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## Bioconversion of 6-*epi*-Notoamide T Produces Metabolites of Unprecedented Structures in a Marine-derived *Aspergillus* sp

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### Abstract

We previously described the bioconversion of Notoamide T into (+)-Stephacidin A and (–)-Notoamide B, which suggested that Versicolamide B (**8**) is biosynthesized from 6-*epi*-Notoamide T (**10**) via 6-*epi*-Stephacidin A. Here we report that [<sup>13</sup>C]<sub>2</sub>-**10** was incorporated into isotopically enriched **8** and seven new metabolites, which were not produced under normal culture conditions. The results suggest that the addition of excess precursor activated the expression of dormant tailoring genes giving rise to these structurally unprecedented metabolites.

### Keywords

Notoamide; *Aspergillus* sp; Bioconversion; Alkaloid

The Notoamides are a family of prenylated indole alkaloids produced by the marine-derived *Aspergillus* sp. MF297-2,<sup>1</sup> and have also been detected as natural metabolites of *A. versicolor* NRRL 35660.<sup>2</sup> These natural substances contain a unique bicyclo[2.2.2]diazaoctane core structure and have attracted significant attention from the synthetic community.<sup>3</sup> Previously, Sammes<sup>4</sup> and Birch<sup>5</sup> suggested that the bicyclo[2.2.2]diazaoctane core of these alkaloids is likely to arise from a biosynthetic intramolecular hetero Diels-Alder (IMDA) reaction. Initially, we isolated Notoamides A–D

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(**1–4**) and Stephacidin A (**5**) from the fungal extract<sup>1a</sup> and proposed a biogenetic pathway,<sup>6</sup> in which a putative biosynthetic precursor was postulated to be Notoamide E (**6**) (Scheme 1). The precursor incorporation experiment utilizing [<sup>13</sup>C]-**6** afforded **3** and **4** but, metabolites containing the bicyclo[2.2.2]diazaoctane core, such as **1**, **2**, and **5**, were not obtained.<sup>1c</sup>

We then speculated that Notoamide S (**7**) would be the progenitor of **6** and converted to **5/2** and Versicolamide B (**8**) by an enzyme-catalyzed IMDA reaction through Notoamide T (**9**) and 6-*epi*-Notoamide T (**10**), respectively. Although we initially reported that **8** isolated from *Aspergillus* sp. MF297-2 was the (–)-enantiomer,<sup>1d</sup> careful, recent re-analysis of the natural metabolite revealed that the absolute configuration should be revised to the (+)-form. We have also carefully confirmed that the absolute configurations of Stephacidin A and Notoamide B are as originally assigned. Thus, the marine-derived *Aspergillus* sp. MF297-2 produces (+)-Stephacidin A, (–)-Notoamide B, and (+)-Versicolamide B and the terrestrial *A. versicolor* NRRL 35660<sup>2</sup> produces the enantiomers: (–)-Stephacidin A, (+)-Notoamide B, but the same enantiomer of (+)-Versicolamide B as that produced in the marine-derived fungus.<sup>7</sup> These stereochemical facts present numerous fascinating biogenetic puzzles that have yet to be unraveled. In our ongoing efforts toward understanding the molecular bases for the biogenesis of the Stephacidins, Notoamides, and Versicolamide B we have previously described the bioconversions of **5** into **2**<sup>8</sup> and of **9** into **5/2**.<sup>9</sup> These results clearly suggested that (+)-**8** would be biosynthesized from **10** via 6-*epi*-Stephacidin A (**11**).<sup>10</sup> In order to verify this hypothesis, we evaluated the detection of **11** in the culture of the marine-derived *Aspergillus* sp. MF297-2 and the bioconversion of **10** into **11** and **8**.

Since 6-*epi*-Stephacidin A (**11**) was expected to be a short-lived precursor, the time-course of the metabolite profile was analyzed by HPLC. The fungus was cultured on agar medium in petri dishes and the extract was semi-purified by ODS column chromatography. To monitor the presence of **11**, the <sup>1</sup>H NMR spectrum of the fraction, of which retention time corresponded to authentic, synthetic **11**, was measured. The spectra revealed that **11** existed only in the culture incubated for six days and subsequently disappeared. The CD spectrum of **11** revealed that this substance possesses the 11*S*,17*S*-configuration (Supplementary data, Figure S1).<sup>11</sup> The experimental data clearly indicated that **11** was produced by the fungus in the early phase of growth and was then rapidly converted to other downstream metabolites. We have previously demonstrated that Notoamide E (**6**) was also a short-lived endogenous precursor and was rapidly converted into Notoamide C (**3**) and Notoamide D (**4**).<sup>1c</sup>

Next, we conducted a precursor incorporation experiment using synthetic, [<sup>13</sup>C]<sub>2</sub>-(±)-6-*epi*-Notoamide T (**10**).<sup>9</sup> The fungal cells of *Aspergillus* sp. MF297-2 were incubated in a trace element solution with [<sup>13</sup>C]<sub>2</sub>-(±)-**10**. After fourteen days, the culture was extracted with *n*-BuOH and the extract was purified by ODS column chromatography followed by ODS HPLC to afford [<sup>13</sup>C]<sub>2</sub>-Versicolamide B (**8**) and very surprisingly, seven new metabolites, [<sup>13</sup>C]<sub>2</sub>-6-*epi*-Notoamides T3–T8 (**12–17**) and [<sup>13</sup>C]<sub>2</sub>-6-*epi*-Notoamide I (**18**) (Scheme 2).

A molecular formula of [<sup>13</sup>C]<sub>2</sub>-6-*epi*-Notoamide T3 (**12**) was determined to be C<sub>25</sub><sup>13</sup>C<sub>2</sub>H<sub>30</sub>N<sub>4</sub>O<sub>4</sub> on the basis of the HRESIMS, which indicated the presence of additional carbon and nitrogen atoms in [<sup>13</sup>C]<sub>2</sub>-**12**. Analysis of NMR spectra in DMSO-*d*<sub>6</sub>

(Supplementary data, Table S1) indicated that [ $^{13}\text{C}$ ] $_2$ -**12** possessed a *spiro*-oxindole structure, similar to that which constitutes Versicolamide B (**8**). The isoprene unit still remained at C-7 yet, the pyran ring was absent in [ $^{13}\text{C}$ ] $_2$ -**12**. In addition, instead of *ortho*-coupled aromatic protons,  $\delta$  7.11 (H-4) and  $\delta$  6.37 (H-5), observed in **8**, a single aromatic proton at  $\delta$  7.75 (H-4, singlet) and a quaternary carbon at  $\delta$  133.8 (C-5) were observed in [ $^{13}\text{C}$ ] $_2$ -**12**. These data clearly suggested that the remaining CHNO unit was attached to C-5. The HSQC spectrum of this substance revealed that a single proton ( $\delta$  8.60, H-30, singlet) was directly attached to a carbon at  $\delta$  152.0 (C-30), and the carbon was attached to hetero atoms as judged by the chemical shift. HMBC cross peaks from  $\delta_{\text{H}}$  8.60 to  $\delta_{\text{C}}$  133.8 (C-5) and 148.0 (C-6) and the chemical shifts of these carbons suggested that an oxazole ring was fused to the tryptophan moiety as shown in Figure 1. The presence of an oxygen atom bonded to C-6 is firmly explained by the biosynthetic relationship with the precursor, [ $^{13}\text{C}$ ] $_2$ -**10**.

[ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamide T4 (**13**) has a molecular formula of  $\text{C}_{25}^{13}\text{C}_2\text{H}_{30}\text{N}_4\text{O}_5$  as established by HRESIMS, and possesses one oxygen atom more than [ $^{13}\text{C}$ ] $_2$ -**12**. The NMR spectra of [ $^{13}\text{C}$ ] $_2$ -**13** in DMSO- $d_6$  was almost superimposable on those of [ $^{13}\text{C}$ ] $_2$ -**12** except for the high-field shift of H-4 ( $\delta$  7.03 in [ $^{13}\text{C}$ ] $_2$ -**13**;  $\delta$  7.75 in [ $^{13}\text{C}$ ] $_2$ -**12**) (Supplementary data, Table S1). Two carbon signals at C-5 and C-30 of **13** were not detected in the HMBC spectrum in DMSO- $d_6$ , suggesting the possibility of two rapidly equilibrating isomers; an HMBC spectrum was measured in acetone- $d_6$  at 5 °C with optimized  $J$  value of 4 Hz. Under this condition, cross peaks from H-4 ( $\delta$  7.20) to C-5 ( $\delta$  135.2) and C-30 ( $\delta$  156.8) were observed (Figure 1). These data suggested that [ $^{13}\text{C}$ ] $_2$ -**13** possess an 2-oxazolone unit instead of the oxazole unit present in [ $^{13}\text{C}$ ] $_2$ -**12**.

Analyses of NMR spectra of [ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamides T5-T8 (**14**–**17**) (Supplementary data, Tables S2 and S3) readily indicated the presence of *spiro*-oxindole structures with various biosynthetic modifications derived from the isoprene unit at C-7 and the hydroxyl group at C-6. [ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamide T5 (**14**) has a formula of  $\text{C}_{24}^{13}\text{C}_2\text{H}_{31}\text{N}_3\text{O}_5$ , suggesting that the attached portion at C-6 and C-7 was composed of  $\text{C}_5\text{H}_{10}\text{O}_2$ . The COSY spectrum showed a -CH $_2$ CH- unit and the methylene signals (H $_2$ -25) showed HMBC correlation to C-7 ( $\delta$  108.0) (Figure 2). An exchangeable proton at  $\delta$  4.58 (27-OH) showed HMBC correlations with carbons at  $\delta$  24.6 (CH $_3$ , C-28), 26.1 (CH $_3$ , C-29), 69.6 (C, C-27), and 89.2 (CH, C-26). A singlet methyl signal at  $\delta$  1.12 (H $_3$ -29) showed HMBC correlations with carbons at  $\delta$  24.6, 69.6 and 89.2. These data suggest that [ $^{13}\text{C}$ ] $_2$ -**14** contains a substituted dihydrofuran ring instead of the pyran ring present in **8**. Although H-26 was observed as a single signal, [ $^{13}\text{C}$ ] $_2$ -**14** might be a mixture of 26-epimers. [ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamide T6 (**15**) has a formula of  $\text{C}_{24}^{13}\text{C}_2\text{H}_{29}\text{N}_3\text{O}_5$ ; one H $_2$  unit less than [ $^{13}\text{C}$ ] $_2$ -**14**. An olefin proton at  $\delta$  6.59 (H-25) showed HMBC correlations with three quaternary carbons at  $\delta$  113.7 (C-7), 155.0 (C-6), and 164.6 (C-26) (Figure 2). Therefore, the structure of [ $^{13}\text{C}$ ] $_2$ -**15** corresponds to a dehydrogenated derivative of [ $^{13}\text{C}$ ] $_2$ -**14**. [ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamides T7 (**16**) and T8 (**17**) were afforded in the ratio of 1:1 and they possess an identical formula  $\text{C}_{24}^{13}\text{C}_2\text{H}_{29}\text{N}_3\text{O}_4$ , an oxygen less than [ $^{13}\text{C}$ ] $_2$ -**15**. Their NMR spectra were almost superimposable, and differences were observed at proton signals at  $\delta$  10.40/10.42 (H-1, **16/17**), 5.04/5.05 (H-28), and 1.70/1.72 (H $_3$ -29). Interpretation of 2D NMR spectra implicated that they corresponded

to dehydro derivatives of [ $^{13}\text{C}$ ] $_2$ -**14** and are C-26-epimers of each other. The structure of [ $^{13}\text{C}$ ] $_2$ -6-*epi*-Notoamide I (**18**) was deduced by the comparison of NMR data of Notoamide I<sup>b</sup> and a formula  $\text{C}_{24}^{13}\text{C}_2\text{H}_{27}\text{N}_3\text{O}_4$  shown by HRESIMS. The diversity of structures involving prenyl group ring closures on a terminal benzyl group is reminiscent of a recent report of novel Aspulvinones, where similar open ring, pyran, and furan ring systems were described.<sup>13</sup>

Although the precursor incorporation experiment was performed with a synthetic, racemic precursor [ $^{13}\text{C}$ ] $_2$ -(±)-**10**, metabolites [ $^{13}\text{C}$ ] $_2$ -**12–18** and [ $^{13}\text{C}$ ] $_2$ -**8** were found to be optically inactive, as revealed by the CD spectra of these substances (Supplementary data, Figures S47–S54) compared with a natural sample of (+)-Versicolamide B (Supplementary data, Figure S55). These results definitively suggest that the fungus converted both enantiomeric precursors, [ $^{13}\text{C}$ ] $_2$ -(±)-**10**, which clearly suggested that enzymes involved in these biotransformations were effective for these exogenic substrates.

Since **11** was proposed to be a precursor of **8**, we expected to detect [ $^{13}\text{C}$ ] $_2$ -(±)-**11** in precursor incorporation experiments of [ $^{13}\text{C}$ ] $_2$ -(±)-**10** but we did not observe this species. We then performed precursor incorporation experiments of non-labeled-(±)-**10** on minimal media agar plates. After sixteen days, the culture was extracted with *n*-BuOH and purification of the extract yielded **11** and four new metabolites, 6-*epi*-Notoamides T9–T12 (**19–22**) (Scheme 3; Supplementary data, Table S4). The CD spectra revealed that the metabolites were all racemates (Supplementary data, Figures S56–S60).

Biosynthetically, [ $^{13}\text{C}$ ] $_2$ -**12** would be derived from [ $^{13}\text{C}$ ] $_2$ -**10** by oxygenation at C-2 and successive pinacol rearrangement followed by an attachment of a CHN unit (Scheme 4). Glycine is one possible donor unit of both the new nitrogen and carbon in the oxazolidine and oxazolidinone units of **12** and **13**. It is also possible that the nitrogen and carbon atoms are derived separately from distinct biosynthetic donors. While beyond the scope of this communication, we are seeking to elucidate the genes responsible for the production of these novel metabolites, the primary metabolic donor units and the mechanism for the formation of the new heterocyclic units of **12** and **13**. At present, however, the origin of this unit, and particularly, the origin and mechanism for the addition of the new nitrogen atom remains unclear. To the best of our knowledge, the oxidative amination of an aromatic ring is an unprecedented biochemical transformation. Based on our previous work to obtain the complete sequence for the *Aspergillus* sp. MF297-2 fungal genome,<sup>12</sup> we anticipate a thorough transcriptome analysis will enable initial identification of gene products expressed following introduction of [ $^{13}\text{C}$ ] $_2$ -(±)-6-*epi*-Notoamide T (**10**) into the culture medium leading to the reported series of novel alkaloids.

In conclusion, we have demonstrated the bioconversion of 6-*epi*-Notoamide T (**10**) to 6-*epi*-Stephacidin A (**11**) and Versicolamide B (**8**). In the precursor incorporation experiment of [ $^{13}\text{C}$ ] $_2$ -(±)-**10** in liquid media, seven new metabolites were unexpectedly obtained, [ $^{13}\text{C}$ ] $_2$ -**12–18**, which are not produced by the fungus (500 petri dishes) under normal culture conditions to any detectable extent. Among the metabolites, [ $^{13}\text{C}$ ] $_2$ -**12** and [ $^{13}\text{C}$ ] $_2$ -**13** contain unprecedented structures, including the oxazole and 2-oxazolone units, respectively, derived from the oxidative amination of the tryptophan moiety. On the other hand, the precursor

incorporation experiment of ( $\pm$ )-**10** on agar medium, four new oxidized metabolites **19–22** were obtained. These data suggest that the exogenous substrate, 6-*epi*-Notoamide T (**10**), selectively activates the expression of dormant tailoring genes that significantly expands the metabolome of the producing organism. Our laboratories are actively investigating the origins of the carbon and nitrogen atoms of the oxazole and 2-oxazolone units. We are also pursuing identification of the unique tailoring enzymes that catalyze construction of these unprecedented substances and how the exogenous substrate, [ $^{13}\text{C}$ ] $_2$ -( $\pm$ )-**10**, has triggered gene expression. This study supports the growing body of evidence that the secondary metabolome of fungi may be considerably more plastic and subject to considerable chemical diversification through addition of both endogenous, as well as exogenous substances.<sup>14</sup> Efforts to capitalize on these findings is under intensive investigation in our laboratories and will be reported in due course.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

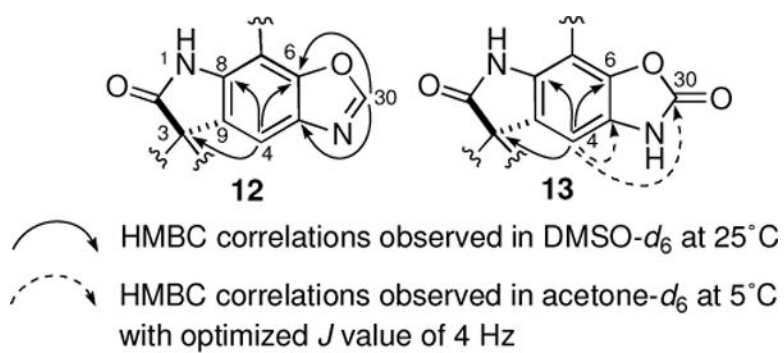
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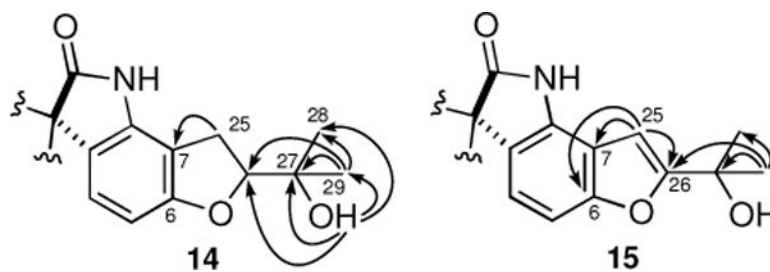
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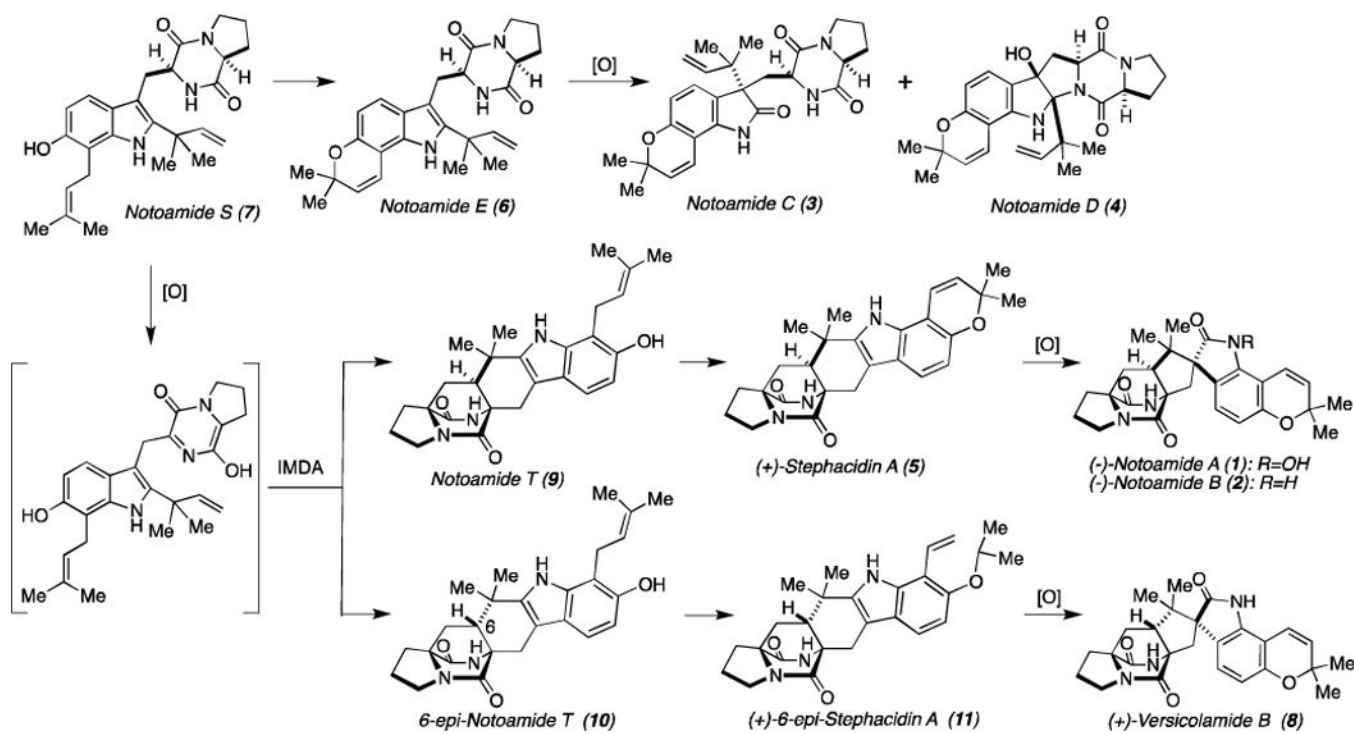


**Figure 1.**  
HMBC correlations observed for **12** and **13**.

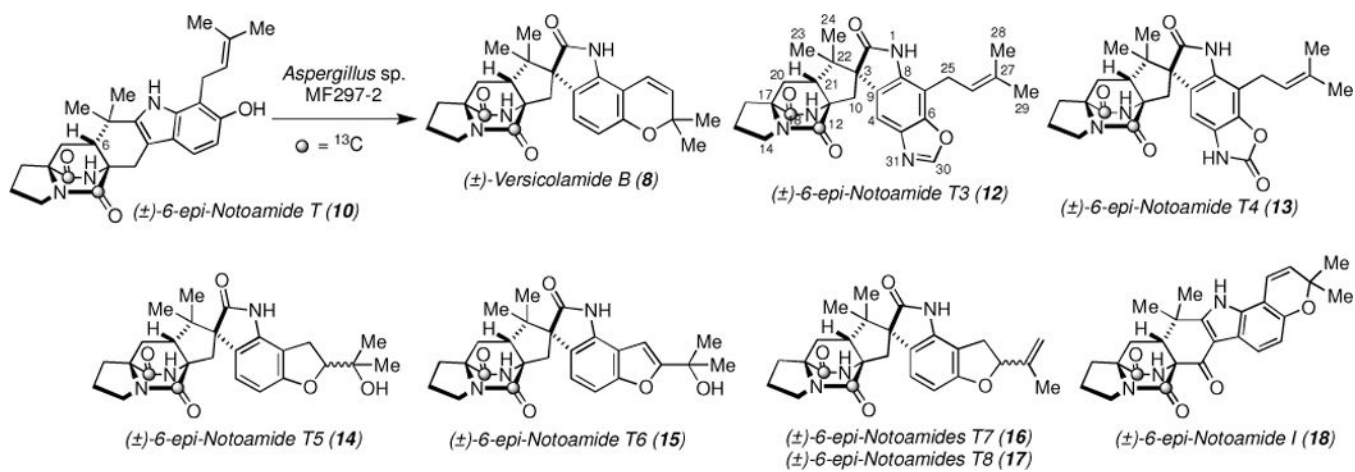


**Figure 2.**  
HMBC correlations observed for **14** and **15**.

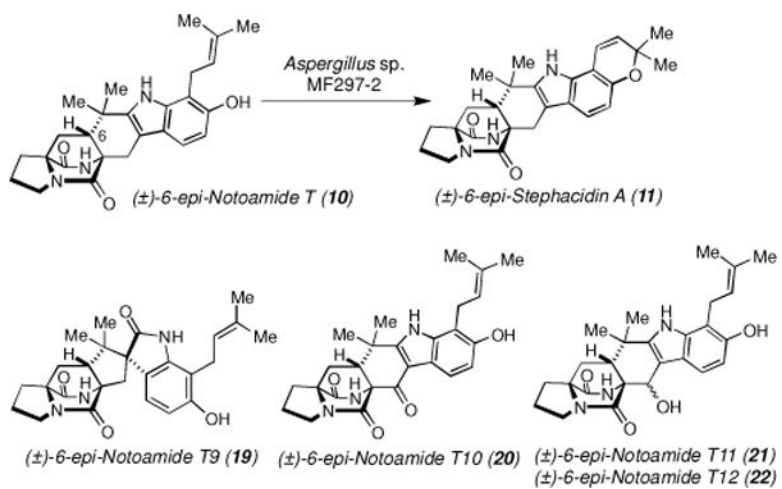




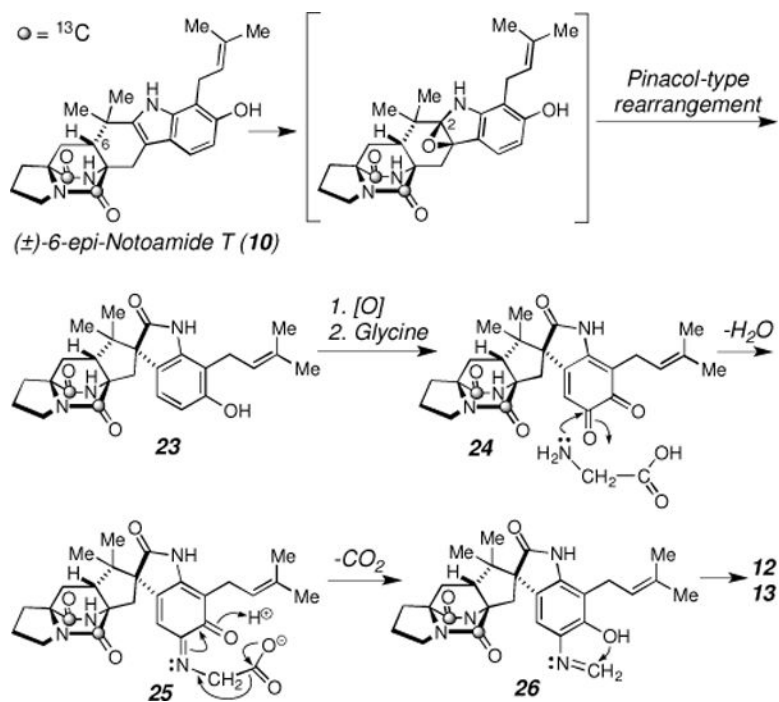
**Scheme 1.**  
Biosynthetic Pathway for the Notoamides, Stephacidin A, and Versicolamide B

**Scheme 2.**

Bioconversion of 6-epi-Notoamide T into Versicolamide B and seven new metabolites

**Scheme 3.**

Bioconversion of (±)-6-*epi*-Notoamide T into (±)-6-*epi*-Stephacidin A and four new metabolites on the agar minimal media.



**Scheme 4.**  
 Possible biosynthetic pathway of **12** and **13** from **10**.