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In Vitro Stepwise Reconstitution of Amino Acid Derived Vinyl Isocyanide Biosynthesis: Detection of an Elusive Intermediate

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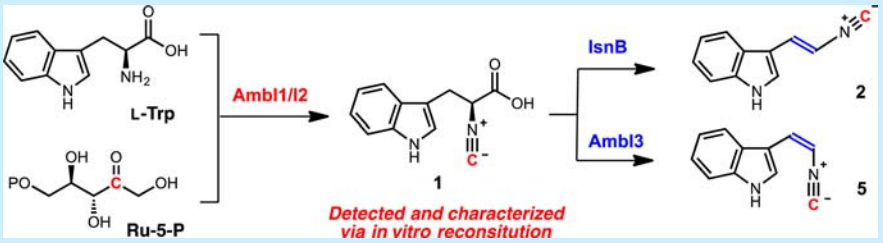
Supporting Information



ABSTRACT: In vitro reconstitution of a newly discovered isonitrile synthase (AmbI1 and AmbI2) and the detection of an elusive intermediate (*S*)-3-(1*H*-indol-3-yl)-2-isocyanopropanoic acid **1** in indolyl vinyl isocyanide biogenesis are reported. The characterization of iron/2-oxoglutarate (Fe/2OG) dependent desaturases IsnB and AmbI3 sheds light on the possible mechanism underlying stereoselective alkene installation to complete the biosynthesis of (*E*)- and (*Z*)-3-(2-isocyanovinyl)-1*H*-indole **2** and **5**. Establishment of a tractable isonitrile synthase system (AmbI1 and AmbI2) paves the way to elucidate the enigmatic enzyme mechanism for isocyanide formation.

Natural products containing vinyl isonitrile (isocyanide) functionality have been isolated from a diverse array of microbial organisms and possess distinct structural complexities and biological activities.[1,2] As isocyanide represents a unique class of reactive functional groups,[3,4] its presence in natural products has raised considerable interest in delineating its biosynthetic origin.[5] The genetic basis for vinyl isocyanide biogenesis was initially disclosed by the analysis of environmental DNAs.[6] A putative isonitrile synthase (IsnA) and an Fe(II)/2-oxoglutarate(2OG)-dependent oxygenase (IsnB) derived from *isnA* and *isnB* genes were shown to collectively convert L-tryptophan (L-Trp) and ribulose-5-phosphate (Ru-5-P) to (*E*)-3-(2-isocyanovinyl)-1*H*-indole (**2**).[7] Heavy-isotope feeding studies established that the terminal *sp*-hybridized isocyanide carbon appended to the L-Trp α-amine is derived from the carbonyl carbon of Ru-5-P.[7] In addition, feeding the methyl ester derivative of (*S*)-3-(1*H*-indol-3-yl)-2-isocyanopropanoic acid **1** to *E. coli* cells overexpressing *isnB* led to the production of **2**.[7] These observations led to a proposal on the molecular basis for vinyl isocyanide biogenesis that proceeds via intermediate **1** by the sole action of IsnA on L-Trp, followed by hydroxylation and decarboxylative desaturation catalyzed by IsnB (Figure 1, eq 1).[7] In addition to the *isn* operon, two vinyl isocyanide biogenetic pathways were identified. XnPvcA and XnPvcB were shown to produce (*E*)-4-(2-isocyanovinyl)phenol (**4**) from L-tyrosine (L-Tyr) via the presumptive intermediate (*S*)-3-(4-hydroxyphenyl)-2-isocyanopropanoic acid **3** (Figure 1, eq 3).[8,9] More recently, enzymes for the biogenesis of (*Z*)-3-(2-isocyanovinyl)-1*H*-indole **5** were identified in the biosynthetic machinery for the hapalindole-type alkaloids,[10,11] typified by

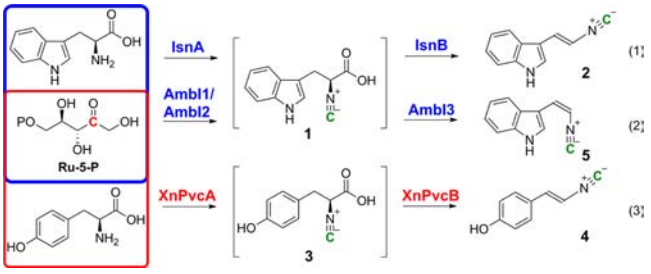


Figure 1. Proposed biosynthetic pathways for vinyl isocyanide containing **2**, **5**, and **4** by (1) IsnA and IsnB; (2) AmbI1, AmbI2, and AmbI3; and (3) XnPvcA and XnPvcB proteins. Equations 1−3 show those proposals outlined in the literature.[7,8,10]

AmbI1, AmbI2, and AmbI3 proteins and their close homologues WelI1, WelI2, and WelI3. AmbI1 and AmbI2 are protein homologues of IsnA.[10] It was proposed they act in complex to produce **1** as an intermediate, analogous to the proposal for IsnA, which is stereoselectively converted to **5** by AmbI3, an IsnB homologue (Figure 1 eq 2).

Although **1** and **3** have been proposed as the direct enzymatic product of IsnA (AmbI1 and AmbI2) and XnPvcA respectively,[7−11] they have not been detected in any vinyl isocyanide producing system either *in vivo* or *in vitro*. Thus, one cannot completely rule out the involvement of an additional protein, such as IsnB or AmbI3, in isocyanide formation. The

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conversion of the carbon atom from the carbonyl group in Ru-5-P to that in an isocyanide moiety requires a 2-e oxidation. It is conceivable that IsnA/IsnB, AmbI1/AmbI2/AmbI3 or XnPvcA/XnPvcB act cooperatively, where the Fe/2OG-dependent oxygenases IsnB/AmbI3/XnPvcB catalyze the required 2-e oxidation to elevate the oxidation state of the carbonyl carbon via unknown intermediate(s). The observed *in vivo* or *in vitro* conversions of **1** to **2**/**5** or **3** to **4** by IsnB/AmbI3 or XnPvcB can be attributed to the dual functionality of IsnB/AmbI3/XnPvcB. Similar observations have been made for several members in this enzyme family, such as H6H, AsqJ, and IPNS in scopolamine, methoxyviridicatin, and isopenicillin pathways, respectively.[12−14]

To unambiguously distinguish and define the roles of each protein component in vinyl isocyanide biogenesis and to elucidate the enigmatic enzymatic mechanism for isocyanide formation, the establishment of a tractable *in vitro* or *in vivo* system(s) to detect the intermediacy of **1** or **3** will be required. In this work, we demonstrate that the newly discovered AmbI1, AmbI2, and AmbI3 system fulfills this requirement, and for the first time, the *in vitro* enzymatic generation of **1** from L-Trp and Ru-5-P was achieved using AmbI1 and AmbI2. We further demonstrate the production of **2** using a mixture of purified AmbI1, AmbI2, and IsnB *in vitro*, confirming the functional complementation of IsnA by AmbI1 and AmbI2 and supporting the hypothesis that **1** is a common intermediate for the biogenesis of **2** and **5**. Finally, we show that IsnB is *E*-specific, solely producing **2** from **1**, while AmbI3 generates **5** stereoselectively. Collectively, this study provides conclusive evidence that IsnA or AmbI1 and AmbI2 are the default stand-alone isonitrile synthases that can covert L-Trp with Ru-5-P to **1**.

We initially chose to work on *isn* and *ambI* pathways as they generate **2** and **5**, a pair of diastereomers, in a stereospecific manner via a postulated common intermediate **1**. This constitutes a complementary system that is deemed suitable for the detection of **1** and for understanding how stereoselectivity arises from **1** by protein homologues IsnB and AmbI3. While heterologous overexpressions in *E. coli* and affinity column purifications led to sufficient quantities of N-His_6-tagged AmbI1, AmbI2, AmbI3, and IsnB (SI Methods), we were unable to procure IsnA after numerous attempts. The putative intermediate **1** was procured by chemical synthesis from L-Trp. Esterification of L-Trp was followed by the introduction of a formyl group upon the α-amine.[15,16] The formyl moiety was then dehydrated to generate the isocyanide group, and the deprotection of the carboxylate yielded **1** in circa. 40% overall yield (SI Methods). This sequence is different from the published route where the formylation was carried out prior to methylation.[9] In the reported sequence, due to reactivity of the formyl group, the methyl group was introduced using trimethylsilyl diazomethane. Installation of the methyl ester prior to formylation allows for the use of acidic methanol and avoiding diazomethane. Using this modified synthetic sequence, compound **1** was procured in a gram scale. Compound **1** was stable as a solid at −78 °C and has a half-life >4 days in water (4 °C) that enables us to use it both as a standard and as a substrate for subsequent investigation.

To assess the proposed function of AmbI1 and AmbI2 on the biogenesis of **1**, deuterium-enriched d_5-L-Trp was incubated in a reaction mixture containing Ru-5-P, AmbI1, and AmbI2 (Figure 2). The reaction was monitored using liquid chromatography−mass spectrometry (LC-MS). After a 20 min incubation at ambient temperature, a new product peak with the identical retention time as synthetic **1** was observed (Figure 2a, lanes 1/4). The peak associated with the enzymatic reaction product exhibits the expected mass shift of +5, establishing that this product arises from the isotopically labeled L-Trp. This peak was not detected in chromatographs of control experiments where proteins (AmbI1 and AmbI2) or substrates (d_5-L-Trp and Ru-5-P) were absent (Figure 2a, lanes 2/3). The peak was also not seen when Ambl3 and 2OG/Fe(II) were included in the reaction with molecular oxygen (Figure 2a, lane 5), suggesting that **1** was rapidly utilized by AmbI3. The enzymatic conversion of L-Trp and Ru-5-P to **1** by AmbI1 and AmbI2 is a single turnover event under our assay conditions (SI Methods), implying it is likely the rate-limiting step in the vinyl isocyanide formation. Based on the isotope tracer experiment result,[7] where the terminal carbon of isocyanide **1** originates from the keto-center of Ru-5-P, we speculate the enzymatic reaction by AmbI1 and AmbI2 is initiated by forming an imine intermediate to connect two substrates (L-Trp and Ru-5-P) (Figure 2b). While how this postulated imine intermediate is transformed to **1** is a subject for future studies, one plausible route is outlined in Figure 2c. After formation of the imine intermediate, the reaction may proceed through β-keto imine formation generated through loss of the phosphate group and followed by enol−keto tautomerization. Carbon−carbon bond cleavage is triggered by nucleophilic attack of the nitrogen lone pair to generate the future isocyanide fragment and hydroxyl acetone. A subsequent "retro aldol type" reaction would give **1** with the concomitant formation of formaldehyde.

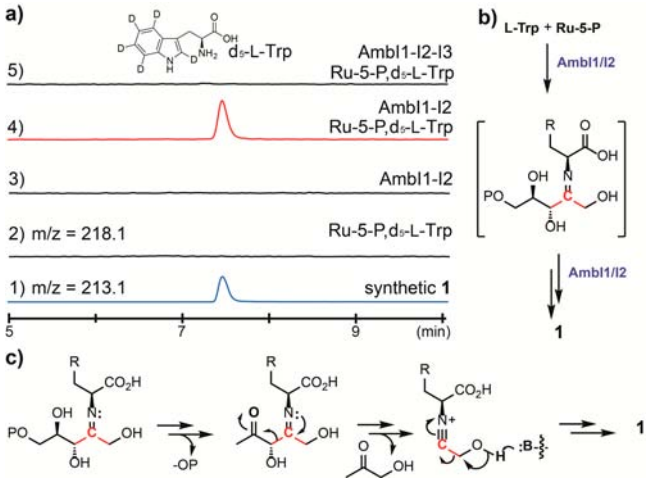

Figure 2. (a) Detection of the elusive intermediate **1** from *in vitro* assay using AmbI1 and AmbI2. The LC-MS EIC chromatograms of synthetic **1** (m/z = 213.1) and d_5-**1** (m/z = 218.1) from enzymatic assay of AmbI1 and AmbI2 in the presence/absence of substrates Ru-5-P and d_5-L-Trp. (b and c) Formation of the imine intermediate by reacting the amine of L-Trp with the ketone of Ru-5-P and a plausible mechanism accounts for the formation of **1** from the imine intermediate.

Having confirmed **1** is the enzymatic product of AmbI1 and AmbI2, we proceeded to validate the functional roles of AmbI3 and its homologue IsnB in the stereodivergent generation of **2** and **5** (Figure 3). First, the products of a reaction containing all three AmbI proteins, d_5-L-Trp, Ru-5-P, and 2OG/Fe(II), were characterized (Figure 3a, lane 4). The peak that corresponds to **5**, but not its diastereomer **2**, was readily detected on an LC-

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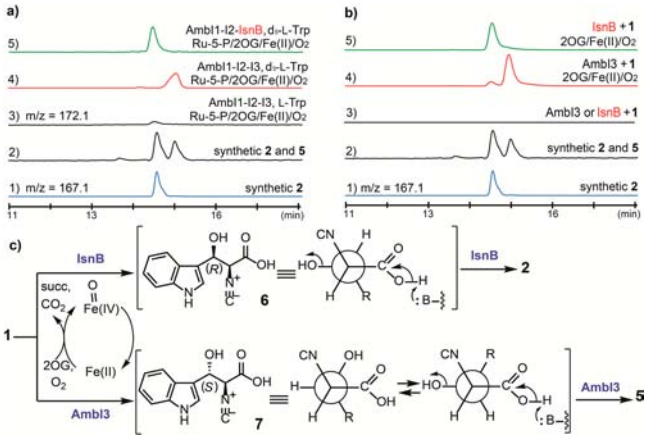

Figure 3. Stereodivergent alkene formation in vinyl isocyanide biogenesis by AmbI3 and IsnB and its potential enzymatic mechanism. The LC-MS EIC chromatograms of **2**/**5** (m/z = 167.1) and d_5-**2**/**5** (m/z = 172.1) from enzymatic assays of the following: (a) AmbI1 and AmbI2 in combination of AmbI3 or IsnB in the presence of substrates (d_5-)L-Trp, Ru-5-P, O_2, and 2OG/Fe(II); (b) AmbI3 or IsnB with Fe(II) and substrate **1** in the presence/absence of O_2, and 2OG. (c) Proposed IsnB and AmbI3 mechanism accounts for the stereoselective alkene formation.

MS chromatogram (Figure 3a, lanes 4 vs 1/2) and exhibits the expected +5 shift in m/z value. When unlabeled (all protium) L-Trp was used, the enzymatic product with the +5 shift in m/z was not seen (Figure 3a, lane 3). Instead, the peak with the same retention time and m/z value as the synthetic standard **5** was detected (data not shown). These results are consistent with the retention of all deuteria from d_5-L-Trp. As we were unable to obtain IsnA protein in a stable form by heterologous expression, we proceeded to probe the function of IsnB by coupling with AmbI1 and AmbI2 that are IsnA homologues. A reaction containing AmbI1, AmbI2, and IsnB proteins, along with all relevant substrates, was analyzed (Figure 3a, lane 5). The peak having m/z = 172.1 and the same retention time of **2** was evident in the LC-MS chromatograms. In the reaction using AmbI1, AmbI2, and IsnB, the peak for the (*Z*)-isomer **5** was not observed. This result is in agreement with prior reports concerning the geometric isomers production from *isn* and *ambI* pathways.[7,10] It further suggests that **1** is a common substrate of IsnB and AmbI3 and likely a true product of the IsnA reaction.

To further probe the stereoselective formation of **2** and **5** by IsnB and AmbI3, we incubated synthetic **1** with IsnB and AmbI3 in the presence of O_2 and 2OG/Fe(II) (Figure 3b). In the case of IsnB, **2** was the only product detected (Figure 3b, lanes 5 vs 1/2). Accumulation of **2** was not detected when O_2 and 2OG were omitted (Figure 3b, lane 3). In the reaction of AmbI3, **5** was observed as the major product. Surprisingly, a minor amount (circa. 5%) of **2** was also detected (Figure 3b, lane 4). These results collectively imply that the stereoselective alkene formation in vinyl isocyanide biogenesis is dominantly governed by the intrinsic differences between AmbI3 and IsnB. Both proteins are homologues of Fe/2OG-dependent oxygenases that typically catalyze hydroxylation via aliphatic C−H bond cleavage using an Fe(IV)-oxo species.[17−19] The observed stereodivergence can be viewed as a downstream result of stereoselective hydroxylation as depicted in Figure 3c. Specifically, to form **2**, the hydroxylation is likely to proceed through pro-*R* C−H cleavage of **1** to produce intermediate **6**. The $C_{sp^2}-C_{sp^2}$ bond formation is then triggered by a decarboxylation via an E2 pathway where the carboxylate and the expelling hydroxyl group are in an anti-coplanar conformation (**6** → **2**). Analogously, the formation of **5** will go through pro-*S* C−H cleavage to form intermediate **7** prior to decarboxylation (**7** → **5**).

The partial erosion of stereoselectivity by AmbI3 when using **1** as the substrate can be attributed to the cooperative nature of isonitrile synthase systems discovered to date. That is, they likely act as a complex to catalyze the stereospecific vinyl isocyanide formation from the corresponding amino acids and Ru-5-P. This is consistent with the observation that while the generation of diffusible **1** by AmbI1 and AmbI2 is merely a single turnover event, when coupled to AmbI3, the system can generate ca. 30 turnovers (unoptimized) to give **5** within 25 min when the enzyme substrate ratio is set at 1/100 (SI Methods), suggesting protein−protein interactions between AmbI1 and/or AmbI2 with AmbI3 is likely operant to shuttle the intermediate **1** efficiently for the following stereospecific alkene installation. When IsnB is coupled with AmbI1 and AmbI2 under the same conditions, ca. 10 turnovers (unoptimized) that led to the production of **2** were observed.

In conclusion, by utilizing a newly discovered isonitrile synthase system (AmbI1, AmbI2, and AmbI3), we achieved the stepwise reconstitution of indolyl vinyl isocyanide biosynthesis *in vitro* from L-Trp and Ru-5-P. This endeavor led to the detection of an elusive biosynthetic intermediate **1** and provided conclusive evidence that **1** is the enzymatic product of standalone isonitrile synthase AmbI1 and AmbI2. Further characterization of Fe/2OG dependent desaturases IsnB and AmbI3 in combination with AmbI1/AmbI2 and **1** sheds light on the origin of stereodivergent biogenesis of indolyl vinyl isocyanide by *isn* and *ambI* pathways. The establishment of AmbI1 and AmbI2 as a tractable isonitrile synthase paved the way to elucidate the enigmatic enzymatic mechanism of the isocyanide formation, a subject of continuing studies by our groups.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b00258.

Detailed experimental procedures, including the synthetic preparation of **1**, purifications of AmbI1, AmbI2, AmbI3, and IsnB proteins, and subsequent enzymatic assays (PDF)

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Notes

The authors declare no competing financial interest.

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