Studies on the Biosynthesis of the Stephacidin and Notoamide Natural Products: A Stereochemical and Genetic Conundrum

James D. Sunderhaus,[a] David H. Sherman,[b] and Robert M. Williams*[a, c]

Dedicated to Stephen F. Martin on the occasion of his 65th birthday.

Abstract: The stephacidin and notoamide natural products belong to a group of prenylated indole alkaloids containing a bicyclo[2.2.2]diazaoctane core. Biosynthetically, this bicyclic core is believed to be the product of an intermolecular Diels–Alder (IMDA) cycloaddition of an achiral azadiene. Since all of the natural products in this family have been isolated in enantiomerically pure form to date, it is believed that an elusive Diels–Alderase enzyme mediates the IMDA reaction. Adding further intrigue to this biosynthetic puzzle is the fact that several related *Aspergillus* fungi produce a number of metabolites with the opposite absolute configu-ration, implying that these fungi have evolved enantiomeri-cally distinct Diels–Alderases. We have undertaken a pro-gram to identify every step in the biogenesis of the stephaci-dins and notoamides, and by combining the techniques of chemical synthesis and biochemical analysis we have been able to identify the two prenyltransferases involved in the early stages of the stephacidin and notoamide biosyntheses. This has allowed us to propose a modified biosynthesis for stephacidin A, and has brought us closer to our goal of find-ing evidence for, or against, the presence of a Diels–Alder-ase in this biosynthetic pathway.

Keywords: *Aspergillus* · biosynthesis · cycloaddition · Diels–Alder · indoles
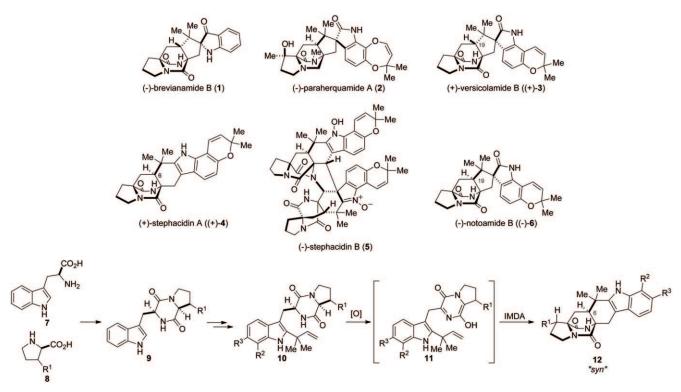
1. Introduction

Fungal-derived natural products are a diverse class of compounds with interesting structural features and biological activities that have allowed them to account for a significant number of clinical therapeutics.[1] For this reason, an increased effort has been placed on the isolation of new members of this class of natural products, and in screening these secondary metabolites for useful biological activities. The diverse class of prenylated indole alkaloids isolated from the *Aspergillus* and *Penicillium* genera of fungi have received increasing levels of attention due to their interesting chemical architectures and biological activities. Structurally, the paraherquamide,[2] brevianamide,[3] stephacidin,[4] and notoamide[5] natural products are unique in that many of them contain a bicyclo[2.2.2]diazaoctane core structure (Scheme 1). These families of alkaloids have attracted biomedical attention and have been reported to display antitumor,[4] in-secticidal,[6] antihelmintic,[2d] calmodulin-inhibitory,[7] and antibacterial properties.[4] These intriguing structural and biological properties have lead to a number of synthetic endeavors towards this class of natural products.[8]

The pioneering work from Sammes[9] and Birch,[10] along with more recent work from our laboratories,[11] has shown that these natural products are derived from L-tryptophan, a cyclic amino acid residue such as proline, β-methylproline, or pipecolic acid, and one or two isoprene units. Furthermore, the current experimental evidence suggests that the bicyclo[2.2.2]diazaoctane ring system arises from an intramolecular hetero Diels–Alder reaction (IMDA) of an achiral 5-hydroxypyrazin-2(1H)-one.[11,12] Since all of the members of this family of natural products have been isolated in entiomerically pure form, the putative biosynthetic IMDA reaction must proceed in a stereo- and enantio-controlled fashion, implying that this cycloaddition is most likely enzyme-mediated. Although enzyme-mediated Diels–Alder reactions have

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Scheme 1. Representative prenylated alkaloids and their proposed biosynthesis.

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been proposed in the biosyntheses of a number of natural products, the existence of a Diels–Alderase remains controversial.[13] In providing corroborating support for this biosynthetic proposal, we have shown that the IMDA is an effective approach for the laboratory synthesis of the bicyclo[2.2.2]diazaoctane core, and have applied this methodology to the biomimetic syntheses of a number of natural products.[14–18]

Since the isolation of (+)-stephacidin A ((+)-**4**) and B (**5**) in 2002 by Bristol-Meyers-Squib from *Aspergillus*

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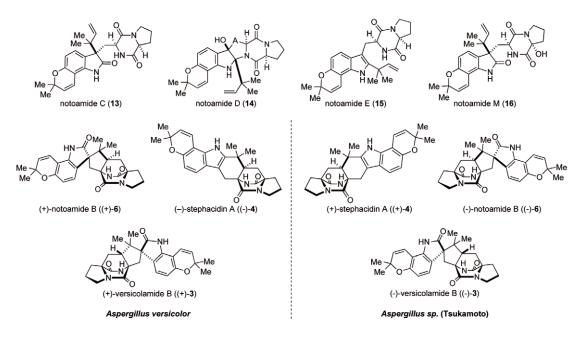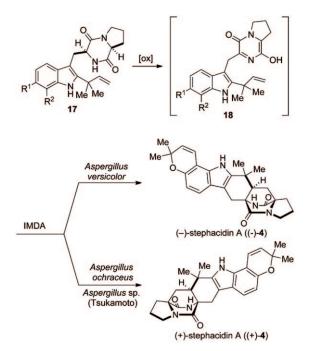
Figure 1. Representative notoamide alkaloids and the enantiomeric forms of stephacin A, notoamide B, and versicolamide B.

ochraceus,[4] our group has taken an interest in both the synthesis[14,19] and biosynthesis of this secondary metabolite. Our interest in the biosynthesis of **4** grew further when Tsukamoto and co-workers isolated the structurally related notamide natural products, as well as (+)-stephacidin A and (−)-versicolamide B ((−)-**3**), from a marine-derived *Aspergillus* sp.[5] (Figure 1). Whereas the notoamide alkaloids are of interest because they may provide clues to the biogenesis of stephacidin A, the isolation of versicolamide B was of particular interest due to its stereochemical relationship to the other stephacidin and notoamide metabolites. The majority of the members of the paraherquamides, stephacidins, and notoamides (i.e., **2**, **4**, and **6**) have a bridgehead hydrogen (C6 in stephacidin A) with a *syn-* relative configuration, while in versicolamide B (C19 in versicolamide B) and the brevianamides (i.e., **1**) the bridgehead hydrogen is in an *anti-* configuration. This difference in the relative stereochemistry about the bicyclo[2.2.2]diazaoctane core poses a number of fascinating questions relating to the biosynthesis of both **3** and **4**.

Adding further intrigue to the biogenesis of the stephacidins and notoamides is the fact that in 2006 the enantiomers of stephacidin A ((−)-**4**), notoamide B ((+)-**6**), and versicolamide B ((+)-**3**) were isolated from the terrestrial fungus *Aspergillus versicolor*.[20]

Although examples of antipodal natural products are known to be produced by Nature, given the vast number of naturally occurring secondary metabolites, examples of this phenomena are exceedingly rare. In terms of the biosynthesis of **4**, the isolation of the antipodal forms of stephacidin A implies that the different species of *Aspergillus* have evolved enantiomerically distinct genes for the

biosyntheses of the stephacidin and notoamide natural products. Moreover, because the chirality of the bicyclo-[2.2.2]diazaoctane core is believed to be formed from an achiral aza-diene via the putative IMDA, the implication is that the *Aspergillus* species may have evolved enantiomerically distinct Diels–Alderases (Scheme 2). Thus, stephacidin A would arise from an appropriate reverse-pre-

Scheme 2. The proposed biogenesis of both enantiomers of stephacidin A.

nylated *cyclo*-L-typtophan-L-proline, which would undergo oxidation to give the achiral azadiene **18**, which would undergo an IMDA to give the two enantiomers of stephacidin A in each specific species, respectively.

To answer these intriguing questions about the biosynthesis of the stephacidins and notoamides, our research group has embarked on a program to elucidate every stage of their biosynthetic constructions. The goal of this project is both to identify every intermediate in the biosynthesis, and to identify and assign function to every gene in the notoamide gene cluster. To do this a number of chemical and biochemical techniques have been utilized, and our current efforts to determining the complete biosynthetic pathway are summarized herein.
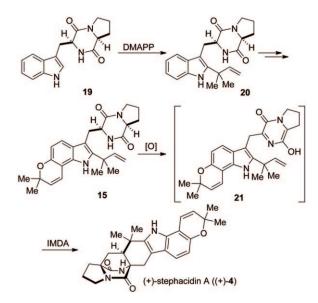
2. Investigations Into the Biosynthesis of the Stephacidin and Notoamide Alkaloids

2.1. Originally Proposed Biosynthesis of Stephacidin A

In 2009, our laboratory had inadvertently synthesized a prenylated indole alkaloid that would later be assigned the name notoamide E (**15**). We provided an authentic sample of this substance to the Tsukamoto laboratory wherein this synthetic material was used to isolate and identify this short-lived metabolite (notoamide E, **15**) from a marine-derived *Aspergillus* sp.[5b] This led to our first proposed biogenesis of stephacidin A, which is outlined in Scheme 3. Starting from brevianamide F (**19**), reverse prenylation would give deoxybrevianamide E (**20**), a known metabolite in stephacidin-producing organisms.[5a] Further prenylation and oxidation could then provide notoamide E. Oxidation of **15** would provide the transient *achiral* azadiene (**21**), which could undergo the IMDA cyclization to give stephacidin A. In order to determine if notoamide E was indeed the precursor to **4** we undertook the synthesis of both unlabeled **15** and doubly ^{13}C-labeled **15** for use in biosynthetic incorporation studies.

The synthesis of notoamide E[21] started from 6-hydroxyindole, which was *O*-protected to give **23** (Scheme 4). Subsequent chlorination and reverse prenylation following conditions first reported by Danishefsky[22] provided the C2-reverse-prenylated **26**. The Boc-group was then removed with TFA, and the resulting phenol was propargylated to give **28** in good yield. Heating **28** in 1,2-dichlorobenzene resulted in the formation of the pyran ring. Tryptophan **32** was then readily obtained via a three step procedure involving formation of gramine **30**, Somei-Kametani[23] coupling with the glycine benzophenone imine **31**, and subsequent hydrolysis. The free amine was then Fmoc-protected, and Me3SnOH mediated hydrolysis of the ethyl ester provided the racemic acid **33**.

The acid **33** was then coupled with proline ethyl ester to give a mixture of diasteriomeric dipetides **35**. Treatment of **35** with morpholine resulted in removal of the
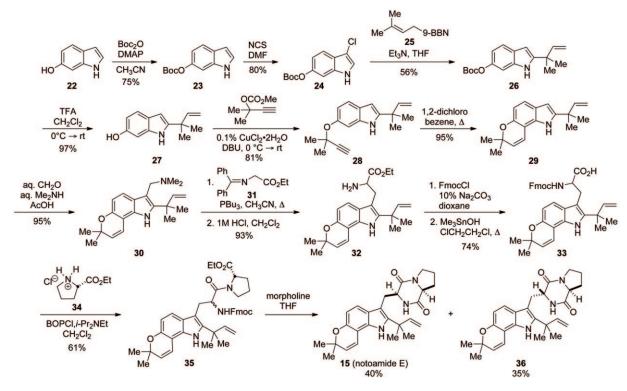

Scheme 3. Originally proposed biosynthetic route to stephacidin A via notamide E.

Fmoc-group and subsequent cyclization to provide notoamide E and *epi*-notoamide E in 40% and 35% yields, respectively. Thus, the total synthesis of notoamide E was completed in 13 steps from commercially available 6-hydroxyindole. This route also provided access to doubly ^{13}C-labeled notoamide E (**37**), which was synthesized for use in biosynthetic incorporation studies.
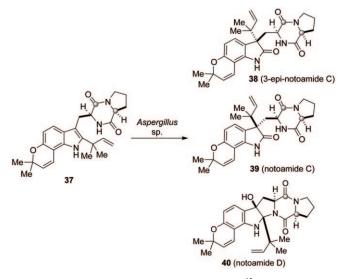
2.1.2. Notoamide E Incorporation Studies

The synthetic doubly ^{13}C-labeled **37** was incubated with the marine derived *Aspergillus* sp., and the fungal extracts were analyzed for the presence of ^{13}C-labeled metabolites. If notoamide E was indeed the precursor to stephacidin A, then the stephacidin A isolated from this experiment should exhibit some level of ^{13}C incorporation. However, upon analyzing the fungal abstracts, the major metabolites found containing ^{13}C enrichment were notoamides C, 3-*epi*-notoamide C, and notoamide D. To our surprise, no ^{13}C-labeled **3**, **4**, or **6** could be detected (Scheme 5).[5b]

These results suggested that notoamide E is not the precursor to stephacidin A, and that our originally proposed biogenesis was incorrect. If notoamide E is not the precursor to stephacidin A, and if the bicyclo-[2.2.2]diazaoctane ring does indeed arise from an IMDA reaction, then the order of steps in our original biosynthetic proposal needed to be re-evaluated. One plausible alternative envisioned that the formation of the bicyclo-[2.2.2]diazaoctane core occurred before the formation of the pyran ring. With this in mind, we set out to develop and test a new biogenetic hypothesis.
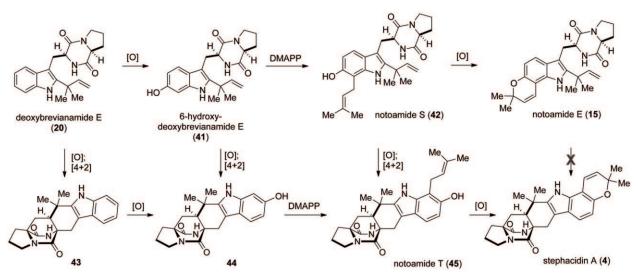
Scheme 4. Synthesis of notoamide E.

Scheme 5. Incorporation studies with doubly ^{13}C-labeled notoamide E.

2.2. Alternative Biogenesis of Stephacidin A

The formation of notoamide E from **20** requires three distinct steps, namely, an oxidation, a prenylation, and a final oxidation/cyclization to form the pyran ring, with the order of the initial oxidation and prenylation stages being unknown. The IMDA could potentially occur before each of these steps, which leads to a number of biosynthetic possibilities that are outlined in Scheme 6.

Deoxybrevianamide E (**20**) can undergo either oxidation to **41**, or oxidation and IMDA cyclization to give **43**. If **20** undergoes an oxidation and IMDA reaction to give **43**, then a straightforward oxidation/prenylation/cyclization (**43**→**44**→**45**→**4**) pathway would lead to stephacidin A. However, if **20** undergoes oxidization to **41**, two pathways to stephacidin A are possible. 6-Hydroxydeoxy-brevianamide E (**41**) could be prenylated to give **42**, which must then undergo the IMDA to give **45**. Alternatively, **41** could undergo the IMDA reaction to give hexacycle **44**, which could then be prenylated to give **45**. Additionally, since the exact order of oxidation and prenylation steps is unknown, it is possible that prenylation of **20** or **43** happens prior to the azadiene oxidation. Regardless of the pathway, compound **45**, which we have named notoamide T, would be the last [stable] intermediate in the biosynthesis of stephacidin A.

In order to ascertain which of these pathways is operable in the biosynthesis of stephacidin A, we set out to synthesize all of the remaining potential biosynthetic intermediates for use as authentic samples in identifying hitherto unknown, trace or short-lived metabolites. We additionally required that access to ^{13}C-labeled isotopomers of these substances for incorporation studies be readily adaptable from the synthetic routes. In addition to this, we have initiated efforts into the genome-based characterization of the producing *Aspergillus* sp. to help us fully elucidate the biogenesis of the notoamide and stephacidin alkaloids.
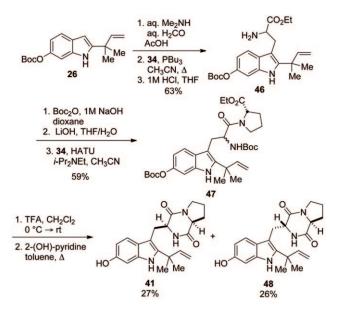
Scheme 6. Potential biosynthetic pathways to stephacidin A.

2.3. Syntheses of the Proposed Biosynthetic Intermediates to Stephacidin A

2.3.1. Synthesis of 6-Hydroxydeoxybrevianamide E

In addition to being a potential intermediate in the biosynthesis of stephacidin A, 6-hydroxydeoxy-brevianamide E is also believed to be the precursor to notoamide J, and it was in this context that the synthesis of **41** was developed.[24] The synthesis of **41** began from the reverse prenylated 6-hydroxyindole **26** and followed a route similar to that described for the synthesis of notoamide E (Scheme 7). Thus, indole **26** was converted to the corresponding gramine derivative and reacted in a Somei–Ka-

metani coupling to provide tryptophan **46** in good yield. Subsequent re-functionalization of the amino acid and coupling with (*S*)-proline ethyl ester gave the dipeptide **47**. The Boc-groups were then removed with TFA, and the resultant crude amine was heated in toluene to provide **41** in 27% yield along with an equal amount of the *trans*-diketopiperazine (DKP).
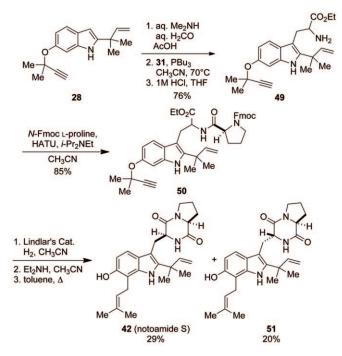
2.3.2. Total Synthesis of Notoamide S

The synthesis of notoamide S[25] was initiated from the propargylated indole **28**, which was elaborated into tryptophan **49** as previously described (Scheme 8). Tryptophan **49** was then coupled with *N*-Fmoc proline to give **50** in high yield. Subsequent Lindlar's reduction and removal of the Fmoc-group provided the free amine, which upon heating in toluene underwent an aromatic Claisen rearrangement and cyclization to give **42** in 29% yield over the three steps. Thus, the synthesis of notoamide S was completed in twelve steps from commercially available 6-hydroxyindole.

Although at the outset of the synthesis the natural origin of notoamide S was unknown, the authentic sample was later used in helping to prove the existence of **42** as an intermediate in the biosynthesis of stephacidin A. In addition, this route is currently being utilized for the synthesis of double ^{13}C-labeled notoamide S needed for future incorporation studies.

2.3.3. Synthesis of Substrate 43

The simple hexacycle **43** represents the earliest possible bicylo[2.2.2]diazaoctane-containing compound in the biosynthesis of stephacidin A. This substance (also named ketopremalbranchiamide) was first synthesized in our laboratory some twenty years ago,[26] and more recently has

Scheme 7. The synthesis of 6-hydroxydeoxybrevianamide E (**41**).

Scheme 8. The total synthesis of notoamide S (**42**).
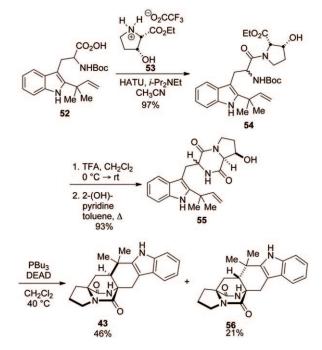
Scheme 9. The synthesis of substrate **43**.

been employed as an intermediate in the synthesis of ^{13}C-labeled compounds that were utilized in the interrogation of the biogenesis of the structurally related malbranchia-mides.[27,28]

Our biomimetic synthesis[14b] of **43** began with the coupling of the reverse-prenylated tryptophan **52**[16a] with hydroxyproline **53** to give a diastereomeric mixture of dipeptides **54** (Scheme 9). Subsequent removal of the Boc-group and cyclization to the DKP provided **55**. The reaction of **55** under Mitsunobu conditions in warm CH₂Cl₂ provided the cycloadducts **43** and **56** in 46% and 21% yields, respectively, thus providing us with ample amounts of **43** for further biological studies.
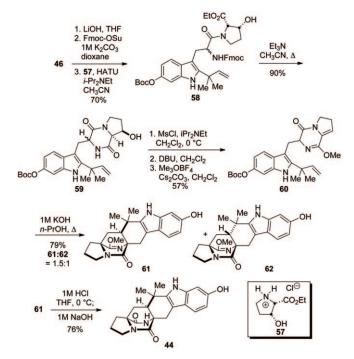
2.3.4. Synthesis of Substrate 44

The synthesis of substrate **44**[29] was modeled after our first reported biomimetic synthesis of stephacidin A.[14a] Thus, the tryptophan **46** was re-functionalized and coupled with hydroxyproline derivative **57** to give **58**. Treatment of **58** with Et₃N in refluxing acetonitrile resulted in cleavage of the Fmoc-group and cyclization to the diketopiperazine. Subsequent elimination of the alcohol moiety in **59**, and conversion of the resulting enamide to its lactim ether provided **60**. Upon treatment with aqueous KOH, **60** underwent the desired isomerization and cyclization to give a separable mixture of the *syn-* and *anti-*products **61** and **62**. Final hydrolysis of the lactim ether in **61** provided the desired substrate **44** (Scheme 10).

With the syntheses of **41**, **42**, **43**, and **44** we have accomplished the synthesis of a majority of the potential

Scheme 10. The synthesis of substrate **44**.

plausible intermediates for the biosynthesis of stephacidin A. As seen in the next section, these authentic samples proved crucial in determining the early steps of the biosynthetic pathway.

Figure 2. The notoamide (*not*) biosynthetic gene cluster.

2.4. Genome-Based Characterization of the Early Steps in the Stephacidin A Biosynthesis
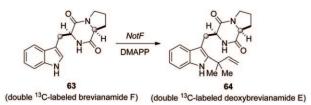
While we were in the process of synthesizing all of the potential intermediates on the stephacidin A biosynthetic pathway, we also undertook a genome-based characterization of the notoamide and stephacidin biosynthetic pathways. To this end, the genome of the marine derived *Aspergillus* sp. was sequenced and the notoamide (*not*) gene cluster was identified (Figure 2).[29]

Bioinformatics analysis helped to predict the function of the majority of the *Not* gene products, and this led to the identification of two predicted aromatic prenyltransferases, NotC and NotF. These two prenyltransferases would presumably catalyze the two key prenylation reactions in the biosynthesis of stephacidin, and further analysis of their function led to the elucidation of the early stages of the pathway.

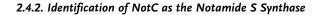### 2.4.1. Identification of NotF as the Deoxybrevianamide E Synthase

NotF was first identified as a prenyltransferase due to it showing a high identity (40%) to a putative dimethylallyl tryptophan synthase (EER24759) found in *Coccidioides posadasii*. The NotF gene was expressed in *E. coli* and the purified enzyme was first tested with doubly ¹³C-labeled brevianamide F (**63**). The results of this experiment revealed that NotF was effective in converting brevianamide F into deoxybrevianamide E (Scheme 11), thus confirming the role of NotF as a reverse prenyltransferase.

The substrate specificity was then tested by reacting NotF with tryptophan, *cyclo*-(L-Phe-L-Pro), *cyclo*-(L-Trp-L-Trp), and *cyclo*-(L-Trp-L-Tyr). In each of these examples, no prenylated products were detected. These indicate that NotF shows selectivity for **19** and that the reverse prenylation must be an early step in the biosynthetic pathway as initially assumed.
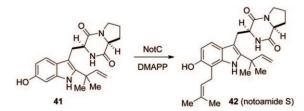

Scheme 11. Reaction of ¹³C-labeled brevianamide F with NotF.

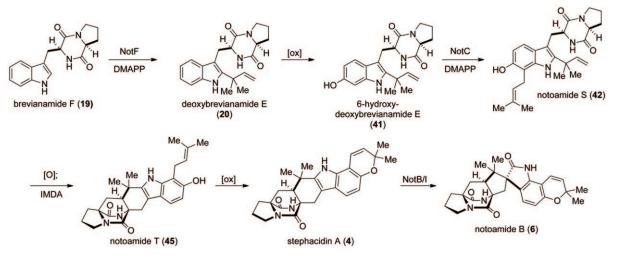### 2.4.2. Identification of NotC as the Notamide S Synthase

The role of NotC as a prenyltransferase was also first predicted because of its sequence identity (50%) to another known prenyltransferase, FtmH in *A. fumigatus*.[30] To determine the function of NotC, it was similarly expressed in *E. coli* and the recombinant protein was isolated and purified. The activity of NotC was then tested with four putative substrates, **20**, **41**, **43**, and **44**. In the cases of **20**, **43**, and **44** no prenylated products could be detected. However, when **41** was subjected to NotC, notoamide S was cleanly produced, indicating that NotC catalyzes a normal prenyltransferase at C7 of the indole ring (Scheme 12). Similar to NotF, NotC is also highly substrate-selective, and this specificity has allowed us to identify the early stages of stephacidin biogenesis.

Scheme 12. The prenylation of 6-hydroxydeoxybrevianamide E by NotC.

2.5. Revised Biogenesis of Stephacidin A

By combining the data garnered from our notoamide E feeding studies with that obtained from our genomic characterizations, we are able to propose a revised biogenesis of stephacidin A and notoamide B (Scheme 13). Starting from brevianaimde F (**19**), the NotF gene product catalyzes the reverse-prenylation of brevianamide F, providing deoxybrevianamide E (**20**). Then, an as-of-yet unidentified oxidase converts deoxybrevianamide E into **41**, which is prenylated in a normal sense by the NotC gene product to give notoamide S (**42**). Notoamide S is then presumed to be oxidized to the azadiene, which undergoes the IMDA reaction to provide notoamide T (**45**). Notoamide T would then be oxidized by another currently unidentified oxidase that mediates fashioning of the pyran ring to give stephacidin A. We have very recently applied the same methodology used to prepare **43**, **44**, and stephacidin A, to prepare notoamide T.[31] We are currently conscripting this synthesis to prepare double

Scheme 13. The revised biosynthesis of stephacidin A.

[13]C-labeled notoamide T to validate its intermediacy as the penultimate precursor to stephacidin A.

Further oxidation of **4** and subsequent Pinacol rearrangement would then provide notoamide B (**6**); a sequence that we have recently confirmed with synthetic, double ^{13}C-labeled stephacidin A.[32]

The identification and characterization of NotF and NotC have allowed us to determine the timing of the two prenylation steps in the biogenesis of stephacidin A and to propose a revised biogenetic pathway. However, the enzymes involved in a number of other steps are still undetermined, and until these are identified, the exact biosynthesis of **4** remains speculative.

2.6. Proposed Biosynthesis of Versicolamide B

Based on the available experimental results, we are tempted to suggest that there is a branch point in the biosynthetic pathway of stephacidin A that also accommodates the construction of versicolamide B that we currently suspect to be at the stage of notoamide S. As mention previously, versicolamide B is unique among the stephacidin and notoamide natural products due to the *anti-* configuration of the bridgehead hydrogen (C19) found in its bicyclo[2.2.2]diazaoctane core. To account for the stereochemical differences between **3** and **4**, three possible biosynthetic pathways to versicolamide B have been proposed, as outlined in Scheme 14.
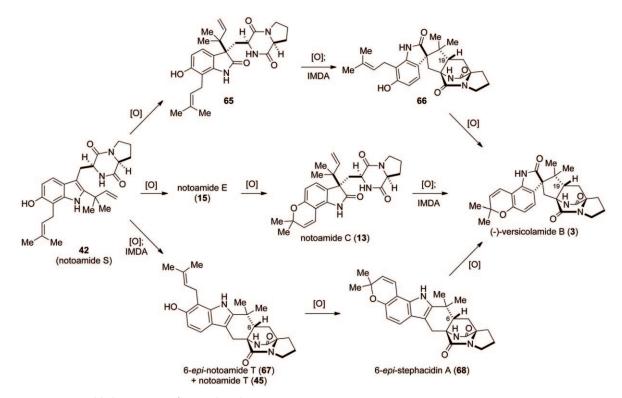
In the first possible route to **3**, notoamide S could undergo two sequential oxidations to give notoamide C (**13**) via **15**. Notoamide C could then undergo oxidation to the azadiene, possibly through the intermediacy of notoamide M (**16**), and subsequent IMDA cyclization would give (−)-versicolamide B. In order for this route to be viable, the IMDA reaction has to occur to provide the required *anti* stereochemistry at C19. Fortunately, both theoretical calculations[33] and experimental results[18] have shown

that the IMDA reaction of an oxindole should proceed to give the *anti-* product exclusively, lending support to this biogenetic pathway. The presence of notoamide M (**16**) among the naturally occur metabolites of *Aspergillus* sp.[5d] lends support to this pathway, as **16** could be the direct oxidation product of notoamide C, and the loss of water from **16** could lead directly to the requisite azadiene. For the biosynthesis of the antipodal (+)-versicolamide B, 3-*epi*-notoamide C would presumably be the corresponding branch point as the absolute stereochemistry at the *spiro*-oxindole stereogenic center would correlate to that in (+)-versicolamide B.

A similar pathway can be envisioned where the IMDA occurs prior to the formation of the pyran ring. In this route **42** is first converted to the oxindole **65**, which can then undergo the IMDA reaction to give **66**. Subsequent cyclization to form the pyran ring would then provide versicolamide B. This pathway is analogous to the stephacidin A pathway in that the IMDA reaction occurs prior to the formation of the pyran ring. However, to date there has been no evidence suggesting the presence of **65** and/ or **66** in the fungal broth, and the previously mention route seems more likely.

A third pathway involving the formation of 6-*epi*-stephacidin A (**68**) is also possible. Thus, if the IMDA reaction of the azadiene derived from **42** is not completely diastereoselective, a mixture of notoamide T (**45**) and 6-*epi*-notoamide T (**67**) would be produced. 6-*epi*-Notoamide T could then be converted to 6-*epi*-stephacidin A, which could be readily oxidized into **3**.

However, since 6-*epi*-stephacidin A, or any other related 6-*epi*-metabolites, have not been detected as natural metabolites of any *Aspergillus* species to date, the likelihood of this pathway being operable is greatly diminished. Although there is currently a high level of uncertainty in the final steps of the versicolamide B biosynthesis, future work with incorporation studies and characteri-

Scheme 14. Some possible biogeneses of versicolamide B.

zation of the other *Not* enzymes should allow for a complete, high-resolution elucidation of the entire biosynthetic pathway.

3. Outlook

The identification and characterization of the NotF and NotC enzymes from a marine-derived *Aspergillus* sp. have allowed us to determine the timing of the two prenylation steps in the biogenesis of stephacidin A, and to propose a revised biogenetic pathway. However, the enzymes involved in a number of other steps are still undetermined, and until these are identified, the exact pathway remains uncertain. To this end, efforts are ongoing to identify the remaining genes involved in the pathway and parallel efforts in the genomic mining of *Aspergillus versicolor*, which produces the antipodal natural products, stephacidin A, notoamide B, and versicolamide B, are being actively investigated. We have also undertaken the syntheses of ^{13}C-labeled notoamides S and T, to aid in determining if **45** is truly the final precursor to both (+)- and (−)-stephacidin A.

This program has relied heavily on a robust technical platform we have built over the past two decades, to achieve the chemical synthesis of virtually every conceivable substrate and product for every stage of the biosynthetic pathways to these prenylated indole alkaloids. The discovery that Nature produces some of these metabolites in

antipodal forms (and optically pure), has greatly added to the intrigue associated with how Nature fashions such beautiful structures. The technical advances in genome sequencing technology, proteomics, and genomics, have provided us with the powerful tools necessary to fully understand the fascinating biogenesis of these natural substances. We are at a very exciting stage of this work and look forward to the discoveries that lie ahead.

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