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Heterocyclic Aromatic *N*-Oxidation in the Biosynthesis of Phenazine Antibiotics from *Lysobacter antibioticus*

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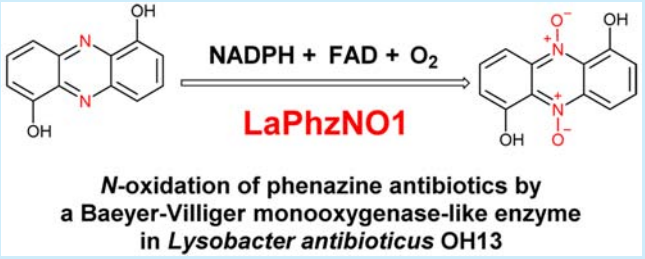
Supporting Information

ABSTRACT: Heterocyclic aromatic *N*-oxides often have potent biological activities, but the mechanism for aromatic *N*-oxidation is unclear. Six phenazine antibiotics were isolated from *Lysobacter antibioticus* OH13. A 10 gene cluster was identified for phenazine biosynthesis. Mutation of *LaPhzNO1* abolished all *N*-oxides, while non-oxides markedly increased. LaPhzNO1 is homologous to Baeyer−Villiger flavoproteins but was shown to catazlye phenazine *N*-oxidation. LaPhzNO1 and LaPhzS together converted phenazine 1,6-dicarboxylic acid to 1,6-dihydroxyphenazine *N*5,*N*10-dioxide. LaPhzNO1 also catalyzed *N*-oxidation of 8-hydroxyquinoline.



Heterocyclic aromatic *N*-oxides are rare in natural products but often possess potent biological activities. These include orellanine, a deadly poison from the toadstool mushroom *Cortinarius orellanus*,[1a] canthin-6-one *N*-oxide, a plant alkaloid from *Eurycoma harmandiana* with a broad spectrum of antibiotic activities,[1b] cortamidine oxide, a novel disulfide metabolite from the mushroom *Cortinarius* sp. with significant antimicrobial activity and cytotoxicity,[1c] piericidin B1 *N*-oxide, a novel inhibitor of phosphatidylinositol turnover from *Streptomyces* sp.,[1d] and phenazine oxides, a group of broad spectrum antibiotics from several bacteria (Figure 1).[2] Among phenazine oxides, myxin (1-hydroxy-6-methoxyphenazine-*N*5,*N*10-dioxide, **1**) is most noteworthy for its potent antimicrobial activity and unique mode of action among antibiotics.[3] Cuprimyxin, a copper(II) complex of myxin, was used for decades as a topical broad spectrum veterinary antibiotic and antifungal drug. Clofazimine, a synthetic phenazine, is a member of the World Health Organization's List of Essential Medicines.[4] Phenazine natural products also function as virulence factors in some bacteria.[2] Recently, it was shown that halogenated phenazines eradicate biofilms, methicillin-resistant *Staphylococcus aureus* (MRSA) persister cells, and *Mycobacterium tuberculosis*.[5] Despite the diverse activities of aromatic *N*-oxides, the mechanism for *N*-oxidation in these natural products has not been reported. Herein, we report the isolation of six phenazines including four *N*-oxides, identification of the biosynthetic gene cluster, and characterization of the phenazine *N*-oxidation enzyme in *Lysobacter antibioticus* OH13.

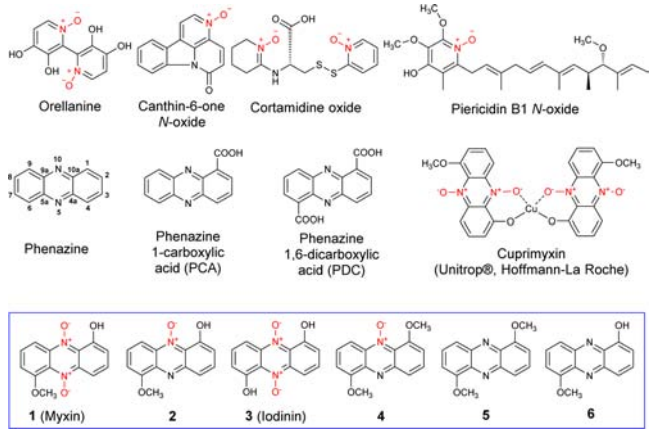


Figure 1. Structures of selected heterocyclic aromatic *N*-oxide natural products and phenazine natural products. Compounds **1**−**6** are isolated from *L. antibioticus* OH13 in this study.

OH13 was initially isolated from the rhizosphere of rice and exhibited potent biocontrol activity against bacterial and fungal pathogens of plants. We subsequently purified six compounds (**1**−**6**) through large-scale fermentation, column chromatography, and bioassay-guided fractionation and HPLC. **1** and **2** are the main products in the OH13 culture (Figure S1). We obtained 16 mg of **1** and 20 mg of **2** from 10 L of OH13 cultures. Their

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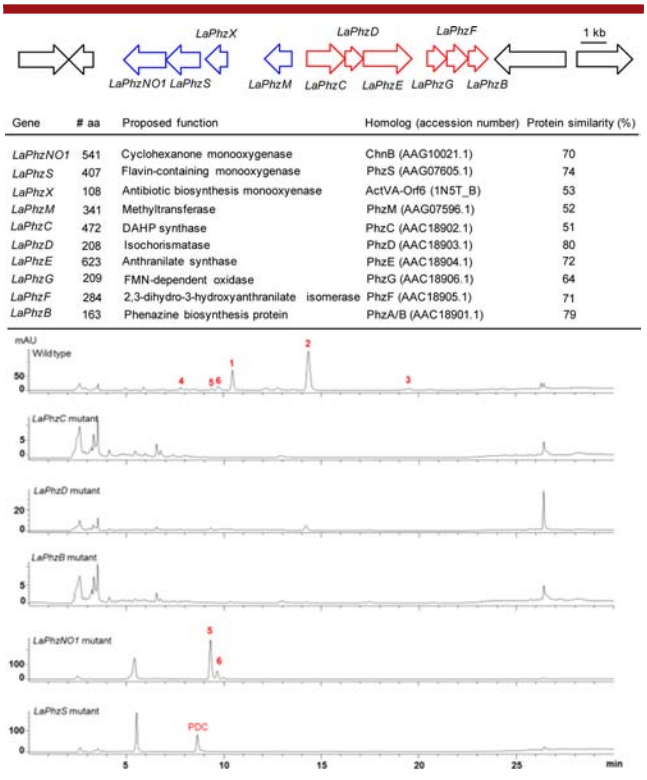
structures were determined by mass spectrometry and NMR (Supporting Information). These compounds were tested for activity against a panel of bacteria. While **1** generally exhibited the strongest activity, the other compounds also showed a varied degree of potency (Figure S2). In addition, we found that **1** was also highly effective against MRSA (MIC 0.05 μg/mL, 10 times more active than vancomycin under the tested conditions).

Although phenazines have been isolated from many bacteria, it is unusual for a single culture to simultaneously produce so many different analogues, particularly multiple *N*-oxides. We assumed that the genome of OH13 would contain not only the common *Phz* genes for synthesizing the phenazine core (PCA or PDC) (Figure 1) but also genes for modifications of the core, including *N*-oxidation. Subsequently, we sequenced and analyzed the genome of OH13 (5.8 M, 4754 genes, 67% average GC content) and found a gene cluster (*LaPhz*) containing orthologues of the previously reported core *Phz* genes.[2] The *LaPhz* cluster contains six putative core genes, *LaPhzB*−*G*, and four putative modification genes, *LaPhzNO1*, *LaPhzS*, *LaPhzX*, and *LaPhzM* (Figures 2 and S3).



Gene	# aa	Proposed function	Homolog (accession number)	Protein similarity (%)
LaPhzNO1	541	Cyclohexanone monooxygenase	ChnB (AAG10021.1)	70
LaPhzS	407	Flavin-containing monooxygenase	PhzS (AAG07605.1)	74
LaPhzX	108	Antibiotic biosynthesis monooxyenase	ActVA-Orf6 (1N5T_B)	53
LaPhzM	341	Methyltransferase	PhzM (AAG07596.1)	52
LaPhzC	472	DAHP synthase	PhzC (AAC18902.1)	51
LaPhzD	208	Isochorismatase	PhzD (AAC18903.1)	80
LaPhzE	623	Anthranilate synthase	PhzE (AAC18904.1)	72
LaPhzG	209	FMN-dependent oxidase	PhzG (AAC18906.1)	64
LaPhzF	284	2,3-dihydro-3-hydroxyanthranilate isomerase	PhzF (AAC18905.1)	71
LaPhzB	163	Phenazine biosynthesis protein	PhzA/B (AAC18901.1)	79

Figure 2. *LaPhz* gene cluster and predicted function of the genes from *L. antibioticus* OH13 (top). HPLC analysis of phenazines in OH13 and *LaPhz* mutants. **1**−**6** and phenazine 1,6-dicarboxylic acid (PDC) are indicated.

The six core genes showed a close similarity to the phenazine biosynthetic genes from *Pseudomonas fluorescens* 2−79.[6] *LaPhzC* is predicted to encode a 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase that catalyzes the first step of the shikimate pathway in chorismate biosynthesis.[7] LaPhzD is similar to isochorismatase that converts 2-amino-2-desoxyisochorismate to *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA).[8] LaPhzE is similar to anthranilate synthase that converts chorismate into aminodeoxyisochorismate.[9] LaPhzG is similar to FMN-dependent oxidase, which is the terminal enzyme in phenazine core biosynthesis to generate PCA or PDC.[10] LaPhzF is similar to 2,3-dihydro-3-hydroxyanthranilate isomerase, which isomerizes DHHA to the ketone 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid (AOCHC).[11] LaPhzB is similar to enzymes responsible for condensing two AOCHC to produce the first tricyclic system in the biosynthetic pathway.[12]

To verify the *LaPhz* gene cluster, we first mutated three core genes, *LaPhzC*, *LaPhzD*, and *LaPhzB* (Figures S4 and S5). HPLC analysis showed that phenazines were abolished from mutants *LaPhzC* and *LaPhzB* and mutant *LaPhzD* produced a trace amount of **2** (Figure 2). We also examined the antibiotic activity of the mutants. Consistent with the HPLC results, the tests showed that mutants *LaPhzC* and *LaPhzB* lost the antibacterial activity and mutant *LaPhzD* exhibited trace activity (Figure S7). These results demonstrated that the core genes are required for phenazine biosynthesis in OH13.

The mechanism for *N*-oxidation is the least understood part of the phenazine biosynthetic pathway.[2] The identification of four phenazine *N*-oxides and the *LaPhz* cluster provide an excellent opportunity to investigate *N*-oxidation of aromatic *N*-oxides. Among the four putative phenazine modification genes, *LaPhzS* is similar to *PhzS* encoding a flavin-containing monooxygenase responsible for converting PCA to 1-hydroxyphenazine or PDC to 1,6-dihydroxyphenazine.[13] *LaPhzM* resembles *PhzM* encoding a methyltransferase catalyzing methylation of the hydroxy groups or the N atom of the phenazine ring.[14] Notably, *LaPhzNO1* and *LaPhzX* do not have known *Phz* orthologues whose function has been experimentally characterized. LaPhzNO1 has a 70% sequence similarity to ChnB cyclohexanone monooxygenases (Figure 2 and Figure S3A), flavoproteins that catalyze the Baeyer−Villiger-type oxidation of cyclic ketones to produce lactones.[15] LaPhzX is similar to ActVA-Orf6 and TcmH, which are small monooxygenases that catalyze oxidation of phenolic compounds to corresponding quinones and do not require any metal ion or cofactor (Figure S3B).[16] We therefore focused our efforts on LaPhzNO1 as a possible novel *N*-monooxygenase. First, we inactivated the *LaPhzNO1* gene and analyzed the metabolites in the mutant (Figure S6). The result showed that none of the *N*-oxides (**1**−**4**) was detectable in the mutant, while two peaks that had the same retention time as **5**−**6** were dramatically increased when compared to the wild type (Figure 2). The identity of these two peaks was confirmed to be **5**−**6** by LC-MS and NMR. Antibacterial assays showed that the *LaPhzNO1* mutant still possessed activity, but the activity was clearly lower than that of the wild type (Figure S7). This result is consistent with the loss of the most potent antibacterial components in the mutant, such as **1**, but the presence of the less potent phenazines such as **5**−**6**. The results indicate that *LaPhzNO1* is indeed involved in *N*-oxidation of phenazines.

To further verify the function of *LaPhzNO1*, we conducted in vitro studies of *LaPhzNO1* with **5** and **6** as the substrate. *LaPhzNO1* was cloned from OH13 genome and expressed in *E. coli* (Figure S8). The expected 60.2 kDa protein LaPhzNO1 was purified and used in enzyme reactions containing **5** or **6**, as well as NADPH and FAD. LC-MS analysis of the reaction products showed a distinct peak (14.3 min) that was absent in the control reaction when **6** was the substrate (Figure 3). This peak gave m/z 243.47, which was consistent with the expected $[M + H]^+$ of **2** (Figure S9). NADPH is absolutely required for the *N*-oxidation activity, and the enzyme activity was significantly decreased when NADH was used (Figure S10). Pure LaPhzNO1 appeared as a light yellow protein with absorptions at 442 and 382 nm (Figure S11), and the addition of FAD or FMN did not significantly change the enzyme activity (Figure S10). These features are

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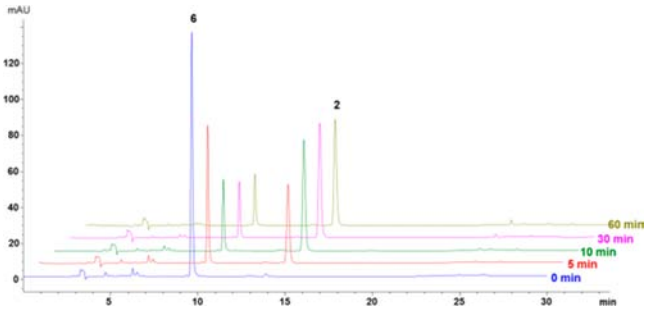

Figure 3. HPLC analysis of the reaction of LaPhzNO1 with **6** as substrate, in the presence of NADPH and FAD.

consistent with LaPhzNO1 being a flavoprotein.[15] Interestingly, when **5** was used as the substrate, no new peak was detected in LC-MS. Besides, only N10, but not N5, was oxidized when **6** was the substrate. These results suggest that the methoxy groups at C1 and C6 are important for the substrate selectivity of LaPhzNO1. To test this idea, we mutated *LaPhzS* and found that the mutant produced a new peak at 8.6 min, which gave *m*/*z* of 269.27 for PDC (Figures 2 and S6). We then expressed and purified the 44.1 kDa LaPhzS (Figure S8). When LaPhzS was incubated with PDC in the presence of NADH and FAD, a new peak at 10.0 min was produced, which gave *m*/*z* of 213.27 for 1,6-dihydroxyphenazine (DHP) (Figure 4). The results not only

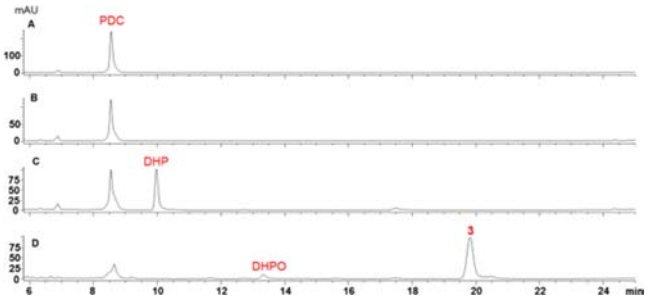

Figure 4. HPLC analysis of coupled reaction between LaPhzS and LaPhzNO1 with PDC as a substrate. (A) Boiled enzymes with PDC, NADH, NADPH, and FAD. (B) LaPhzNO1 with PDC, NADPH, and FAD. (C) LaPhzS with PDC, NADH, and FAD. (D) LaPhzS + LaPhzNO1 with PDC, NADH, NADPH, and FAD. DHP, 1,6-dihydroxyphenazine; DHPO, *N*-monooxide of DHP; **3**, *N*5,*N*10-dioxide (iodinin).

confirmed LaPhzS's function in decarboxylative hydroxylation of PDC to produce the intermediate DHP (Figure 5) but also provided a non-methylated substrate for LaPhzNO1, presumably the direct substrate leading to the dioxide product at both N5 and N10. We subsequently coupled the reactions of LaPhzS and LaPhzNO1 using PDC as a substrate. A new peak at 19.8 min that co-migrated with **3** was produced in the coupled reactions (Figure 4D). The reaction also produced a minor peak at 13.3 min that had *m*/*z* of 229.47, which is consistent with the *N*-monooxide of DHP (DHPO). The results demonstrate that LaPhNO1 is able to catalyze *N*-oxidation at both N5 and N10 and also suggest that methoxy at C1 and C6 can block the *N*-oxidation (Figure 5). Finally, our data also support PDC as the precursor of myxin, which has been proposed but to our knowledge never experimentally confirmed.

Phenazines are unique among antibiotics due to their bioreductive activity.[3a,17] So far, the function of the six core genes, *LaPhzB*−*G*, have been extensively studied in other microorganisms.[2] These genes make up the biosynthetic pathway that leads to PDC formation (or PCA in some organisms) (Figure 5). PCA and PDC are considered as

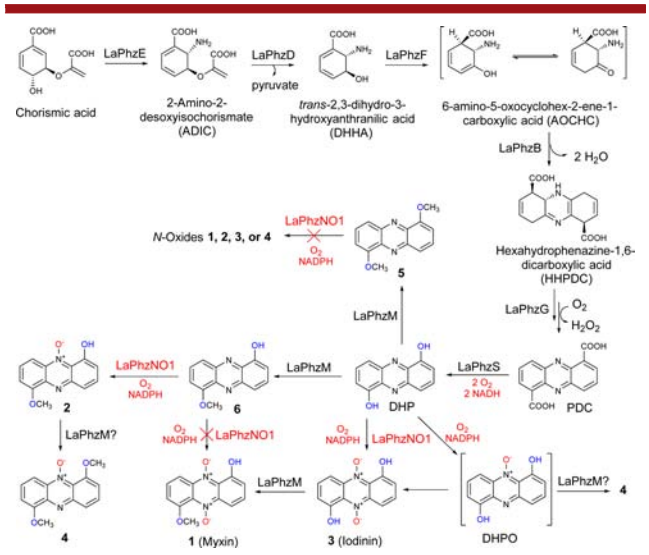

Figure 5. Proposed biosynthetic mechanism for phenazine compounds isolated from *L. antibiotius* OH13. The cross symbols indicate that the reactions did not take place in enzyme assays.

common precursors for more complex phenazines. However, the molecular mechanism for biosynthesis of the vast complex phenazines from these two common precursors is relatively unclear, and the enzyme responsible for *N*-oxidation of phenazines had not been identified. Besides, five of the six compounds contain one or two methoxy groups, implying that LaPhzM may have a relaxed substrate selectivity when catalyzing *O*-methylation and the timing of methylation could be before and after *N*-oxidation (Figure 5). Interestingly, we did not observe a product that is both dimethylated and dioxidated, although both dimethylation (**4** and **5**) and dioxidation (**1** and **3**) apparently have occurred in the biosynthesis. How the *O*-methylation influences the *N*-monooxygenase and the *N*-oxidation affects the *O*-methyltransferase needs further investigation. LaPhzX shares a similarity to small monooxygenases that catalyze oxidation of phenolic compounds to corresponding quinones.[16] We have not isolated phenazines containing quinone functionality, and the function of LaPhzX is unclear.

Orthologues of *LaPhzNO1* and *LaPhzS* are present in other phenazine gene clusters.[18] However, none of the genes had been experimentally investigated for *N*-oxidation. Now we have obtained both in vivo and in vitro evidence to show that LaPhzNO1 is responsible for *N*-oxidations of phenazines. Our data demonstrate that LaPhzNO1 is an NADPH-dependent, flavin-containing *N*-monooxygenase in phenazine biosynthesis. Although LaPhzNO1 shows a sequence similarity to cyclohexanone monooxygenase, which is a member of the Baeyer−Villiger monooxygenase family, it apparently has acquired the ability to use phenazine as a substrate and to add an oxygen to the nitrogen atom. A structure modeling of PhzNO1 based on the crystal structure of cyclohexanone monooxygenase (PDB 3GWD) revealed a well-conserved NADPH/FAD binding pocket in PhzNO1 (Figure S11).[15e] Based on the results, we propose a mechanism for the *N*-oxidation of **6** by LaPhzNO1 (Figure S12). The enzyme-bound FAD is reduced to $FADH_2$ by NADPH. $FADH_2$ then reacts with molecular oxygen to form

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flavin hydroperoxide FADH−OOH, which is attacked by the aromatic nitrogen of **6**, resulting in release of FADH−OH and **2**. A dehydration of FADH−OH regenerates the oxidized FAD.

In the enzyme assays, LaPhzNO1 was unable to convert **5** to any of the *N*-oxide products (Figure 5). Moreover, LaPhzNO1 could only convert **6** into **2** but not **1**. These results suggest that the *N*-oxidation steps must take place before the adjacent *O*-methylation steps. Once the hydroxy groups at the C1 and C6 positions are methylated, LaPhzNO1 is no longer able to oxidize the nitrogen atom. Using LaPhzS and LaPhNO1 coupled reactions, we confirmed the substrate selectivity of LaPhzNO1. To further prove this point, we tested a group of *N*-containing aromatic compounds as potential substrates, including 8-hydroxyquinoline (8-HQ), 6-hydroxyquinoline (6-HQ), quinoline, quinoxaline, quinine, and 2-phenylpyridine (Figure S13). LaPhzNO1 was able to convert 8-HQ into its oxide in the presence of NADPH but unable to use any of the other compounds as substrates. This result not only confirms the importance of the β-hydroxy for the LaPhzNO1-catalyzed *N*-oxdidation but also shows a potential use of this enzyme in the chemoenzymatic synthesis of aromatic *N*-oxides, as there are over 6000 synthetic phenazine compounds and over 150 natural phenazines.[2] Further investigation is needed to elucidate the molecular basis of the observed substrate selectivity. In conclusion, LaPhzNO1 represents the first experimentally characterized aromatic *N*-monooxygenase. This study may access to other aromatic *N*-oxide natural products that possess fascinating chemical structures and potent biological activities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01089.

Details of experimental procedures, construction of gene inactivation plasmids and verification of mutants, protein expression in *E. coli* and enzyme purification, reactions and activity assay, and spectroscopic data (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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