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Discovery of (Dihydro)pyrazine *N*-Oxides via Genome Mining in *Pseudomonas*

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Abstract

Overexpression of the *Pseudomonas virulence factor* (*pvf*) biosynthetic operon led to the identification of a family of pyrazine *N*-oxides (PNOs), including a novel dihydropyrazine *N,N'*-dioxide (dPNO) metabolite. The nonribosomal peptide synthetase responsible for production of (d)PNOs was characterized, and a biosynthetic pathway for (d)PNOs was proposed. This work highlights the unique chemistry catalyzed by *pvf*-encoded enzymes and sets the stage for bioactivity studies of the metabolites produced by the virulence pathway.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Detailed experimental procedures, supplemental figures, and spectral data (PDF)

The authors declare no competing financial interest.

Pseudomonas bacteria thrive in diverse environments and interact extensively with eukaryotic hosts and other microbes. These bacteria produce a variety of small molecules that possess unique structures and functions, including siderophores, phytotoxins, antibiotics, and quorum-sensing molecules. The genomes of these bacteria contain many uncharacterized biosynthetic gene clusters, including the *Pseudomonas* virulence factor (*pvf*) cluster. The *pvf* cluster was identified through genetic screens and is implicated in the virulence of the animal pathogen *Pseudomonas entomophila* L48 and the plant pathogen *Pseudomonas syringae* pv. *syringae* UMAF0158. Disruption of *pvfB*, *C*, and *D* in *P. entomophila* and *P. syringae* significantly decreases the virulence of these strains against *Drosophila* and tomato plants, respectively. The *pvf* cluster was also found to be important for the biocontrol activity of a *Pseudomonas fluorescens* strain. Additional studies found that the *pvf* cluster does not encode enzymes that synthesize toxins to directly harm the host, but rather synthesize signaling molecule(s) that regulate virulence factors, including the pore-forming toxin monalysin in *P. entomophila* and the phytotoxin mangotoxin in *P. syringae*.

The *pvf* operon contains four genes: a putative nonribosomal peptide synthetase (NRPS, *pvfC*), a putative diiron *N*-oxygenase (*pvfB*), and two genes of unknown function (*pvfA* and *pvfD*, Figure 1A, Table S1). NRPSs are a large family of multidomain enzymes that are responsible for the production of many bioactive natural products such as the antibiotics vancomycin and daptomycin and the virulence factor pyoverdine. PvfB shares homology with AurF and CmlI, which convert aryl amines into aryl nitro compounds in the biosynthesis of the antibiotics aureothin and chloramphenicol, respectively.

Homologues of *pvfA–D* were identified in *Burkholderia cenocepacia* H111 as part of the ham cluster (*hamA–G*, Figure S1), which was recently shown to be responsible for the synthesis of fragin, an antifungal molecule. Deletion of the fragin biosynthetic gene *hamF* that is absent in the *pvf* cluster led to the discovery of valdiazene, a valinol diazeniumdiolate. Valdiazene exhibits moderate signaling activity toward the *hamA* promoter reporter strain and was proposed to be the signaling molecule that autoregulates the expression of *ham* genes.

However, this study also found that valdiazene could not be identified in *pvf*-containing *Pseudomonas* strains that do not contain *hamF* or *hamG* homologues. Therefore, we propose *pvf*-encoded enzymes from *Pseudomonas* produce different bioactive molecule(s) than valdiazene and set out to identify these small molecules using *P. entomophila* as a model.

Through overexpression of the *pvf* operon, we identified and characterized a new family of natural products, (dihydro)-pyrazine *N*-oxides ((d)PNOs). We found that PvfC and PvfB are responsible for the biosynthesis of this unique family of compounds. With this evidence, we propose a biosynthetic pathway for (d)PNOs and suggest a shared biosynthetic intermediate for (d)PNOs and valdiazene.

Many bacterial small molecules, especially signaling molecules, are natively produced at low levels. To enhance the production of small molecules made by *pvf*-encoded enzymes, we engineered an inducible expression system for the *pvf* cluster and identified small molecules unique to *pvf*-expressing strains. The expression plasmid containing *pvfABCD* under an IPTG-inducible lacUV5 promoter was introduced into a *P. entomophila* mutant from which the NRPS gene *pvfC* has been deleted (*pvfC*⁻), generating the native *pvf* overexpression strain (*pvfC*⁻ + pPSV-*pvfABCD*). The culture supernatants of this *pvf* expression strain and the *pvfC*⁻ control were extracted using dichloromethane. The extracts were analyzed using liquid chromatography-coupled high-resolution mass spectrometry (LC-HRMS). Comparison of the metabolic profiles revealed several unique compounds. Three major metabolites are present at high levels in the extract of *pvfC*⁻ + pPSV-*pvfABCD*, but are absent in the extract of the *pvfC*⁻ control: **1** ($[M+H]^+$, 199.145 *m/z*, C₁₀H₁₈N₂O₂), **2** ($[M+H]^+$, 197.126 *m/z*, C₁₀H₁₆N₂O₂), and **3** ($[M+H]^+$, 181.135 *m/z*, C₁₀H₁₆N₂O) (Figures 1B, S2). The *pvf* plasmid was also introduced into *Pseudomonas aeruginosa* PAO1, which does not natively harbor the *pvf* cluster, yielding a heterologous *pvf* expression strain (PAO1 + pPSV-*pvfABCD*). Metabolites **1–3** were also detected in the organic extract of PAO1 + pPSV-*pvfABCD*, but not in the extract of the control strain carrying the empty expression vector (Figures 1B, S2). These results indicate that **1**, **2**, and **3** are synthesized by the *pvf*-encoded enzymes.

We isolated **1**, **2**, and **3** from 8 L cultures of *pvfC*⁻ + pPSV-*pvfABCD* for structure elucidation. The major metabolites were isolated at a yield of 5 mg/L for **1** and 1–2 mg/L for **2** and **3**. The structures of these metabolites were analyzed and characterized using a combination of NMR, UV, and IR spectroscopies (Figure 2, Table 1, Tables S4–S5, Figures S3–S22).

The ¹H spectrum in conjunction with the molecular formula indicates that **2** is symmetric. The COSY correlations between six methyl protons and a single multiplet proton indicate the presence of an isopropyl group (Figure 2). The shift at δ_H 8.09 and UV spectra suggest a heterocyclic pyrazine core (Table S4, Figures S3, S13). The oxygens are likely attached to pyrazine as *N*-oxides. Together, the structure of **2** was assigned as 2,5-diisopropylpyrazine *N,N'*-dioxide (PNO A).

The chemical formula of **3** contains one less oxygen than **2**, and the NMR spectrum suggests that **3** is asymmetric. The ¹H shifts of **3** are largely similar to those of **2**, suggesting **3**

possesses a related structure but contains a single *N*-oxide. The COSY, (¹H, ¹³C)-HMBC, and (¹H, ¹⁵N)-HMBC NMR correlations support the pyrazine core and were used to assign the isopropyl groups to the 2,5 positions of the pyrazine (Figure 2, Table S5). The structure of **3** was assigned as 2,5-diisopropylpyrazine *N*-oxide (PNO B). The ¹H and ¹³C shifts of PNO A and PNO B are consistent with the shifts reported for these compounds, which have been previously synthesized.[–]

The chemical formula indicates that **1** has one less degree of unsaturation than **2** and **3**, which may be due to hydration within the pyrazine ring. The ¹H NMR spectrum of **1** contains an additional multiplet proton at 63.89 and two diastereotopic protons at 64.16 and 64.49 (Table 1). The connectivity of these atoms was determined based on COSY, (¹H, ¹³C)-HSQC, (¹H, ¹³C)-HMBC, and (¹H, ¹H)-TOSCY NMR correlations. These correlations and proton splitting patterns support the presence of a 2,3-dihydropyrazine core, and the (¹H, ¹⁵N)-HMBC correlations indicate the positions of 2,5-diisopropyl groups (Table 1, Figure 2). Thus, the structure of **1** was assigned as 2,5-diisopropyl-2,3-dihydropyrazine *N,N'*-dioxide (dPNO).

The structure of **1** contains two conjugated nitrones and is highly unusual. We conducted UV absorbance and IR spectroscopy to further characterize this molecule. We found that **1** exhibits two UV absorbance maxima at 219 and 348 nm (Figure S3) and distinctive IR peaks at 1564, 1435, and 1398 cm^{–1} (Figure S5), which is consistent with the reported UV maxima in the 345–370 nm range and strong IR bands at 1530–1570 cm^{–1} and 1440–1470 cm^{–1} for synthetic dinitrone compounds.[–] These results further support the proposed structure for **1**. The stereochemistry at C₂ remains to be determined.

We synthesized **2** and **3** to confirm the proposed structures and obtain materials for biological studies (Scheme S1). Products **2** and **3** were synthesized starting with L-valinol to yield a 2,5-diisopropylpyrazine intermediate that was oxidized to yield **2** and **3**. The ¹H and ¹³C NMR and UV spectra of synthetic **2** and **3** are consistent with previous reports[–] and are nearly identical to the spectra for **2** and **3** isolated from *P. entomophila* (Figures S23–27, Tables S4–S5), confirming the structural assignments.

We conducted biochemical and genetic studies to characterize the biosynthesis of (d)PNOs. Overexpression of different combinations of *pvf* genes revealed that *pvfB* and *pvfC* are necessary and sufficient for the production of (d)PNOs (Figure S28). The NRPS PvfC contains three domains: adenylation (A), thiolation (T), and reductase (R) (Table S1, Figure 3A). The adenylation activity of PvfC was investigated *in vitro* using a ATP-[³²P]PP_i exchange assay. PvfC selectively activates L-Val among the 20 proteinogenic amino acids (*K*_M = 3.4 ± 0.7 mM, *k*_{cat} = 3.4 ± 0.3 s^{–1}, Figures 3A and B, S29). The observed *K*_M is 2–3-fold higher than that of typical NRPS adenylation domains, but is generally consistent with the mM cellular concentration of L-Val in bacteria. The incorporation of Val was also investigated in cells using isotopically labeled Val ([²H_{1–8}]DL-Val). An increase of +7 and +14 (and +15 for **1**) was observed for **1**, **2**, and **3** (Figures 3C, S30), when the overexpression cultures were supplemented with [²H_{1–8}]DL-Val, indicating the incorporation of one or two [²H_{1–8}]DL-Val. This result suggests that L-Val is the physiological substrate for PvfC and is likely the precursor for (d)PNOs.

A PvfB homologue from *P. syringae* pv. *phaseolicola* was previously reported to convert *p*-aminobenzoic acid to *p*-nitrobenzoic acid. However, neither (d)PNOs nor valdiazene contains a *p*-nitrobenzoic acid. Thus, *p*-aminobenzoic acid may not be the physiological substrate for PvfB. The structures of (d)PNOs and valdiazene suggest that a *N*-hydroxy-L-Val derivative exists as a precursor to both sets of molecules. PvfB may install the hydroxylamine on PvfC-linked L-Val instead.

We propose a biosynthetic pathway for (d)PNOs (Figure 4). First, PvfC activates and loads L-Val onto the T domain. The amine of the tethered L-Val is oxygenated by PvfB to generate a hydroxylamine. A two-electron reduction of the *N*-hydroxy-L-Val linked to the T domain by PvfC R domain releases the intermediate as an aldehyde. Two molecules of this aldehyde cyclize and dehydrate to create the 2,4-dihydropyrazine *N,N'*-dioxide. Isomerization forms dPNO, while aromatization forms PNO A. Production of the monoxide PNO B may result from transformation from dPNO (Figure S31) or cyclization between a *N*-hydroxy-L-Val aldehyde and a L-Val aldehyde. Valdiazene produced by *Burkholderia* may result from a four-electron reduction of the T-domain-linked *N*-hydroxy-L-Val to generate *N*-hydroxy-L-valinol. This molecule can be further modified to the diazoniumdiolate (Figure 4).

We evaluated the activity of (d)PNOs to restore the virulence of the *pvfC* mutant using a fly infection assay. Cultures of the *pvfC* mutant were supplemented with isolated **1**, isolated or synthetic **2**, or isolated or synthetic **3**. After 24 h of growth, these cultures were concentrated and fed to adult female flies. These molecules do not restore the virulence of *pvfC* against adult flies individually or combined (Figure S32), while the extracts of the *P. entomophila* wildtype culture restored the virulence of *pvfC* to wild type levels. Furthermore, the biosynthesis of (d)PNOs does not require PvfD (Figure S28). Previously, *pvfD* and a homologue were shown to be essential for signaling activity. Together, the evidence suggests that (d)PNOs are potential biosynthetic intermediates or shunt products of the *pvf* pathway. As some signaling pathways require the synergistic action of multiple metabolites, additional metabolites produced by *pvf*-encoded enzymes may be necessary for the full signaling activity. Identifying the additional metabolites required for the signaling activity will be the focus of future studies.

Although (d)PNOs do not restore the signaling activity of *pvfC*, their unique structures suggest other potential activities toward microbes or multicellular organisms. Volatile pyrazines are well-established pheromones for insects and are distributed in environmental niches ranging from *Pseudomonas* spp. on wine corks to bacteria associated with leaf cutter ants. Recently, a 3,5-dimethylpyrazin-2-ol was identified as a quorum-sensing molecule for *Vibrio cholerae*. In regard to the *N*-oxides, only a few examples have been identified in natural products, such as 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) and iodinin (1,6-dihydroxy phenazine *N,N'*-dioxide), which possess signaling, antimicrobial, and redox cycling activities. The *N*-oxide derivatives of pyrazines are rare; in particular, the 2,3-dihydropyrazine *N,N'*-dioxide moiety of **1** is unprecedented in natural products, to the best of our knowledge. Unlike iodinin, dPNO does not have connected aromatic rings, yet it is stable at the pH range 1–11 (Figure S33) and for several months at room temperature (less than 15% was converted to PNO B after one year in storage, Figure S31). Attempts to synthesize dPNO have been unsuccessful; however, overexpression of *pvf* allows the

isolation of this unique compound in relatively high yields for further exploration of its chemical properties and biological activities.

We have identified a new biosynthetic pathway for pyrazine derivatives involving a noncanonical NRPS, PvfC, and an iron-dependent *N*-oxygenase, PvfB. The A-T-R domain structure of PvfC resembles two fungal NRPSs, one involved in a general biosynthetic pathway for lysine and the other specific for the biosynthesis of a dityrosyl-pyrazine in *Aspergillus flavus*. Interestingly, a *N,N'*-dioxide species of dityrosyl-pyrazine has also been isolated, but no PvfB homologue was found in the gene cluster and the biosynthetic origin of this *N*-oxidation is unknown. Reactions conducted by PvfC and PvfB and their homologues provide essential chemistries for divergent biosynthesis of a variety of structures, including diazeniumdiolates in fragin and valdiazene and (dihydro)pyrazine *N*-oxides.

In summary, through genome-mining in *Pseudomonas* and overexpression of the *pvf* gene cluster, we have identified and characterized a family of pyrazine *N*-oxides, including a novel dihydropyrazine *N,N'*-dioxide metabolite. This work identifies the role of a noncanonical NRPS in the biosynthesis of pyrazine derivatives and suggests a common biosynthetic origin for two families of molecules. Our work sets the stage for understanding the biosynthesis and bioactivity of (d)PNOs and identifying new molecules involved in *Pseudomonas* signaling and virulence.

Supplementary Material

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Figure 1.

Identification of new metabolites produced by *pvf*-encoded enzymes using comparative metabolomics. (A) The *pvf* biosynthetic cluster from *P. entomophila*. (B) Overexpression of *pvfABCD* on a pPSV35 vector in *P. entomophila* *pvfC* (green) led to the production of three new metabolites 1–3, which are absent from *P. entomophila* *pvfC* (red). These metabolites were also identified when *pvfABCD* was heterologously expressed in *P. aeruginosa* PAO1 (blue).

Figure 2. Structural characterization of dPNO (1), PNO A (2), and PNO B (3). COSY correlations are indicated by bonds in bold, and key (¹H, ¹⁵N)-HMBC and (¹H, ¹³C)-HMBC correlations are indicated by red and blue arrows, respectively.

Figure 4. Proposed biosynthesis of (dihydro)pyrazine *N*-oxides in *P. entomophila* L48 and valdiazene in *B. cenocepacia* H111.

Table 1.

NMR Assignments and Correlations for dPNO (1)

position	δ_C	proton	δ_H (multiplicity, J [Hz])	COSY	$(^1H, ^{13}C)$ HMBC	δ_N	$(^1H, ^{15}N)$ HMBC
1-N						-94 (286)	
2	72.0	CH	3.89 (td, [6.5, 4.4])	3a, 3b	6, 10, 11, 12		1, 4
3a, b	57.7	CH ₂	4.49 (dd, [15.9, 6.8]), 4.16 (dd, [15.9, 4.4])	2, 3b, 3a	2, 5, 6, 10		1, 4
4-N						-121 (259)	
5	145.2						
6	126.8	CH	7.39 (s)		2, 5, 7		1, 4
7	26.4	CH(CH ₃) ₂	3.42 (hept, [7.0])	8, 9	5, 6, 8, 9		4
8, 9	18.4, 18.7	CH ₃	1.15 (d, [7.0])	7	5, 7, 8, 9		
10	28.9	CH(CH ₃) ₂	2.48 (m, [13.5, 6.8])	11, 12	2, 3, 11, 12		1
11	19.0	CH ₃	1.09 (d, [7.1])	10	2, 10, 12		
12	17.7	CH ₃	1.03 (d, [6.8])	10	2, 11, 10		