

# Biosynthetic Studies of the Notoamides: Isotopic Synthesis of Stephacidin A and Incorporation into Notoamide B and Sclerotiamide

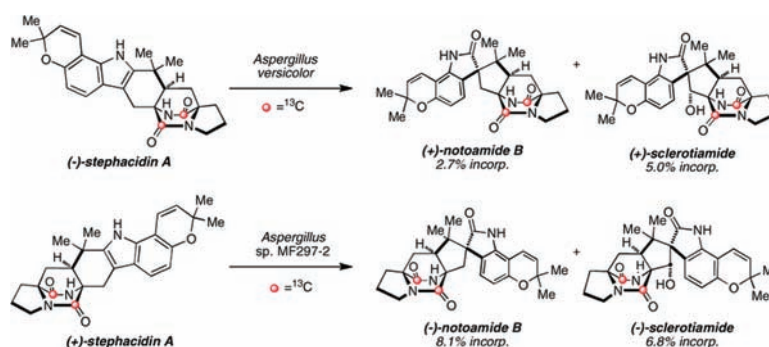
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## ABSTRACT



The advanced natural product stephacidin A is proposed as a biosynthetic precursor to notoamide B in various *Aspergillus* species. Doubly <sup>13</sup>C-labeled racemic stephacidin A was synthesized and fed to cultures of the terrestrial-derived fungus, *Aspergillus versicolor* NRRL 35600, and the marine-derived fungus, *Aspergillus* sp. MF297-2. Analysis of the metabolites revealed enantiospecific incorporation of intact (–)-stephacidin A into (+)-notoamide B in *Aspergillus versicolor* and (+)-stephacidin A into (–)-notoamide B in *Aspergillus* sp. MF297-2. <sup>13</sup>C-Labeled sclerotiamide was also isolated from both fungal cultures.

Elucidation of the biosynthesis of prenylated indole alkaloids containing a unique bicyclo[2.2.2]diazaoctane ring system has been an area of intense research for several

years.<sup>1</sup> Mainly produced by fungi in the genera *Aspergillus* and *Penicillium*, the stephacidins,<sup>2</sup> notoamides,<sup>3</sup> paraherquamides,<sup>4</sup> malbrancheamides,<sup>5</sup> marcfortines,<sup>6</sup> asperparalines,<sup>7</sup> and brevianamide<sup>8</sup> families of natural products are biosynthetically derived from three different building blocks, tryptophan, a cyclic amino acid, and one or two

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mevalonate-derived isoprene units.<sup>9</sup> Early studies by Sammes<sup>10a</sup> and Birch,<sup>10b</sup> as well as by our laboratory,<sup>1</sup> have suggested that the bicyclo[2.2.2]diazaoctane core found in these families of secondary metabolites is formed biosynthetically via an intramolecular hetero-Diels–Alder (IMDA) reaction of a 5-hydroxy-pyrazin-2(1*H*)-one. The proposed biosynthetic IMDA reaction has been extensively validated by its application to the biomimetic total synthesis of several members of this family of prenylated indole alkaloids including brevianamide B,<sup>11</sup> VM55599,<sup>12</sup> marcfortine C,<sup>13</sup> stephacidin A,<sup>14</sup> malbrancheamide,<sup>15</sup> and versicolamide B.<sup>3c,16</sup> Nonetheless, the complete biosynthetic pathways of these secondary metabolites are yet to be fully elucidated.

In 2007, Tsukamoto and co-workers isolated the known metabolites stephacidin A and sclerotiamide from a marine-derived *Aspergillus* sp. MF297-2.<sup>3a</sup> Along with these metabolites, Tsukamoto reported the isolation of several new metabolites, some of which possess the same pyraindoles moiety and/or core bicyclo[2.2.2]diazaoctane ring system as stephacidin A and sclerotiamide, and named this new family of prenylated indole alkaloids the

notoamides. One novel metabolite in particular, notoamide B, contains a similar structural core to both stephacidin A and sclerotiamide, which suggested that notoamide B might serve as a biosynthetic metabolite derived from stephacidin A. Thus, questions arose as to whether or not the stephacidins and notoamides share a common biogenesis and if stephacidin A could be a direct precursor to notoamide B.

Elucidation of the biosynthesis of the stephacidins and notoamides garnered additional intrigue when Gloer et al. reported the isolation of the corresponding enantiomers of stephacidin A and notoamide B from the closely related fungus, *Aspergillus versicolor* NRRL 35600.<sup>3c</sup> In addition, the individual (+)- and (–)-enantiomers of versicolamide B were isolated from *Aspergillus versicolor* NRRL 35600 (Gloer) and the marine-derived *Aspergillus* sp. MF297–2 (Tsukamoto). As depicted in Scheme 1, we recently proposed that oxidation and tautomerization of the proposed precursor notoamide S would yield the key *achiral* azadiene species (**1**).<sup>17</sup> Cycloaddition of the azadiene intermediate, followed by oxidative ring closure to the pyran, would produce the (+)- and (–)-enantiomers of stephacidin A in the respective producing organisms. Face-selective oxidative rearrangement of the 2,3-disubstituted indole would then yield the corresponding *spiro*-oxindoles, (+)- and (–)-notoamide B, respectively. A second face-selective oxidation of notoamide B at the C-10 position (sclerotiamide numbering) would give the respective (+)- and (–)-sclerotiamide enantiomers.

Our laboratory recently provided corroborating laboratory support for the proposed biosynthetic conversion of stephacidin A to notoamide B through the efficient biomimetic oxidation of stephacidin A to notoamide B by deploying an oxaziridine.<sup>14</sup> However, it still remained to be demonstrated that this chemically feasible oxidative transformation was indeed valid in Nature. Furthermore, the biosynthetic conversion of stephacidin A to sclerotiamide (which presumably proceeds via notoamide B) was still unknown. To specifically investigate the biosynthetic formation of these metabolites in both *Aspergillus* fungal cultures, we relied on traditional isotopically labeled substrate incorporation techniques. Herein we report the preparation of doubly <sup>13</sup>C-labeled (±)-stephacidin A and the enantioselective biotransformation of this substrate to (+)-notoamide B and (+)-sclerotiamide in *Aspergillus versicolor* NRRL 36500, as well as (–)-notoamide B and (–)-sclerotiamide in *Aspergillus* sp. MF297-2.

Following an unlabeled biomimetic strategy recently described by our laboratory,<sup>14</sup> the synthesis of doubly <sup>13</sup>C-labeled stephacidin A commenced with the amino acid coupling of the <sup>13</sup>C<sub>1</sub>-labeled reverse-prenylated tryptophan derivative **2**<sup>14</sup> with <sup>13</sup>C<sub>1</sub>-labeled L-proline derivative **3** in the presence of HATU and <sup>i</sup>Pr<sub>2</sub>NEt (Scheme 2). Deprotection and cyclization of amide **4** was achieved in one step with a solution of morpholine in THF to afford a

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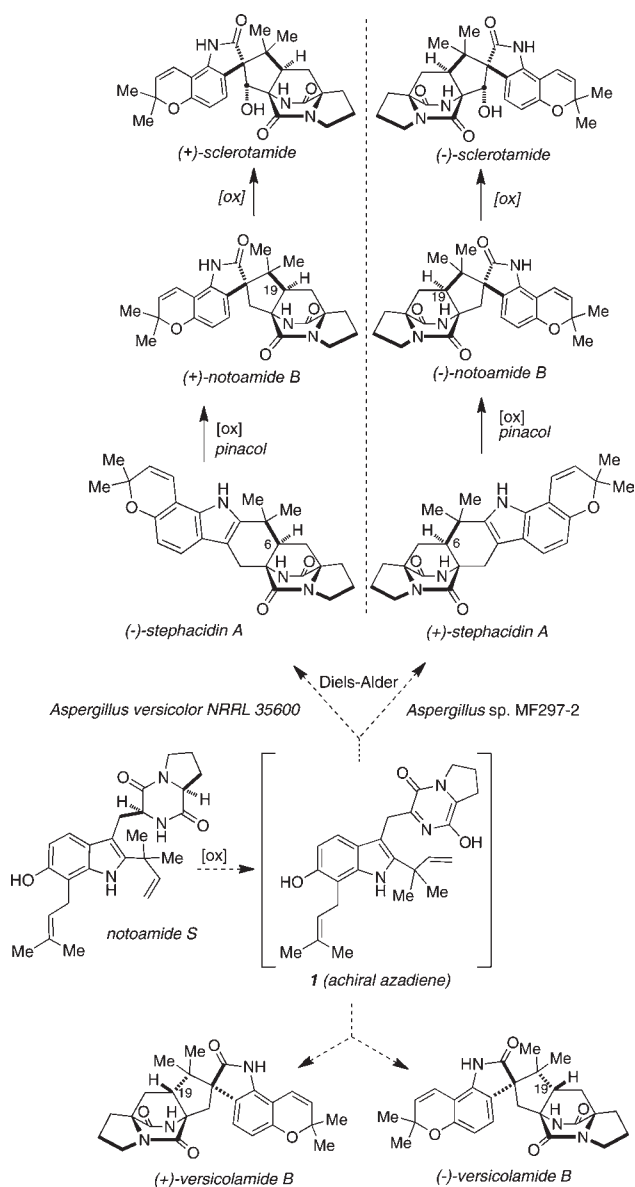
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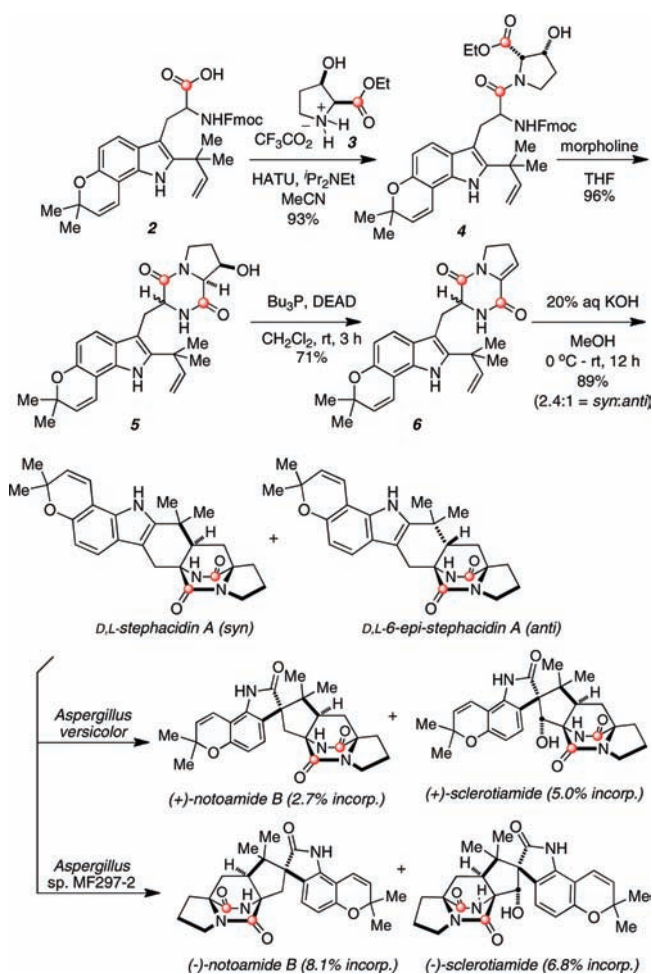
**Scheme 1.** Possible Biosynthetic Relationship of Some Antipodal Metabolites



mixture of diastereomers of dioxopiperazine **5**. Both diastereomers were subjected together through the Mitsunobu-type elimination with  $\text{PBu}_3$  and DEAD to yield enamide **6**. Treatment of **6** with aqueous KOH in MeOH effected the intramolecular hetero-Diels–Alder reaction to produce racemic cycloadducts *D,L*-stephacidin A and *D,L*-6-*epi*-stephacidin A as a 2.4:1 separable mixture of diastereomers favoring the desired *syn*-stereochemistry as previously observed.

With the desired  $^{13}\text{C}$ -labeled substrate in hand, *D,L*-stephacidin A was added to cultures of *A. versicolor* NRRL 35600 in a precursor incorporation experiment. Fungal extracts from this experiment were analyzed by LC-MS and  $^{13}\text{C}$  NMR spectroscopy, and further analysis of the electrospray mass spectrum showed 2.7% intact

**Scheme 2.** Doubly  $^{13}\text{C}$ -Labeled Stephacidin A and Biotransformation to Notoamide B



incorporation of labeled (–)-stephacidin A into (+)-notoamide B.<sup>15b,18</sup> The unreacted stereoisomer (+)-stephacidin A was also isolated from the fungal extract, which was anticipated since it possesses the opposite absolute configuration of the metabolite notoamide B produced by *A. versicolor*. The metabolite (+)-sclerotamide<sup>3a,19</sup> was also found to contain intact double- $^{13}\text{C}$ -labels (5.0% incorporation).

In a similar precursor incorporation study with *D,L*-stephacidin A in the marine-derived *Aspergillus* sp. MF297-2, analysis of the fungal extract via ESI mass spectrometry revealed significant  $^{13}\text{C}$ -incorporation into (–)-notoamide B (8.1%), as well as 6.8% intact incorporation into the known metabolite, (–)-sclerotamide. In addition to the detection and isolation of (–)-notoamide B and (–)-sclerotamide, the unreacted enantiomer, (–)-stephacidin A, was similarly recovered from the precursor incorporation study with *Aspergillus* sp. MF297-2.

(18) Calculated according to the method outlined in: Lambert, J. B.; Shurvell, H. B.; Lightner, D. A.; Cooks, R. G. *Organic Structural Spectroscopy*; Prentice Hall: Upper Saddle River, NJ, 1998; pp 447–448.

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In conclusion, these results provide experimental validation for the hypothesis that complementary, face-selective oxidative enzymes (currently presumed to be flavoenzymes) are present in both *Aspergillus versicolor* NRRL 35600 and *Aspergillus* sp. MF297-2. Specifically in *A. versicolor* NRRL 35600, this oxidase is responsible for the biosynthetic conversion of (–)-stephacidin A into (+)-notoamide B, while a stereochemically complementary oxidase in the Tsukamoto marine-derived *Aspergillus* sp. MF297–2 converts (+)-stephacidin A into (–)-notoamide B. Furthermore, these results show that the known metabolite sclerotiamide is biosynthetically derived from stephacidin A in both *Aspergillus* fungal cultures in an enantioselective manner. While these results provide further insight into the antipodal biogenesis of the stephacidins and notoamides, several questions remain unanswered. In particular, midstage precursors between

notoamide S and stephacidin A remain to be characterized. A combination of genome mining, proteomics, and bio-transformation experiments aimed at elucidating these questions remain the focus of ongoing studies in these laboratories and will be reported in due course.

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**Supporting Information Available.** Synthetic procedures, characterization, and calculation of percent incorporation. This material is available free of charge via the Internet at <http://pubs.acs.org>.