl-Homoserine Lactone Acylase from Pseudomonas aeruginosa PAO1," *Infect. Immun.*, vol. 74, no. 3, pp. 1673–1682, 2006, doi: 10.1128/IAI.74.3.1673-1682.2006.

[15] E. Papaioannou, M. Wahjudi, P. Nadal-Jimenez, G. Koch, R. Setroikromo, and W. J. Quax,

"Quorum-quenching acylase reduces the virulence of Pseudomonas aeruginosa in a Caenorhabditis elegans infection model," *Antimicrob. Agents Chemother.*, vol. 53, no. 11, pp. 4891–4897, 2009, doi: 10.1128/AAC.00380-09.

[16] P. D. Utari, R. Setroikromo, B. N. Melgert, and W. J. Quax, "PvdQ quorum quenching acylase attenuates Pseudomonas aeruginosa virulence in a mouse model of pulmonary infection," *Front. Cell. Infect. Microbiol.*, vol. 8, pp. 1–12, 2018, doi: 10.3389/fcimb.2018.00119.

[17] A. V. Grishin, M. S. Krivozubov, A. S. Karyagina, and A. L. Gintsburg, "Pseudomonas Aeruginosa lectins as targets for novel antibacterials," *Acta Naturae*, vol. 7, no. 2. Russian Federation Agency for Science and Innovation, pp. 29–41, Jun. 15, 2015, doi: 10.32607/20758251-2015-7-2-29-41.

[18] C. Y. O, K. Sanderson, L. F. Roddam, S. M. Kirov, D. W. Reid, and D. W. Reid DECReid, "Iron-binding compounds impair Pseudomonas aeruginosa biofilm formation, especially under anaerobic conditions," *J. Med. Microbiol.*, vol. 58, pp. 765–773, 2009, doi: 10.1099/jmm.0.004416-0.

[19] S. Moreau-Marquis, G. A. O'toole, and B. A. Stanton, "Tobramycin and FDA-Approved Iron Chelators Eliminate Pseudomonas aeruginosa Biofilms on Cystic Fibrosis Cells," *Am. J. Respir. Cell Mol. Biol.*, vol. 41, pp. 305–313, 2009, doi: 10.1165/rcmb.2008-0299OC.

[20] M. H. Ly-Chatain, "The factors affecting effectiveness of treatment in phages therapy," *Front. Microbiol.*, vol. 5, no. 51, pp. 1–7, 2014, doi: 10.3389/fmicb.2014.00051.

[21] A. Vieira, Y. J. Silva, Â. Cunha, N. C. M. Gomes, H. W. Ackermann, and A. Almeida, "Phage therapy to control multidrug-resistant Pseudomonas aeruginosa skin infections: In vitro and ex vivo experiments," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 31, no. 11, pp. 3241–3249, Nov. 2012, doi: 10.1007/s10096-012-1691-x.

[22] E. Morello, E. Saussereau, D. Maura, M. Huerre, and L. Touqui, "Pulmonary Bacteriophage Therapy on Pseudomonas aeruginosa Cystic Fibrosis Strains: First Steps Towards Treatment and Prevention," *PLoS One*, vol. 6, no. 2, p. 16963, 2011, doi: 10.1371/journal.pone.0016963.

[23] E. Waters *et al.*, "Phage therapy is highly effective against chronic lung infections with Pseudomonas aeruginosa," *Thorax*, vol. 72, pp. 666–667, 2017, doi: 10.1136/thoraxjnl-2016-209265.

[24] A. Sulakvelidze, Z. Alavidze, and J. G. Morris, "Bacteriophage Therapy," *Antimicrob. Agents Chemother.*, vol. 45, no. 3, pp. 649–659, 2001, doi: 10.1128/AAC.45.3.649-659.2001.

[25] L. Wang, C. Hu, and L. Shao, "The antimicrobial activity of nanoparticles: present situation and prospects for the future," *Int. J. Nanomedicine*, vol. 12, pp. 1227–1249, 2017, doi: 10.2147/IJN.S121956.

[26] R. Salomoni, A. F. Montemor, B. Rinaldi, and M. Rodrigues, "Antibacterial effect of silver nanoparticles in Pseudomonas aeruginosa," *Sci. Appl.*, vol. 10, pp. 115–121, 2017, doi: 10.2147/NSA.S133415.

[27] P. Papareddy *et al.*, "NLF20: an antimicrobial peptide with therapeutic potential against invasive Pseudomonas aeruginosa infection," *J. Antimicrob. Chemother.*, vol. 71, pp. 170–180, 2016, doi: 10.1093/jac/dkv322.

[28] U. Wnorowska *et al.*, "Bactericidal Activities of Cathelicidin LL-37 and Select Cationic Lipids against the Hypervirulent Pseudomonas aeruginosa Strain LESB58," *Antimicrob. Agents Chemother.*, vol. 59, no. 7, pp. 3808–3815, 2015, doi: 10.1128/AAC.00421-15.

[29] X. Zhu *et al.*, "Bactericidal Efficiency and Modes of Action of the Novel Antimicrobial Peptide T9W against Pseudomonas aeruginosa," *Antimicrob. Agents Chemother.*, vol. 59,

no. 6, pp. 3008–3017, 2015, doi: 10.1128/AAC.04830-14.

[30] S. T. Sultana, D. R. Call, and H. Beyenal, "Eradication of Pseudomonas aeruginosa biofilms and persister cells using an electrochemical scaffold and enhanced antibiotic susceptibility," *npj Biofilms Microbiomes*, vol. 2, pp. 1–8, 2016, doi: 10.1038/s41522-016-0003-0.

[31] G. O. Latunde-Dada and R. J. Simpson, "Regulation of iron absorption and distribution," in *Iron Deficiency and Overload*, S. Yehuda and D. I. Mostofsky, Eds. Humana Press, 2010, pp. 31–49.

[32] C. Ratledge and L. G. Dover, "Iron metabolism in pathogenic bacteria," *Annu. Rev. Microbiol.*, vol. 54, pp. 881–941, 2000, doi: 10.1146/annurev.micro.54.1.881.

[33] P. Visca, F. Imperi, and I. L. Lamont, "Pyoverdine siderophores: from biogenesis to biosignificance," *Trends Microbiol.*, vol. 15, no. 1, pp. 22–30, 2007, doi: 10.1016/j.tim.2006.11.004.

[34] M. T. Ringel and T. Brüser, "The biosynthesis of pyoverdines," *Microb. Cell*, vol. 5, no. 10, pp. 424–437, 2018, doi: 10.15698/mic2018.10.649.

[35] E. J. Drake and A. M. Gulick, "Structural Characterization and High-Throughput Screening of Inhibitors of PvdQ, an NTN Hydrolase Involved in Pyoverdine Synthesis," *ACS Chem. Biol.*, vol. 6, pp. 1277–1286, 2011, doi: 10.1021/cb2002973.

[36] J. M. Wurst *et al.*, "Identification of inhibitors of PvdQ, an enzyme involved in the synthesis of the siderophore pyoverdine," *ACS Chem. Biol.*, vol. 9, no. 7, pp. 1536–1544, 2014, doi: 10.1021/cb5001586.

[37] D. R. Kirienko, D. Kang, and N. V. Kirienko, "Novel Pyoverdine Inhibitors Mitigate Pseudomonas aeruginosa Pathogenesis," *Front. Microbiol.*, vol. 9, no. 3317, pp. 1–14, 2019, doi: 10.3389/fmicb.2018.03317.

Inhibition of PvdP Tyrosinase