

# OxaD: A Versatile Indolic Nitronone Synthase from the Marine-Derived Fungus *Penicillium oxalicum* F30

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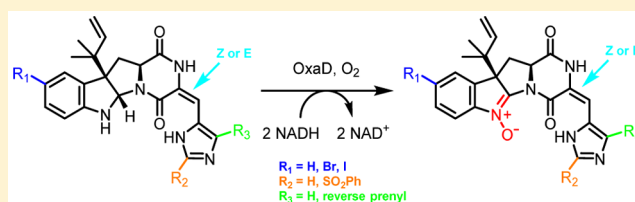
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## Supporting Information

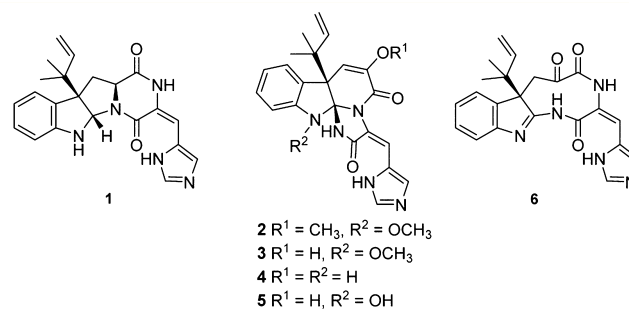
**ABSTRACT:** Indole alkaloids are a diverse class of natural products known for their wide range of biological activities and complex chemical structures. Rarely observed in this class are indolic nitrones, such as avrainvillamide and waikialoid, which possess potent bioactivities. Herein the *oxa* gene cluster from the marine-derived fungus *Penicillium oxalicum* F30 is described along with the characterization of OxaD, a flavin-dependent oxidase that generates roquefortine L, a nitronone-bearing intermediate in the biosynthesis of oxaline. Nitronone functionality in roquefortine L was confirmed by spectroscopic methods and 1,3-dipolar cycloaddition with methyl acrylate. OxaD is a versatile biocatalyst that converts an array of semisynthetic roquefortine C derivatives bearing indoline systems to their respective nitrones. This work describes the first implementation of a nitronone synthase as a biocatalyst and establishes a novel platform for late-stage diversification of a range of complex natural products.



## INTRODUCTION

Prenylated indole fungal alkaloids constitute a structurally complex class of natural products, for which potent antibacterial, anticancer, antiparasitic, and insecticidal activities have been reported.<sup>1,2</sup> These alkaloids are typically derived from a tryptophan–proline diketopiperazine skeleton, with a prenyl group frequently attached in the reverse orientation to the indole moiety. The biosynthesis of fungal prenylated indole alkaloids involves a multistep cascade of reactions beginning with a nonribosomal peptide synthetase (NRPS)-catalyzed assembly of the diketopiperazine core. Subsequent sets of enzymes catalyze various oxidative/reductive reactions, C–C coupling, rearrangements, and in some cases, Diels–Alder reactions.<sup>1,2</sup>

Roquefortines, glandicolines, meleagrins, and oxaline comprise a select group of biosynthetically related prenylated indole alkaloids (Figure 1). The archetypic roquefortine C (1) was first isolated from *Penicillium roqueforti* and has been detected in over 30 different *Penicillium* species.<sup>3–5</sup> Oxaline (2) and meleagrins (3) have been less frequently isolated from fungal cultures.<sup>6,7</sup> Roquefortine C (1) is found in low levels in industrially produced blue cheese,<sup>8</sup> grain,<sup>9–12</sup> and animal feedstocks,<sup>13</sup> and while cytotoxic effects of varying degrees have been observed in mice<sup>14,15</sup> and cockerels,<sup>16</sup> roquefortine C



**Figure 1.** Roquefortine C (1) and derived alkaloids oxaline (2), meleagrins (3), glandicolines A (4), and glandicolines B (5). 1,4-Diazonane-2,5,6-trione (6) was proposed by Steyn and Vlegaar<sup>22</sup> as an intermediate in the biosynthesis of 4.

has shown little to no cytotoxicity in a number of human cell lines.<sup>12,17,18</sup> Roquefortine C is known to bind to iron, inactivating hepatic cytochrome P450s<sup>19</sup> and inhibiting growth of Gram-positive bacteria containing heme proteins.<sup>20,21</sup> Roquefortine C may also be significant in indoor air

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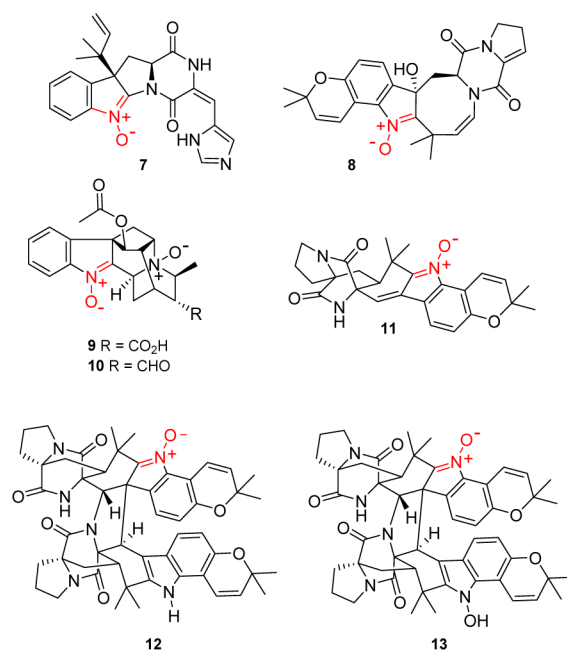
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contamination as it has been isolated from numerous mold-contaminated indoor environments. Inflammatory responses have been reported for mouse lungs intratracheally instilled with roquefortine C.<sup>14,23–25</sup> Despite their threat to human and animal health, as well as the economic loss due to food contamination by these compounds,<sup>26</sup> **1–3** have also attracted interest as potential therapeutics. Recent reports indicate that **3** is a promising lead for the treatment of breast cancer,<sup>27</sup> while meleagrins B, a diterpene-substituted biosynthetic derivative of **3**, was shown to induce HL-60 cell apoptosis.<sup>28</sup> Additionally, **3** has demonstrated inhibition of bacterial type II fatty acid synthesis, while **2** has been shown to inhibit tubulin polymerization in Jurkat cells.<sup>29,30</sup>

Oxaline (**2**) and meleagrins (**3**) feature a triazaspirocyclic skeleton derived from an unusual rearrangement of the diketopiperazine core of **1**.<sup>22</sup> The first proposed mechanism for the biosynthesis of **2** from **1** included the formation of 1,4-diazonane-2,5,6-trione intermediate **6** as the key step, prior to the transannular attack of the indole by the histidine-derived  $\alpha$ -nitrogen, leading to the formation of the trinitrogen-substituted core in **2** and **3**.<sup>22</sup> Experiments performed with cultures of *Penicillium glandicola*, *Penicillium atramentosum*, and *Penicillium farinosum* provided additional evidence that glandicolines A (**4**) and B (**5**), meleagrins (**3**), and oxaline (**2**) are derived from roquefortine C (**1**).<sup>31</sup>

Whole genome sequencing, analysis, and substrate modeling with *Penicillium chrysogenum* Wis54–1255 enabled the determination of the gene cluster that encodes the biosynthesis of **1**.<sup>32</sup> Open reading frames (ORFs) within the roquefortine–meleagrins gene cluster in the *P. chrysogenum* genome have been subjected to a series of RNA silencing experiments, which enabled a putative biochemical assignment for each step of the biosynthetic pathway.<sup>33</sup> Transformation of roquefortine C (**1**) into glandicoline A (**4**) is promoted by a MAK1-like monooxygenase RoqM (encoded by *Pc21g15460*), as evidenced by loss of production of **3–5** upon gene silencing. Subsequent gene deletion analysis of the *P. chrysogenum* strain DSS4555 roquefortine C gene cluster<sup>34</sup> confirmed the results obtained by gene silencing,<sup>33</sup> leading to a mechanism identical to the one previously proposed by Steyn and Vlegaar.<sup>22</sup> More recently, a rigorous structural elucidation of the metabolites of this pathway prompted a revision of the biosynthetic mechanism for this particular rearrangement.<sup>35</sup> The key revision to this newly proposed metabolic pathway is the generation of the nitron-bearing intermediate roquefortine L (**7**) by RoqM from **1** (Figure 2). Roquefortine L (**7**) has a molecular weight identical to that of glandicoline A (**4**), so it was proposed that **7** had been mistakenly characterized as **4** in the initial gene silencing and gene deletion studies. Cytochrome P450 monooxygenase RoqO (encoded by *Pc21g15450*) was proposed to catalyze the rearrangement of **7** to give the triazaspirocyclic core of glandicoline B (**5**) as deletion of this gene resulted in loss of production of **5** and **3**.

Fungal production of roquefortine L (**7**) is particularly relevant as very few indoline nitrones are known as natural products.<sup>36–40</sup> With the exception of the recently isolated versicamide E (**8**), alstoyunine D (**9**), and perakine  $N^1, N^4$ -dioxide (**10**), the remaining fungal indolic nitrones share a common avrainvillamide (**11**) core (Figure 2). Nitron incorporation into these compounds can promote several biologically relevant events. For example, the nitron functional group on avrainvillamide is required for dimerization to stephacidin B (**13**).<sup>41</sup> The nitron function of avrainvillamide



**Figure 2.** Known indolic nitrones. Roquefortine L (**7**) is a labile intermediate in the biosynthesis of **2**. Indolic nitron bearing natural products versicamide E (**8**), alstoyunine D (**9**), perakine  $N^1, N^4$ -dioxide (**10**), avrainvillamide (**11**), waikialoid A (**12**), and stephacidin B (**13**).

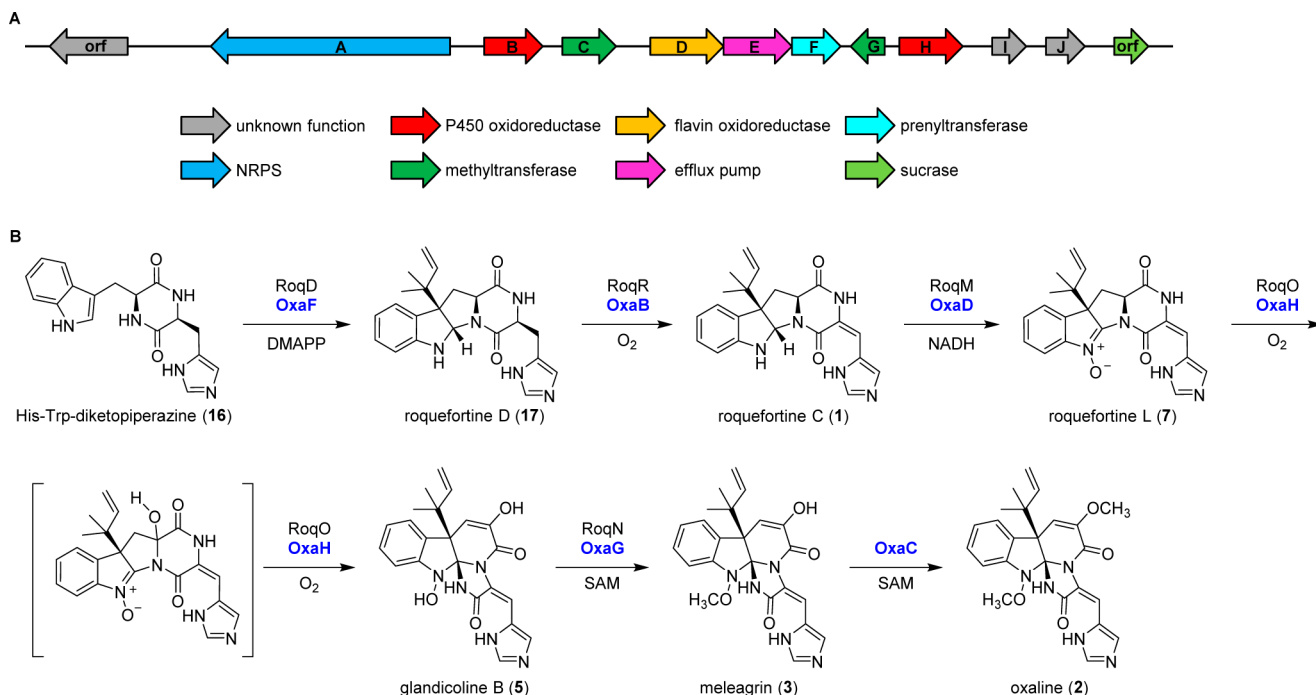
also plays a vital role in its antiproliferative activity, with the proposed mechanism of antiproliferation involving nucleophilic attack of the nitron functionality by cysteine residues in cellular proteins.<sup>40,42–44</sup> Such properties generate great interest in the chemical synthesis of these molecules.<sup>45–47</sup>

Chemical conversion of indolines into their respective nitron has been reported in only a few instances. It requires either mild oxidative conditions to give very moderate yields<sup>46,48</sup> or a multistep procedure affording good yield.<sup>49</sup> Discovery of a biocatalyst that could efficiently perform such a reaction in a single high-yield step would be of major interest for the synthesis of nitron-functionalized indolines. Previous accounts of enzymatic nitron formation have focused on metabolism of xenobiotic secondary amines and hydroxylamines by hepatic microsomal enzymes;<sup>50–57</sup> however, in the majority of these instances, nitron formation was accompanied by a number of additional oxidative side reactions. Therefore, the complex and biologically active indole alkaloids remain important targets for this type of functionalization.

We herein report the isolation, characterization, and enzymatic activity of OxaD, which can be produced in significant yields by heterologous expression. OxaD is a flavoprotein that has been harnessed for the biocatalytic conversion of roquefortine C and roquefortine C semisynthetic derivatives to afford the resulting indoline nitrones in very good yield and in a single step. To the best of our knowledge, this work represents the first biocatalytic synthesis of indoline nitrones.

## RESULTS

**Oxaline Biosynthetic Gene Cluster.** To investigate the biogenesis of oxaline in mechanistic detail, we sequenced the genome of *Penicillium oxalicum* F30 and performed gene cluster analysis using antiSMASH followed by a deep annotation of the oxaline biosynthetic pathway (GenBank: KX601657; Scheme

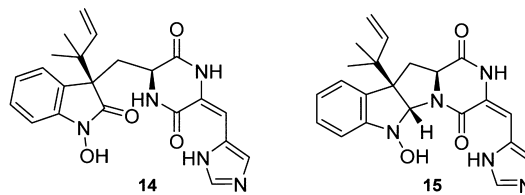
Scheme 1. Oxaline Biosynthetic Gene Cluster<sup>a</sup>

<sup>a</sup>(A) Schematic representation of the ORFs in the oxaline (*oxa*) gene cluster. (B) Proposed biosynthetic pathway in *P. oxalicum* F30. The homologous enzymes from the roquefortine C/meleagrins (*roq*) gene cluster in *P. chrysogenum* are shown. A second methyltransferase, OxaC, lacks a homologue in *P. chrysogenum*.

1A and Table S1). The composition of the oxaline (*oxa*) gene cluster is highly similar to that of the roquefortine/meleagrins (*roq*) cluster from *P. chrysogenum*<sup>33</sup> (Table S2), with an average 78.4% similarity between homologous ORFs in the respective systems. One exception is a second methyltransferase (OxaC), which is absent in *P. chrysogenum*, suggesting that this enzyme is responsible for the enol *O*-methylation that distinguishes 2 from 3. By homology, we reason that the initial biosynthetic steps toward 2 match those previously reported for 3 (Scheme 1B).<sup>34,58,59</sup> Briefly, the first biosynthetic intermediate His-Trp diketopiperazine (HTD, 16) is generated by an NRPS-mediated condensation of the corresponding L-amino acids. HTD undergoes reverse prenylation at C3 catalyzed by OxaF and dehydrogenation catalyzed by OxaB to give 1. The flavin oxidase, OxaD, and the P450 monooxygenase, OxaH, are homologous to *P. chrysogenum* RoqM and RoqO, respectively. RoqM and RoqO have been proposed to generate core intermediate 5 from 1 by proceeding through intermediate 7, as identified by Ries et al.<sup>35</sup> The conversion of 7 to 5 is proposed to arise from a P450-dependent hydroxylation catalyzed by OxaH, although this transformation has not been observed *in vitro*. Two *O*-methylations by OxaG and OxaC generate final product 2 (manuscript in preparation).

**OxaD Is a Flavin-Dependent Nitron Synthase.** OxaD was cloned from a *P. oxalicum* F30 cDNA library and heterologously expressed in *Escherichia coli*. The purified enzyme has an intense yellow color. HPLC-MS analysis of the supernatant of boiled OxaD shows near-quantitative incorporation of flavin adenine dinucleotide (FAD) (Figure S1). To obtain sufficient quantities of starting substrate 1, a fermentation-based isolation using *Penicillium crustosum* was developed.<sup>60</sup> Gram quantities of pure 1 were obtained for enzymatic testing and chemical derivatization. Both NADH and

NADPH were shown to be effective in the oxidation of 1 by OxaD. Incubation of OxaD with 1 and reduced nicotinamide cofactor resulted in the formation of several products (Figure S2). After 4 h of incubation of 1 with OxaD, HPLC-MS analysis (50:50 MeOH/H<sub>2</sub>O) showed peaks with molecular weights corresponding to nitron 7, hydrated product 14, and a presumed MeOH adduct. Low levels of the hydroxylamine 15 (Figure 3) were also detected. Preparative-scale enzymatic

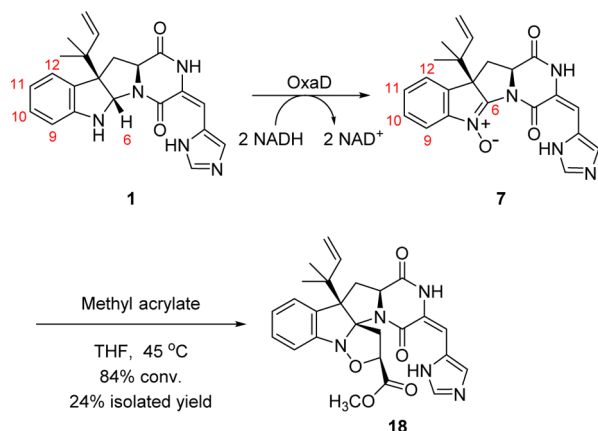


**Figure 3.** Reactions with OxaD and 1 led to the detection of a hydroxylamine intermediate 15 and the hydrolysis product 14.

reactions were conducted using 1 (20 mg) in order to characterize the major reaction product. Reverse-phase C18 chromatography led exclusively to hydrolysis product 14, so subsequent reactions were designed to minimize the exposure of 7 to aqueous conditions, acidic or basic pH, and light. Biocatalytic reactions were performed for 1 h prior to extraction with EtOAc and normal phase chromatography purification on deactivated silica gel in the absence of small alcohols. These conditions led to 7 in a single step in good yield. The presence of the nitron functionality in 7 was confirmed by analysis of <sup>1</sup>H NMR, <sup>13</sup>C NMR, and IR spectra. Additionally, *P. crustosum* was cultured on <sup>15</sup>N-enriched medium to give <sup>15</sup>N-labeled 1 (86% incorporation, Table S4). This material was subjected to the same reaction conditions as 1 to provide <sup>15</sup>N-labeled 7,

which was characterized by  $^{15}\text{N}$  NMR. Comparison with the spectrum for labeled **1** clearly demonstrated the presence of a nitron at N1 in roquefortine L (**7**) (Figure S16–S17). A final confirmation of the nitron functionality in roquefortine L (**7**) resulted from its 1,3-dipolar cycloaddition with methyl acrylate (Scheme 2 and *vide infra*).

### Scheme 2. Roquefortine L (**7**) Undergoes 1,3-Dipolar Cycloaddition with Methyl Acrylate to Generate **18**

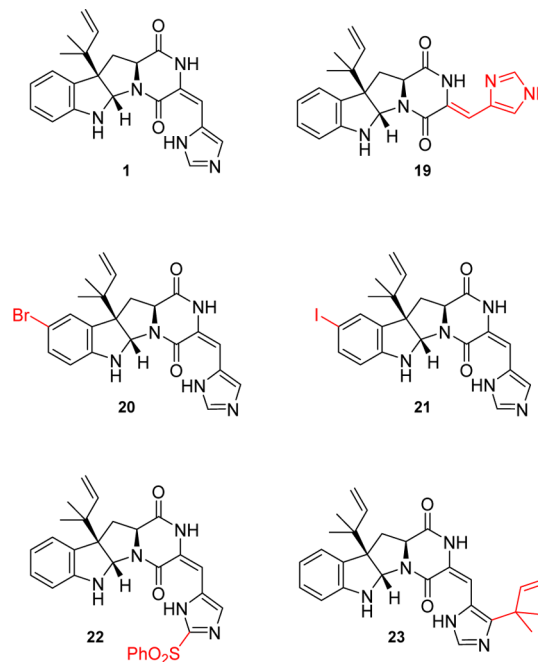


**Investigating the Mechanism of Oxidation.** The formation of hydroxylamine **15** (Figure 3) led us to investigate the mechanism of roquefortine C (**1**) oxidation by OxaD. The full catalytic cycle encompasses a four-electron oxidation, in which 2 equiv of reduced nicotinamide cofactor (NADH) are consumed en route to the nitron product (Figure S3). Therefore, a single turnover experiment was performed, in which only 1 equiv of NADH was provided to equimolar concentrations of OxaD and roquefortine C. Analysis of the reaction products showed an approximate 1:2 ratio of singly oxidized hydroxylamine **15** species relative to the doubly oxidized nitron product (Figure S3), conclusively demonstrating that the reaction proceeds through a hydroxylamine intermediate. The abundance of **15** with respect to **7** under single-turnover conditions also suggested the possibility that oxidation by OxaD is catalyzed in an iterative rather than a processive manner. To further support this proposal, single-turnover reactions were performed using a 10-fold stoichiometric excess of roquefortine C with respect to NADH and OxaD in an effort to preclude rebinding of the dissociated hydroxylamine species. Under these conditions, **15** was the dominant reaction product (Figure S3), further supporting an iterative mechanism for OxaD. This iterative *N*-oxidation mechanism has been previously reported with human FMO3 using various substrates.<sup>61,62</sup> The steady-state kinetic constants of OxaD with roquefortine C were determined using an HPLC-based assay. These data show a  $k_{\text{cat}}$  of  $0.017\text{ s}^{-1}$  with a  $K_{\text{M}}$  of 71 nM resulting in a catalytic efficiency ( $k_{\text{cat}}/K_{\text{M}}$ ) of  $2.3 \times 10^5\text{ M}^{-1}\cdot\text{s}^{-1}$  (Figure S4).

**Roquefortine C Nitron Undergoes 1,3-Dipolar Cycloaddition.** As a means to further validate the enzymatic production of **7** and to explore indoline nitrones as a handle for further indole alkaloid diversification, **7** was screened with known dipolarophiles for 1,3-dipolar cycloaddition reactions. Methyl acrylate showed the best reactivity toward **7**. Optimized reaction conditions generated cycloadduct **18** in a single step with 84% conversion and 24% yield (Scheme 2). The observed

regiochemistry of this reaction is consistent with predictions based on frontier molecular orbital interactions.<sup>63</sup>

**Roquefortine C Derivatives Are Converted to Nitrones by OxaD.** To explore the substrate scope of OxaD, roquefortine C derivatives **19–23** were assayed under the same conditions as described above for native substrate **1**. Compounds **19–23** were generated by semisynthesis from **1** (Figure 4). Iso-roquefortine C (**19**) was obtained by photo-

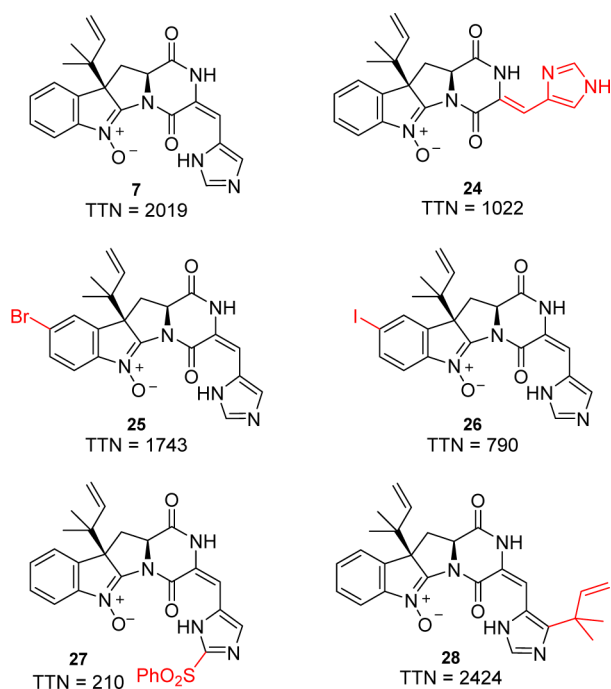


**Figure 4.** Semisynthetic roquefortine C derivatives. Modifications to the roquefortine C scaffold are highlighted in red.

chemical reaction of **1** using broad spectrum UV light.<sup>64</sup> Electrophilic aromatic substitution of **1** using *N*-bromosuccinimide (NBS) and *N*-iodosuccinimide (NIS) yielded 11-bromo-roquefortine C (**20**)<sup>65</sup> and 11-iodo-roquefortine C (**21**), respectively. Phenylsulfonyl-roquefortine C (**22**) was prepared using *N*-fluorobenzenesulfonimide (NFSI) as a phenylsulfonyl group transfer reagent.<sup>66</sup> Roquefortine E (**23**) was purchased from Enzo (Ann Arbor, MI). Processing **19–23** to their corresponding nitron products (**24–28**) demonstrated that OxaD can accommodate variations at several positions in the roquefortine C skeleton.

Total turnover numbers (TTN) were measured in order to quantify the effect of these variations on the OxaD biocatalyst (Figure 5). Modifications to the histidine moiety, including both alkene isomerization (**24**) and reverse prenylation (**28**), were well-tolerated. Only the phenylsulfonyl derivative (**22**) showed a substantial decrease in TTN. To test whether the diminished turnover of **22** was observed due to the poor solubility of this substrate, TTNs were measured using both 10 and 20% DMSO cosolvent. While elevated cosolvent increased the solubilization of **22**, only a modest increase in TTN was observed, likely indicating that the benefit was offset by attenuated enzymatic activity. Modifications to the indole ring via halogenation were also processed efficiently by OxaD.

**OxaD Catalyzes Indole 2,3-Oxidation of Notoamide S.** On the basis of the broad reactivity that OxaD displayed with roquefortine C derivatives, additional indole alkaloids were subjected to the enzyme to examine whether this substrate



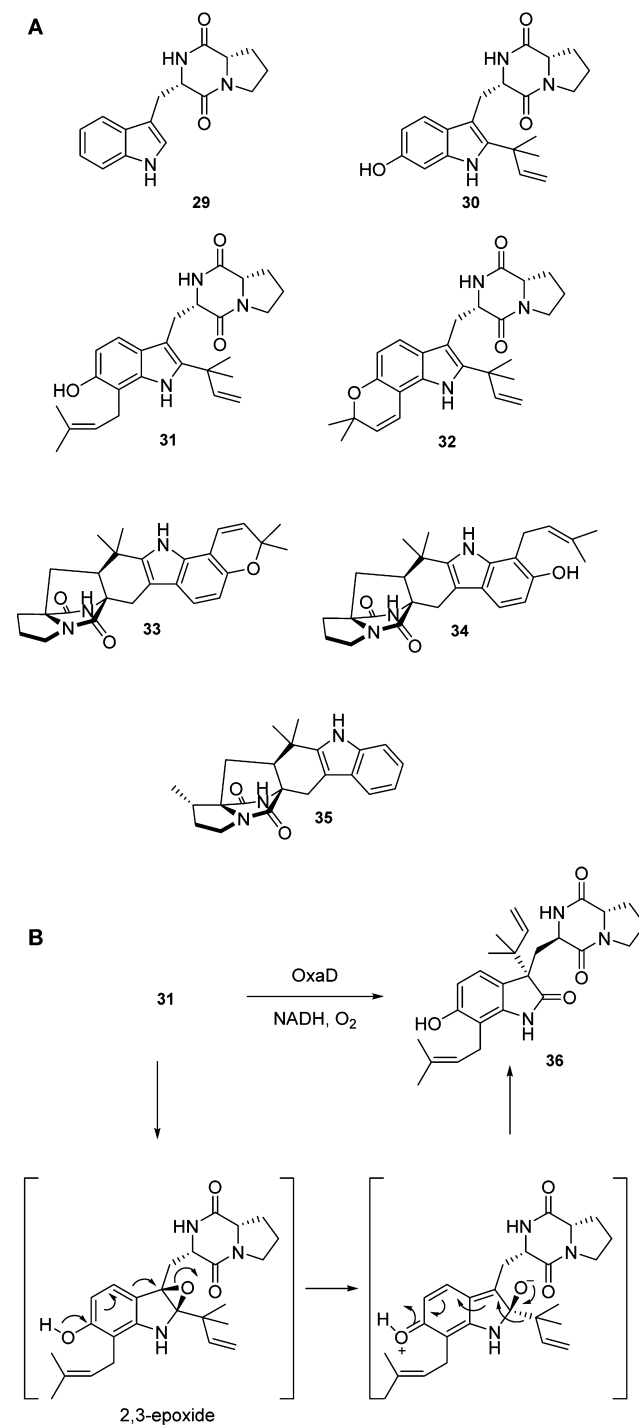
**Figure 5.** Modifications to the native substrate **1** at several positions were processed by OxaD. TTN was calculated as moles product/moles enzyme.

flexibility extended beyond the roquefortine C scaffold. These included brevianamide F (**29**), 6-OH-deoxybrevianamide E (**30**), notoamide S (**31**), notoamide E (**32**), (+)-stephacidin A (**33**), notoamide T (**34**), and preparaherquamide (**35**) (Scheme 3A). Of particular interest was **33**, a presumed biosynthetic precursor to the nitron-bearing avrainvillamide (**11**);<sup>67</sup> however no reaction was observed for substrates **29**, **30**, and **32–35**. Enzyme-dependent generation of a new product with  $m/z +16$  Da was uniquely observed by LC-MS with **31**. The total conversion (20%) was lower than that of the roquefortine C alkaloids. Biocatalytic reaction of **31** with OxaD yielded **36** (Scheme 3B), rather than the expected indole *N*-hydroxylamine. Product **36** is presumed to arise via a pinacol-like rearrangement, following 2,3-epoxidation in an analogous fashion to the NotB-catalyzed formation of notoamides C (**37**) and D (**38**) (Scheme 4).<sup>68</sup>

## DISCUSSION

While the biosynthesis of roquefortine C derived alkaloids **1–5** has been the subject of investigations employing genetic approaches,<sup>33–35</sup> enzymatic characterization relating to assembly and processing steps is completely lacking for these indole alkaloid pathways. By sequencing the *P. oxalicum* F30 genome, we have identified the oxaline biosynthetic gene cluster, which is homologous to the melegarin system.<sup>33</sup> Large-scale heterologous expression of indoline nitron synthase OxaD enabled its complete enzymatic characterization. OxaD catalyzes the key oxidation steps leading to the conversion of **1** to **5**. It is a robust biocatalyst for nitron installation on the wild-type substrate roquefortine C as well as a range of structurally related alkaloids. Large quantities of **1**, obtained by fermentation of *P. crustosum*, enabled semisynthetic preparation of roquefortine C analogues and preparative-scale biocatalytic reactions with these substrates. *P. crustosum* produced **1** for a fraction of the cost of total synthesis<sup>69</sup> and promotes green

## Scheme 3. OxaD Reactivity with Notoamides<sup>a</sup>

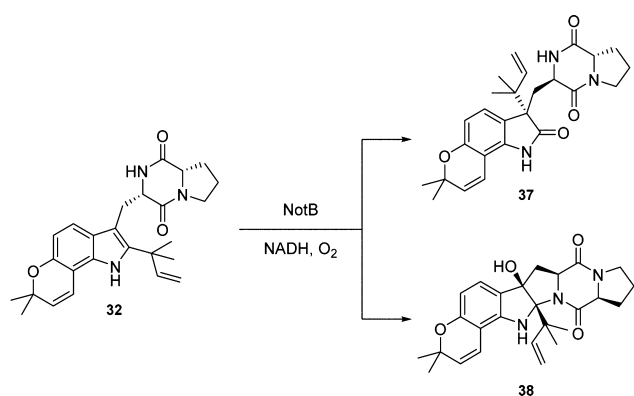


<sup>a</sup>(A) Synthetic notoamides tested with OxaD. Only **31** was converted by the enzyme. (B) Pinacol-like rearrangement of the OxaD-catalyzed 2,3-indole epoxidation on **31** gives rise to the novel product **36**

chemistry practices by avoiding the use of large quantities of organic solvents and unnecessary derivatizations.

Identification of nitrones has long been a challenge with regard to their differentiation from other products of *N*-oxidation, such as imines, amine oxides, or oxaziridines. Nitrones undergo 1,3 dipolar cycloadditions with dipolarophiles such as alkenes, alkynes, and nitriles, so roquefortine L (**7**) was subjected to 1,3-dipolar cycloaddition with methyl acrylate to give **18**, in agreement with the frontier molecular

**Scheme 4. NotB-Catalyzed Formation of Notoamides C (37) and D (38) from Notoamide E (32)**



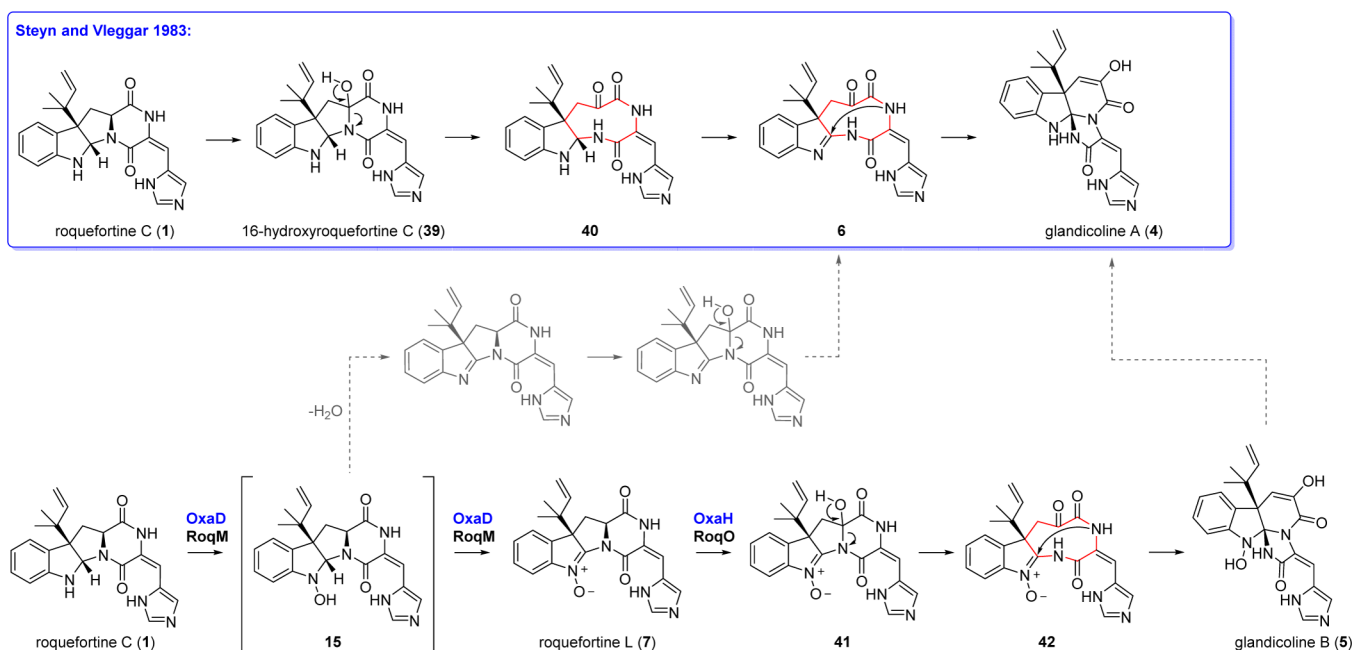
orbital interactions described by Sustmann and Trill.<sup>63</sup> While this cycloaddition is highly indicative of the presence of a nitronone functionality in 7, oxaziridines are also known to thermally rearrange to nitrones before undergoing 1,3-dipolar cycloadditions.<sup>70,71</sup>

Preparative-scale (>20 mg) reactions of 1, <sup>15</sup>N-enriched 1, 19, 20, and 22 with OxaD were conducted to provide sufficient material for spectroscopic analysis of nitrones 7, 24, 25, and 27. Analysis of the <sup>1</sup>H NMR spectrum of 7 confirms the double oxidation event at the indoline nitrogen, demonstrated conclusively by the disappearance of the H6 signal at  $\delta$  6.35 ppm. Moreover, chemical shifts of H9–H12 were observed further downfield. Analysis of the <sup>13</sup>C NMR spectrum of 7 was further indicative of the nitronone formation as the chemical shift of the C6 peak shifted from  $\delta$  78 ppm to  $\delta$  150 ppm. Additionally, previous infrared analysis of *N*-phenyl nitrones shows a very strong band between 1000 and 1100 cm<sup>-1</sup>

corresponding to the N–O stretch.<sup>72</sup> This band disappears upon isomerization of *N*-phenyl nitrones to the corresponding oxaziridines. Following the oxidation of roquefortine C by OxaD, an intense band was observed at 1014 cm<sup>-1</sup> in the IR spectrum of 7, additional support for the presence of a nitronone functionality instead of an oxaziridine. The presence of the nitronone group was also confirmed by analysis of <sup>1</sup>H–<sup>15</sup>N HMBC spectrum of <sup>15</sup>N-enriched 7. Nitrogen atoms in *N*-aryl nitrones display a characteristic peak between  $\delta$  270–300 ppm, while the corresponding amine nitrogens are observed between  $\delta$  0–100 ppm. In the <sup>1</sup>H–<sup>15</sup>N HMBC spectrum of <sup>15</sup>N-enriched 7, the signal at  $\delta$  83 ppm disappeared, and a new peak was observed at  $\delta$  281 ppm, which correlated to H9 and H10.

The enzymatic characterization of OxaD, along with the results of gene disruptions,<sup>34</sup> indicates that formation of roquefortine L (7) is a key biosynthetic intermediate toward formation of the glandicoline core. OxaD conversion of 1 into 7 generates the electrophilic center required for the attack by the amide nitrogen in the proposed 1,4-diazonane-2,5,6-trione intermediate (42, Scheme 5).<sup>35</sup> Hydroxylation at C16 of 1 leading to 39 appears insufficient to induce rearrangement because this metabolite has been previously isolated and identified;<sup>73,74</sup> therefore, both hydroxylation at C16 and nitronone generation to give 41 are likely required for the core rearrangement to occur. Roquefortine L (7) involvement is supported by the fact that glandicoline A (4) has not been observed in *P. chrysogenum*, and that disruption of *roqM* (81% homology with *oxaD*) abolished the production of glandicoline B (5).<sup>35</sup> The first report of 4 in *P. glandicola* is sufficiently supported by spectroscopic data;<sup>75</sup> however, subsequent reports of 4 were based on mass spectrometry analysis, chromatographic retention time (*R<sub>t</sub>*), and/or UV signature, but not by NMR analysis.<sup>31,33,34</sup> Substantial structural characterization of metabolites is imperative in differentiating glandico-

**Scheme 5. Proposed Biogenesis of Glandicoline A (4) and B (5)<sup>a</sup>**



<sup>a</sup>Nitronone intermediate 7 described by Ries et al. and in this work is likely a key priming step for the rearrangement to 5. Compound 4 is not observed in *P. chrysogenum* or *P. oxalicum* F30 but is a known natural product. One possible route to 4 is through dehydration of the hydroxylamine intermediate 15 followed by hydroxylation and rearrangement. Enzymatic processing of 5 to 4 must also be considered.

line A from 7, so we have unequivocally assigned the nitron group in 7 by NMR, IR, and chemical transformation.

At present, the mechanism of formation of 4 in *P. glandicola* is unclear. It could require a yet unknown route to the 1,2-imine 6 proposed by Steyn and Vleggaar<sup>33</sup> or arise by modification of 5 (Scheme 5). Investigation of the biosynthetic gene clusters in strains that produce 4 will be necessary to identify candidate enzymes that could give rise to 4, either through a different oxidation cascade involving 1 or enzymatic processing of 5. Understanding the role of the oxidative enzymes involved in the rearrangement of 1 to 5 will be critical for *in vitro* reconstitution and for future mutasynthetic efforts based on derivatives of 1. There is ample precedence for plasticity in these pathways as mutasynthesis with *roqA* gene disruption mutant strains has been used to generate novel roquefortine D derivatives.<sup>76</sup> Characterization of OxaD reported in this study also reveals the ability of this roquefortine C nitron synthase to accept an array of modified substrates that enables the generation of novel indole nitrones which would be synthetically appealing for the preparation of further elaborated alkaloid scaffolds.

Indole alkaloids 29–35 were tested primarily as a means to define the substrate scope of OxaD and also to explore the possibility that a flexible OxaD enzyme could reveal a functional link between nitron biogenesis in roquefortine L (7) and avrainvillamide (11). While no reaction was observed with OxaD and (+)-stephacidin A (33), OxaD was shown to catalyze oxidation of notoamide S (31) to rearranged product 36. Surprisingly, in this case the enzyme catalyzed a 2,3-indole epoxidation rather than the expected *N*-hydroxylation. Compound 36 is likely derived from a pinacol-type rearrangement, similar to that reported for the homologous NotB, which shows 53% similarity and 37% identity with OxaD (Scheme 3B).<sup>68</sup> Compound 36 was observed as a single reaction product between 31 and OxaD, as opposed to the reaction between notoamide E (32) and NotB, which generated notoamides C (37) and D (38) (Scheme 4). OxaD appears to play a role in directing reaction selectivity, although low enzyme activity with notoamide S (31) could limit the detection of minor reaction products. This outcome shows that OxaD presents a remarkable functional plasticity in addition to its broad substrate scope. Indeed, bioinformatic analysis of OxaD reveals that biochemically characterized homologues promote a variety of oxidative reactions (Table S3), indicating that subtle structural differences likely enable these enzymes to differentially catalyze oxidative processes. Further structural investigation of OxaD or its structural and functional homologues would be required to determine the mechanistic basis of this branch in reaction specificity.

The discovery of OxaD as an indolic nitron synthase enables a unique strategy in late-stage chemical derivatization of nitron-functionalized indolines through 1,3-dipolar cycloadditions. There are limited reports of the occurrence of 1,3-dipolar cycloadditions using natural products or their biosynthetic intermediates as substrates.<sup>77</sup> Roquefortine L (7) undergoes effective 1,3-dipolar cycloaddition with methyl acrylate to give 18 (Scheme 2), a derivatization outcome that may be extended to an array of other dipolarophiles with electron-withdrawing substituents.<sup>78</sup> By coupling this derivatization strategy with the broad substrate scope of OxaD, we have established a platform in which novel indole alkaloid frameworks can be paired with an array of dipolarophiles in a cycloaddition cascade. Recent reports describing the therapeutic

potential of 2<sup>29</sup> and 3<sup>27,30</sup> provide strong motivation to develop these tools. Future efforts in our laboratories will also seek to extend the substrate scope of OxaD beyond alkaloids in the roquefortine family.

## CONCLUSIONS

The results herein illustrate that the combination of biological and chemical approaches for the generation of new and diverse natural products can lead to a number of applications in chemistry and chemical biology. Understanding the genetic and metabolic information on biosynthetic systems can provide access to complex chiral molecular scaffolds, which can be further synthetically elaborated into novel molecules. These natural-product-based modified frameworks are of considerable interest in chemistry, biochemistry, and chemical biology as bioactive chemical entities or as unique biochemical tools. Heterologous production and enzymatic characterization of OxaD constitutes proof of concept that unique biocatalysts can generate versatile and powerful chemical and biochemical tools for structural diversification.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04915.

Full experimental details and NMR spectra (PDF)

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### Notes

The authors declare no competing financial interest.

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