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# Flavin-Dependent Monooxygenases NotI and NotI ´Mediate Spiro-Oxindole Formation in Biosynthesis of the Notoamides

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## Abstract

The fungal indole alkaloids are a unique class of complex molecules that have a characteristic bicyclo[2.2.2]diazaoctane ring and frequently contain a spiro-oxindole moiety. While various strains produce these compounds, an intriguing case involves the formation of individual antipodes by two unique species of fungi in the generation of the potent anticancer agents (+)- and (−)-notoamide A. NotI and NotI ´have been characterized as flavin-dependent monooxygenases that catalyze epoxidation and semi-pinacol rearrangement to form the spiro-oxindole center within these molecules. This work elucidates a key step in the biosynthesis of the notoamides and provides an evolutionary hypothesis regarding a common ancestor for production of enantiopure notoamides.

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Conflict of Interest

The authors declare no conflict of interest.

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## Introduction

The fungal-derived prenylated indole alkaloids are a large class of natural products having a diverse range of biological activities relevant to many human and animal diseases.[1] They are assembled by fascinating biosynthetic mechanisms, and have been the subject of numerous unique and challenging bioinspired total syntheses.[2] This constantly expanding family of compounds includes the anthelmintic paraherquamides,[1e] calmodulin-inhibitory malbrancheamides,[1n] and anticancer notoamides,[3] stephacidins,[1m] and citrinadins,[4] among others. These alkaloids are typically composed of an initial prenylated dipeptide that is modified via a proposed intramolecular Diels-Alder (IMDA) reaction to form the characteristic bicyclo[2.2.2]diazaoctane core.[5],[6],[7],[2f, 8] A semi-pinacol rearrangement is predicted to generate the spiro-oxindole moiety found in the notoamides, paraherquamides, and spiromalbramide.[9],[10],[11] The respective enzymes involved in spirocycle formation are proposed to generate an initial indole-2,3-epoxide with facial selectivity, followed by controlled collapse of the epoxide giving rise to the observed spiro-oxindoles. Very few enzymes responsible for this type of reaction within the bicyclo-ring containing family have been characterized.[12],[13] NotB catalyzes 2,3-β-face epoxidation of notoamide E (**1**) to generate the non-spirocyclized terminal metabolites notoamides C (**2**) and D (**3**) that do not undergo IMDA cyclization. [12] Additionally, biochemical and structural analysis of PhqK provided the first insights into the mechanism of selective spirocyclization.[13] However, there has been little biochemical evidence to support the direct role of specific enzymes in the rearrangement reaction to form spirocyclized notoamide products, leaving an incomplete understanding of the formation of these molecules in secondary metabolism.[14]

The notoamides are a fascinating class of anticancer metabolites in the indole alkaloid family where two phylogenetically related fungal strains have evolved to generate terminal products based on catalytic processes with opposing enantioselectivity (Scheme 1). The natural products (−)-stephacidin A (**4**), (+)-notoamide B (**5**), (+)-6-*epi*-stephacidin A (**6**), (−)-6-*epi*-stephacidin A (**7**), and (+)-versicolamide B (**8**) are produced by the terrestrial strain *Aspergillus amoenus* (formerly *Aspergillus versicolor* NRRL 35600), while (+)-stephacidin A (**9**), (−)-notoamide B (**10**), (+)-6-*epi*-stephacidin A (**6**), and (+)-versicolamide B (**8**) are produced by the marine strain *Aspergillus protuberus* (formerly *Aspergillus sp. MF297–2*).[15],[16] Based on these isolation data, it was proposed that the two strains underwent an enantiodivergence with respect to the production of the notoamides.

The formation of enantiomeric natural products identified from one or more species is highly unusual,[17] and the existence of distinct metabolic systems that form exact antipodal pairs suggests that the strains have evolved one or more biosynthetic gene products that catalyze an identical enzymatic reaction to produce and further modify enantiomeric compounds.[18] Bioinspired synthetic schemes have been devised to generate stephacidin A and notoamide B, supporting the hypothesis that one or more biosynthetic enzymes determine the chirality of the molecule.[2d, 2h, 19] However, the molecular mechanisms that control this divergence within the respective organisms have remained a mystery.

As a starting point for these studies, we sequenced the genomes of *A. amoenus (not′)* and *A. protuberus (not)*.[12] The *not*/*not′* gene clusters were identified through *in silico* database

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mining followed by open reading frame (*orf*) and BLAST analysis to complete a more thorough annotation of the metabolic systems. These data, combined with previous isotopically enriched precursor incorporation studies[20] supported our proposed biosynthetic pathway for the notoamides.[18] Comparison of the *not*/*not′* gene clusters showed an overall 71% DNA sequence identity (between *notA*-not*J*/*notA′-notJ′*), indicating a closely related phylogeny.[21] However, bioinformatic analyses provided only limited understanding of the biosynthetic pathway and little mechanistic information about the sequence divergence observed between the two enantiomeric natural products **7** and **2**. Consequently, we were motivated to investigate the biochemical transformations involved in the formation of the notoamides, and determine the basis for the structural branch-point that generates these antipodal indole alkaloid molecules.

## Results and Discussion

Previous work from our laboratories revealed the role of early steps in notoamide assembly. NotF and NotC were characterized as reverse and normal prenyltransferases, respectively, while NotB was shown to be a FAD-dependent oxidase.[9],[12] Herein, we report the biochemical function of late-stage enzyme NotI as a flavin-dependent monooxygenase (FMO) (Figures S3, S4, **and** S5) that catalyzes the semi-pinacol rearrangement to generate the spiro-oxindole moiety present in many of the bicyclo[2.2.2]diazaoctane fungal indole alkaloids. We also report the biochemical function of NotI′ (85% sequence identity to NotI) as the first heterologously expressed and biochemically characterized gene product from *A. amoenus*.

The functions of NotI and NotI′ were investigated by separately incubating compounds (+)- and (−)- stephacidin A (**9** and **4**) with NotI or NotI′. The reactions were subjected to HPLC analysis and compared directly with synthetic standards or authentic natural products isolated from the respective fungal cultures (Scheme 1 **and** Figure 1). Both NotI and NotI′ catalyzed conversion of (+)- and (−)- stephacidin A (**9** and **4**) to (−)- and (+)- notoamide B (**10** and **5**), although a clear preference was observed for the conversion of (−)-stephacidin A (**4**) to (+)-notoamide B (**5**) (Table S4). Additionally, NotI and NotI′ converted (+)-6-*epi*-stephacidin A (**6**) to (+)-versicolamide B (**8**), but no reaction was observed with (−)-6-*epi*-stephacidin A (**7**) to produce (−)-versicolamide B (**11**). This is compatible with the conversion observed in *A. amoenus* where (+)-versicolamide B (**8**) was produced and (−)-6-*epi*-stephacidin A (**7**) was determined to be a shunt metabolite.[16] To further define this biocatalytic process, the reactions of NotI with (−)-stephacidin A (**4**) to generate (+)-notoamide B (**5**) were fit to Michaelis-Menten model kinetics (Figure S6). The $K_m$ and $V_{max}$ values were determined to be 37.4 ± 14.5 μM and 1.19 ± 0.13 μM/min, respectively. The relatively low conversion observed for this enzyme could be due to decoupling of the flavin redox chemistry from the epoxidation/semi-pinacol rearrangement. The measured rate of product formation ($V_0$) was 0.06 μM/min at a standard substrate concentration of 200 μM, and the rate of NADH consumption was 1.43 μM/min, with an epoxidation efficiency of 4.2%, indicating decoupling (Figure S7). While a comparison of reactivity between the two enzymes would be intriguing, the necessity of using an MBP-tagged NotI′ precluded an accurate determination of the native reaction kinetics. Accordingly, we have confirmed that

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the enzymes may not be optimally folded because the flavin incorporation ratios for both enzymes are quite low (18% for NotI and 0.5% for NotI′).

Next, we sought to assess the timing of epoxidation and semi-pinacol rearrangement in notoamide biosynthesis. Reactions were conducted using NotI and NotI ′with pre-IMDA pathway intermediates including brevianamide F (**12**), deoxybrevianamide E (**13**), 6-OH-deoxybrevianamide E (**14**), notoamide S (**15**), notoamide E (**1**), and post-IMDA intermediates (+)-notoamide T (**16**), and (−)-notoamide T (**17**) and analyzed by QTOF LC-MS (Scheme 2 **and** Figures S12–S24). Both enzymes demonstrated a remarkable range of substrate tolerance, leading to new products with masses indicative of oxidation. In some cases, multiple products were generated, indicating either a loss of stereocontrol for collapse of the epoxide or the generation of an alternative oxidized product.

Based on the analysis above, we expected that the reactions between NotI/NotI′ and notoamide T (**16** and **17**) had formed a new metabolite, thus we determined the structure by NMR spectroscopy (Table S2 **and** Figures S8, S9, S25–S35). Racemic notoamide T was converted by NotI and purified by HPLC to yield approximately 2 mg of final product. The new compound notoamide TI (**18**) was obtained as a white amorphous solid and possessed a molecular formula of $C_{26}H_{31}N_3O_4$ as suggested by HRESIMS based on $[M+H]^+$ ion peak at m/z 450.2414, representing thirteen degrees of unsaturation. Moreover, the UV spectrum in methanol with wavelength of maximum absorbance at 242, 309, and 335 (sh) nm was indicative of aromatic functionality.

Lastly, a comparison of NotI/NotI′ to the recently characterized PhqK,[13] provided insight into the potential reaction mechanism. The enzymes share 35% sequence identity and have highly similar native substrates; thus the Phyre2[22] homology models of NotI and NotI′ were aligned with the crystal structure of PhqK (RMSD = 2.28, PDB ID: 6pvi) to investigate the presence of possible catalytic amino acids (Figure 2). Arginine 195 in NotI/NotI′ aligns with the catalytic arginine in PhqK (Arg192), indicating that it may play a similar role in directing the collapse of the epoxide and spirocyclization. The C-terminus of the enzyme, which is important for binding the substrate and closing off the active site in PhqK, seems to be very different in NotI. This portion of the enzyme shared no homology with FMOs in the Protein Data Bank, including PhqK, indicating that the C-terminus may be a point of divergence in the evolution of these FMOs (Figures S10 **and** S11). While the substrate-binding regions of FMOs vary in structure and function, the architecture of the cofactor binding domain seems to be maintained over time.[23]

Additionally, sequence comparison with homologous enzymes demonstrated that this catalytic arginine is conserved across a range of fungal FMOs, many of which are capable of catalyzing epoxidation and semi-pinacol rearrangement (Table S3 **and** Figure S11). In all cases, the catalytic arginine is bordered by a large N-terminal amino acid (Phe/Tyr/Trp) and a smaller C-terminal amino acid (Ala/Gly/Ser). The steric conservation around this pivotal amino acid indicates that the nearby residues may facilitate the range of motion required for the arginine to direct collapse of the indole epoxide species.

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## Conclusion

We initially hypothesized that NotI/NotI′ would be selective for their respective enantiomeric substrates. However, both NotI and NotI′ accepted either of the stephacidin A enantiomers, with a clear preference for the (−)-isomer (**4**). This indicates that the conversion of (+)-stephacidin A (**9**) may be an evolved trait from an ancestral organism previously only capable of converting (−)-stephacidin A. In contrast, the facial selectivity of the FMO-catalyzed oxidation appears to be highly diastereoselective, delivering the oxygen atom from the least-hindered face of the 2,3-disubstituted indole substrate. These findings support our proposed biosynthesis in which NotI and NotI′ are not candidates for the control of the enantio-divergence in compounds produced by the *Aspergillus* species, and that the formation of antipodal notoamides is instead due to an earlier step in the biosynthetic pathway.[15]

With our current knowledge of the biochemical transformations involved, we reason that the enzyme responsible for the IMDA is the likely candidate for the enantio-divergent step. Additionally, a novel indole alkaloid metabolite notoamide TI was generated through *in vitro* reactions, further expanding the chemical diversity of bicyclo[2.2.2]diazaoctane ring containing molecules. The production of notoamide TI suggests that there may be parallel pathways to the formation of notoamide B through either (+)/(−)-notoamide T (**16**/**17**) or (+)/(−)-stephacidin A (**9**/**4**) (Scheme 2). However, precursor feeding studies with isotopically labelled stephacidin A suggest that the proposed order involving initial pyran ring formation followed by semi-pinacol rearrangement is likely to be the preferred route.[20a] Moreover, we cannot eliminate the possibility of a brevianamide A-like route similar to what we elucidated recently.[24] This would involve the sequential FMO-mediated epoxidation, P450-catalyzed desaturation of the dioxopiperazine ring, and spontaneous IMDA to generate **5** or **10** without passing through **4** or **9** (Scheme 2). In this investigation, two group A FMOs were found to be involved in the formation of the spiro-oxindole center of various bicyclo[2.2.2]diazaoctane fungal indole alkaloids via semi-pinacol rearrangement.[25] The designation of NotI/NotI′ into this group is based on the presence of the DG fingerprint, which is involved in both FAD and NAD(P)H binding (Figure S11). The mechanism of this reaction has recently been investigated in a homologous paraherquamide-producing system and FMO PhqK.[13] It is hypothesized that the C2–C3 bond of the indole is epoxidized by the FMO on the less-hindered face, as has been reported in synthetic approaches,[2d, 15] and suggested for similar molecules such as the taichunamides and paraherquamides.[13, 24] Protonation of the reactive epoxide intermediate leads to ring opening to form a hydroxy cation and the subsequent deprotonation facilitates the 1,2 shift to provide notoamide B (Scheme 3). While the enzymes have evolved a mechanism for stereospecific collapse of the indole epoxide, unnatural substrates seem to evade this catalyst-controlled selectivity. As has been observed with other oxidative enzymes,[26] whether an epoxidation or hydroxylation reaction occurs can depend on positioning of the substrate in the active site. This indicates that, in some cases, the unnatural substrates may not be orientated properly for selective spirocycle formation.

Through homology modelling of NotI and NotI′, and comparison to the high-resolution crystal structures of PhqK, we have determined that the catalytic arginine in PhqK may also

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be present in NotI/NotI′ (Figure 2). Arg195 in NotI/NotI′ is proposed to perform general acid catalysis to mediate the collapse of the epoxide with a stereoselective formation of the spiro-oxindole. These findings demonstrate that a catalyst-controlled semi-pinacol rearrangement reaction is involved in notoamide biosynthesis, and may have important implications for the inherently flexible FMOs in fungal indole alkaloid biosynthesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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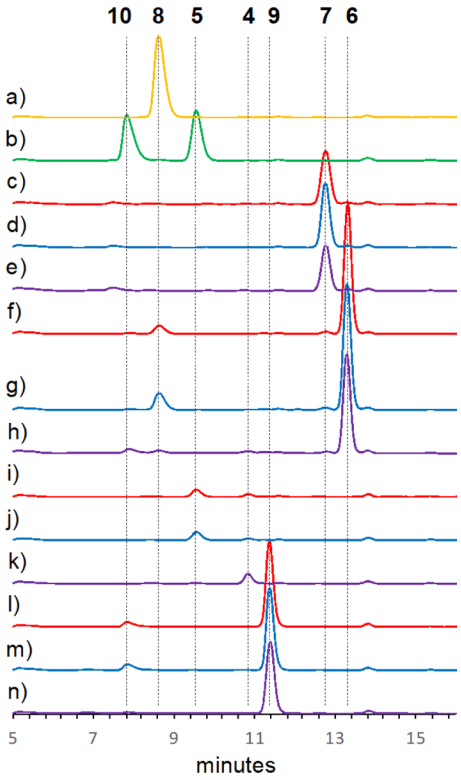


**Figure 1.**

Biochemical activity assays with NotI and NotI′. (a) (+)-versicolamide B (**8**) standard. (b) (+)- and (−)-notoamide B (**5** and **10**) standards. (c) NotI′ reaction with (−)-6-*epi*-stephacidin A (**7**). (d) NotI reaction with **7**. (e) No enzyme control with **7**. (f) NotI′ reaction with (+)-6-*epi*-stephacidin A (**6**). (g) NotI reaction with **6**. (h) No enzyme control with **6**. (i) NotI′ reaction with (−)-stephacidin A (**4**). (j) NotI reaction with **4**. (k) No enzyme control with **4**. (l) NotI′ reaction with (+)-stephacidin A (**9**). (m) NotI reaction with **9**. (n) No enzyme control with **9**. No enzyme controls are shown in purple, reactions with NotI are shown in blue, and NotI′ reactions are shown in red. The HPLC data were collected at a wavelength of 240 nm.

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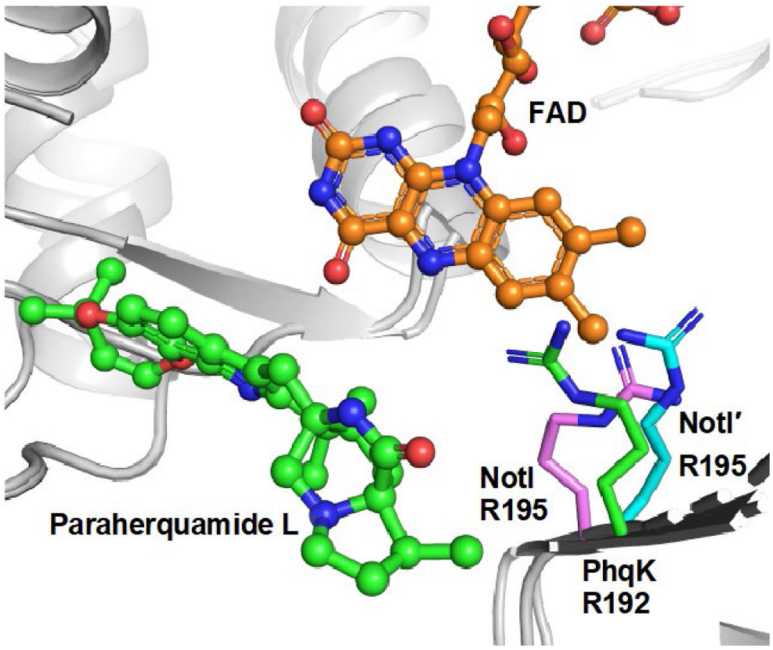
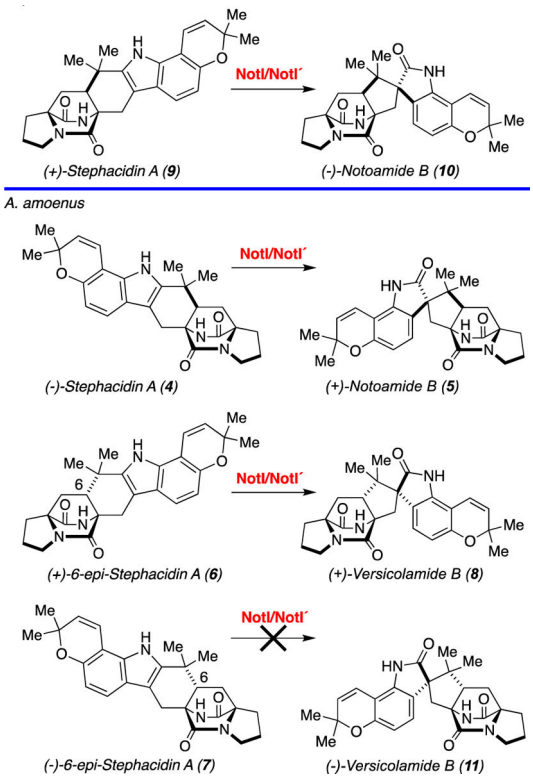



**Figure 2.**

Alignment of PhqK (PDB ID: 6pvi) and Phyre2 models of NotI and NotI ´demonstrating the presence of the catalytic arginine in all three proteins. FAD conformation was modelled from urate oxidase HpxO (PDB ID: 3rp7).




**Scheme 1.**

Reactions catalyzed by NotI/NotI´ *in vitro* and in the respective native fungal species.



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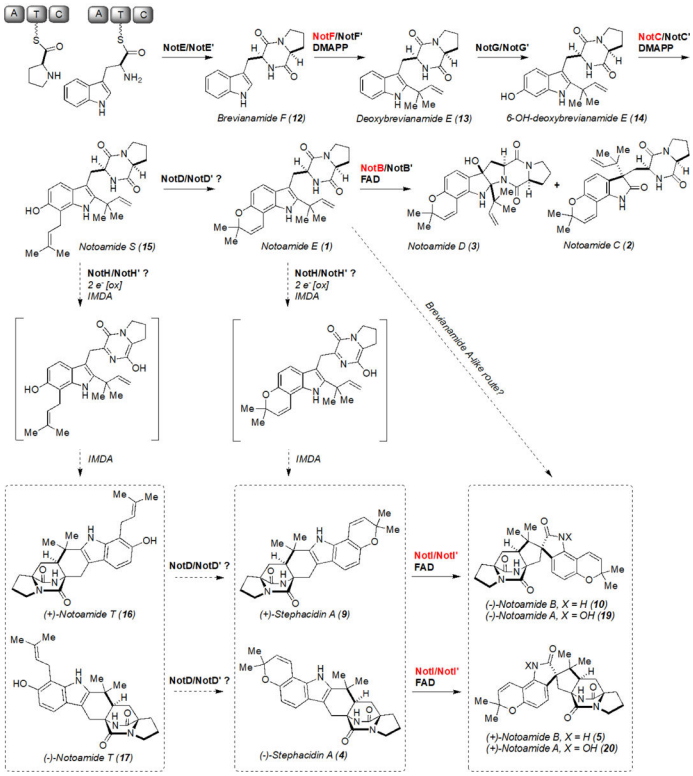


**Scheme 2.**

Proposed (−)/(+)-notoamide A (**19** and **20**) biosynthetic pathways. The functions of enzymes highlighted in red have been experimentally confirmed. The terminal N-hydroxylation to generate **19** and **20** is catalyzed by an additional enzyme subsequent to spirocyclization.



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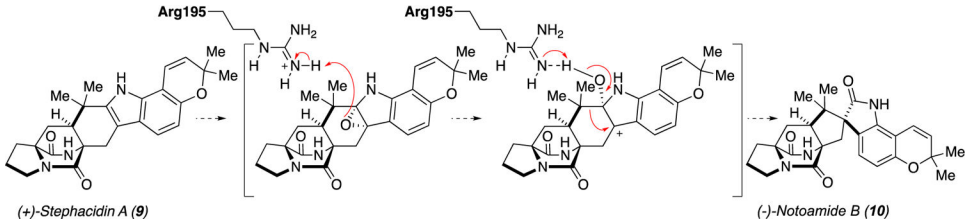


**Scheme 3.**

Proposed mechanism for epoxidation and spirocyclization catalysed by NotI and NotI′.



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