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Biocatalysts from cyanobacterial hapalindole pathway afford antivirulent isonitriles against MRSA

Brittney M Bunn[1], Mizhi Xu[2], Chase M Webb[3] and Rajesh Viswanathan[4]*

[1]*Thermo Fisher Scientific, Cleveland, OH, USA*

[2]*Department of Chemistry, Georgia Institute of Technology, Atlanta, GA, USA*

[3]*Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA, USA*

[4]*Department of Chemistry and Biology, Indian Institute of Science Education and Research Tirupati, Tirupati, India*

**Corresponding author (Email, rajesh@iisertirupati.ac.in)*

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The emergence of resistance to frontline antibiotics has called for novel strategies to combat serious pathogenic infections. Methicillin-resistant *Staphylococcus aureus* [MRSA] is one such pathogen. As opposed to traditional antibiotics, bacteriostatic anti-virulent agents disarm MRSA, without exerting pressure, that cause resistance. Herein, we employed a thermophilic *Thermotoga maritima* tryptophan synthase (*Tm*TrpB1) enzyme followed by an isonitrile synthase and Fe(II)-α-ketoglutarate-dependent oxygenase, in sequence as biocatalysts to produce antivirulent indole vinyl isonitriles. We report on conversion of simple derivatives of indoles to their C3-vinyl isonitriles, as the enzymes employed here demonstrated broader substrate tolerance. *In toto*, eight distinct L-Tryptophan derived α-amino acids (**7**) were converted to their bioactive vinyl isonitriles (**3**) by action of an isonitrile synthase (WelI1) and an Fe(II)-α-ketoglutarate-dependent oxygenase (WelI3) yielding structural variants possessing antivirulence against MRSA. These indole vinyl isonitriles at 10 μg/mL are effective as antivirulent compounds against MRSA, as evidenced through analysis of rabbit blood hemolysis assay. Based on a homology modelling exercise, of enzyme-substrate complexes, we deduced potential three dimensional alignments of active sites and glean mechanistic insights into the substrate tolerance of the Fe(II)-α-ketoglutarate-dependent oxygenase.

Keywords. Antivirulence; Enzymology; Isonitriles; MRSA; Natural products; Tryptophan-derived bioactives

1. Introduction

Emergence of highly resistant strains to frontline antibiotics, coupled with the decline in the discovery of new antibiotics has called in an urgent search for novel agents to combat serious bacterial infections (Gordon and Lowy 2008). Methicillin-Resistant *Staphylococcus Aureus* (MRSA), is one among the most widespread bacterial pathogens in the United States (Tommasi *et al*. 2015) and this trend is slowly spreading to the entire world (Otto 2010). Antivirulence agents present alternatives to conventional antibiotics (Yu *et al*. 2014). In contrast to conventional antibiotics, antivirulence agents are typically not bactericidal. Antivirulence agents, by virtue of not threatening the survival of bacteria, will elicit less evolutionary pressure against the pathogen to develop resistance (Kuo *et al*. 2015). The mechanism of action is based upon curtailing the pathogen's ability to elicit toxins against the host's immune system. An unimpaired immune system may

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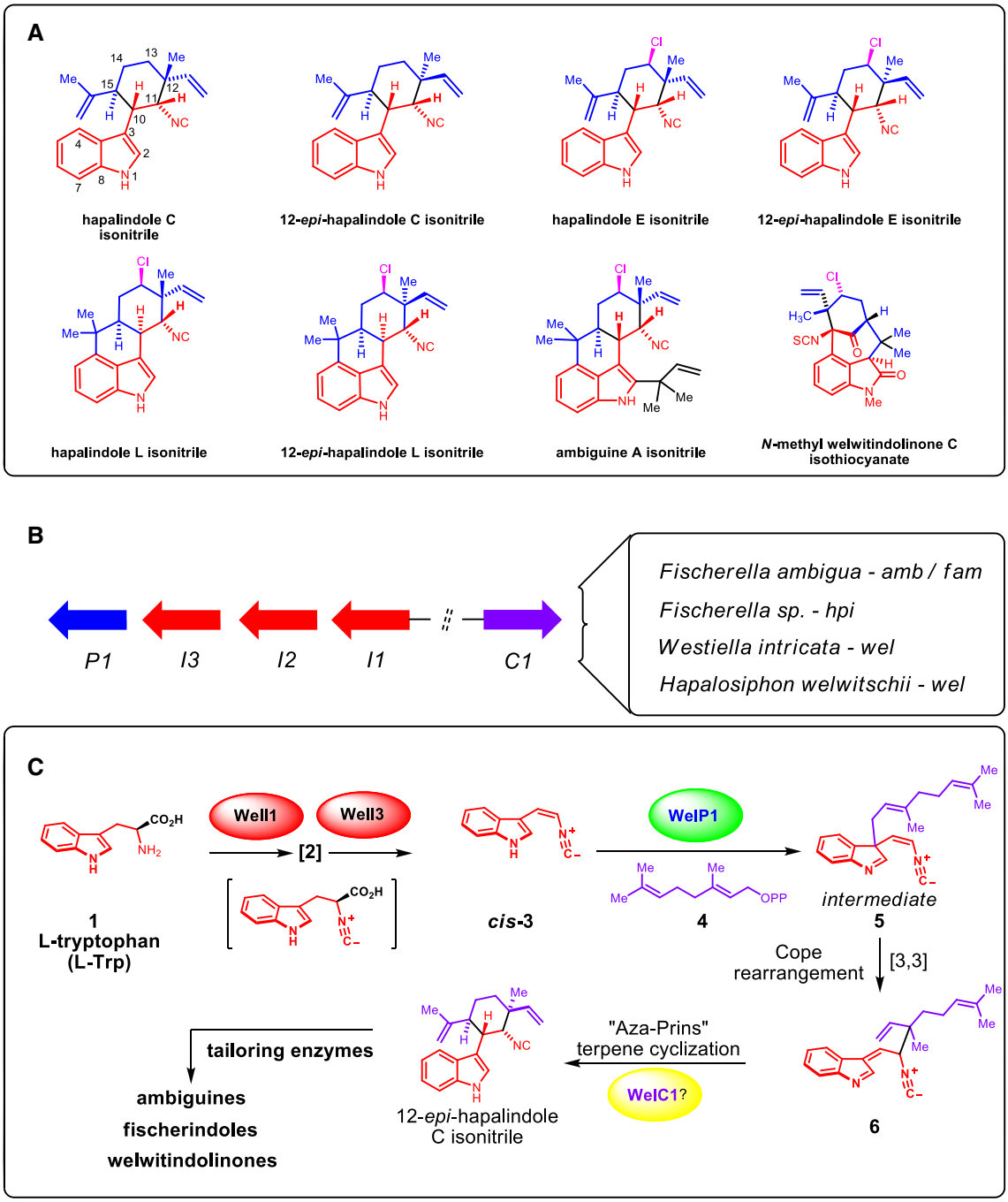
then be able to fight off the infection on its own. Alternatively, a boost in the form of a low-dose conventional antibiotic in combination with an antivirulence agent may become a successful strategy against more invasive infections. Antivirulence therapy offers the attractive prospect of bringing back conventional and affordable antibiotics into the clinic. We have recently reported the design and evaluation of novel antivirulent agents and showed that the dual measurement of pathogen's growth progress in tandem with the agent's ability to disrupt hemolysis (causing a reduced amount of hemoglobin to be ruptured) can serve as a reporter assay to evaluate a novel agent's ability to reduce the pathogen's virulent release of α-toxins (Yu *et al*. 2014). In this regard, due to previously known antibiotic properties, we hypothesized that the novel indole vinyl isonitriles may possess activity worthy of evaluating for potential antivirulence against MRSA.

Synthetic biology continues to enhance the structural and functional novelty of natural products. By recruiting a diverse set of enzymes from secondary metabolic pathways, the repertoire of this field is rapidly expanding (Keasling 2010; Keasling *et al*. 2012). While total chemical synthesis continues to be a tour-de-force for access to scalable quantities of unique chemicals (especially pharmaceuticals), recent advances offers complementary synthetic biological tools to execute chemoenzymatic production of complex molecules (Savile *et al*. 2010; Kwon *et al*. 2012; Ongley *et al*. 2013). Cyanide or isonitrile-containing natural products and their intriguing biosynthetic pathways continue to generate synthetic interest because of their chemical complexity and versatility (Scheuer 1992; Simpson and Garson 2004; Wilson *et al*. 2012). Furthermore, isonitriles have shown promise as imaging probes and have distinct use in chemical biology approaches (Wainman *et al*. 2013).

Since their original discovery by Moore in the 1980's, (Moore *et al*. 1984, 1987) the structurally complex hapalindole isonitriles have expanded to a super family of more than 80 highly bioactive natural products identified to date (Scheme 1A). Based on established molecular and biochemical targets, these alkaloids display an array of bioactivities ranging from antibacterial, antimycotic and multiple drug resistant (MDR) reversal, microtubulin inhibition, vasopressin receptor blockage, RNA polymerase inhibition, NFκβ inhibition and as sodium channel modulators (Walton and Berry 2016). This relatively large family of isoprenoid-indole alkaloids, with exquisite structural diversity, has inspired numerous total synthesis pursuits (> 50 independent routes) and have served as a benchmark for the development of selective methods to orchestrate concise bond-forming chemistries (Bhat *et al*. 2014; Lu *et al*. 2014; Maimone *et al*. 2015). Though several groups advanced their biosynthetic hypothesis underlying nature's strategy for hapalindole construction based on elegant chemistries (Park *et al*. 1992; Richter *et al*. 2008), it wasn't until recently that the genetic and biochemical basis of their biosynthesis have begun to emerge (Micallef *et al*. 2014; Li *et al*. 2015; Liu *et al*. 2016; Hillwig *et al*. 2016). Strain-specific gene clusters were identified and biochemically characterized via heterologous expression of select enzymes, that include a highly conserved set of genes encoding for isonitrile biosynthesis (Scheme 1B). These studies collectively have thrown light at the biocatalytic roles of isonitrile synthases (Ittiamornkul *et al*. 2015), indole N-methyl transferases, free-standing halogenases (Hillwig and Liu 2014; Hillwig *et al*. 2016) and prenyltransferases with their associated cyclase enzymes (Li *et al*. 2015) from the biosynthetic pathway of the hapalindoles (Scheme 1C).

We aimed at the production of novel indole vinyl isonitrile analogs using genes from hapalindole pathway with potential routes to their novel unnatural derivatives. Herein, we report a biocatalytic approach by sequential expression of genes from *T. maritima* and *Westiella intricata* to construct enzymes that produce novel indole vinyl isonitriles (**3**) and their unnatural derivatives (Bunn 2016). Figure 1 illustrates multiple beneficial features of biocatalytic conversions for constructing bioactive molecules, in particular the vinyl indole isonitriles such as **3** (Hoppe and Schöllkopf 1984; Spallarossa *et al.* 2016).

The isonitrile synthase and Fe(II)-α-ketoglutarate-dependent oxygenase genes (*welI1 and welI3*) are encoding for indole vinyl isonitrile biosynthesis and are conserved in every hapalindole-producing cyanobacterial genome sequenced thus far (*hpi*, *fam* or *amb* gene clusters). Previously, we and others have shown that the indole-isonitrile (**3**) (derived from L-tryptophan) is the product of the sequential biocatalysis transformations carried out by isonitrile synthase and Fe(II)-α-ketoglutarate-dependent oxygenase enzymes. (Micallef *et al*. 2014; Hillwig *et al*. 2014) Specifically, in these two steps, the first enzyme is an isonitrile synthase that converts an amino group into its isocyano group and WelI3 is an Fe(II)-α-ketoglutarate-dependent oxygenase that causes decarboxylative desaturation of the Trp backbone. Homologs of these two enzymes (also found from eDNA) are highly conserved in all hapalindole gene clusters identified so far (Brady and Clardy 2005; Brady *et al*. 2007). These enzymes work



Scheme 1. (**A**) Representative hapalindole alkaloid family members. Conventional ring numbering is provided for core structure only. (**B**) Conserved portion of the gene clusters characterized from four distinct cyanobacterial strains. The isonitrile biosynthetic genes are colored in red; prenyltransferase is colored blue and a proposed cyclase associated with ring closure is colored purple. (**C**) Currently accepted operating mechanism for hapalindole core biosynthesis involving early stage isonitrile generation followed by prenyltransfer and a Cope rearrangement of the terpene skeleton ending with an Aza-Prins cyclization to result in the hapalindole core. *Cis-3* is a vital intermediate and is also a known antibiotic.

in tandem to synthesize indole-isonitriles (**3**) and thereby afford a biocatalytic strategy for their synthesis. By isolating metabolites directly from hapalindole-producing *Fischerella* strains and unambiguously characterizing their structural identity, we established indole-isonitriles are bona fide pathway intermediates in cyanobacteria (Micallef *et al.* 2014; Bunn 2016). Considering their chemical and biological versatility of enzymes producing vinyl isonitriles, we probed the innate substrate promiscuity of indole vinyl isonitrile

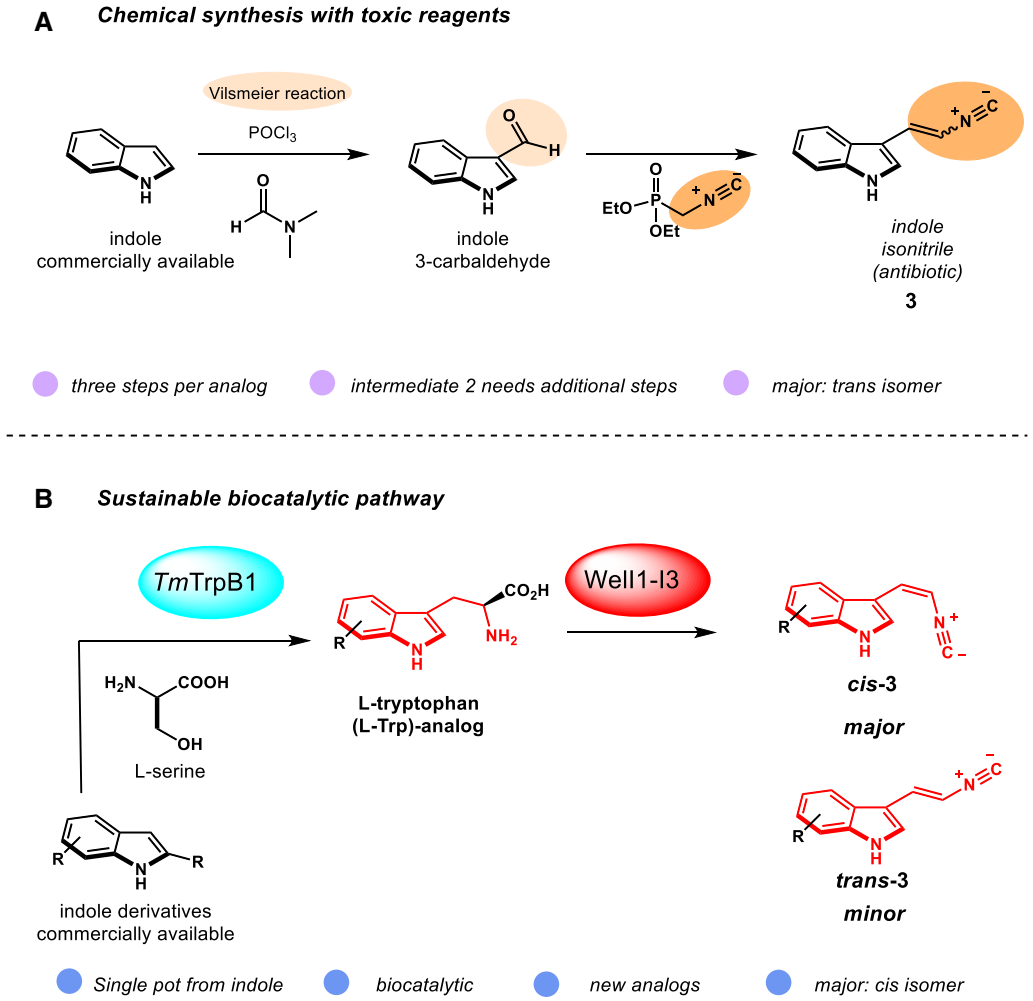


Figure 1. Premise of this work. (**A**) Descriptions and details of chemical synthesis leading to vinyl isonitriles. (**B**) Green and sustainable biocatalysis that can provide access to bioactive isonitriles using enzymes.

synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase involved in hapalindole biosynthesis as a promising pathway for producing isonitrile natural products.

To establish feasibility toward a streamlined assembly of these alkaloids, we developed a two-enzyme-single-assay system with the isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase. By sequencing the pathway with a third hyperthermophilic *Thermotoga maritima* tryptophan synthase (*Tm*TrpB1) enzyme (Hettwer and Sterner 2002) we report on conversion of indoles to yield **3**. We assembled C2-methyl L-tryptophan analog (as an intermediate) from C2-methyl indole and L-Serine, *en route* to novel indole isonitriles. Results from exploring the substrate tolerance of isonitrile synthase (WelI1) and Fe(II)-α-ketoglutarate-dependent dioxygenase (WelI3) through a direct tandem enzymatic assay is reported. Interestingly, antivirulence potential of these novel indole vinyl isonitrile analogs are evaluated revealing an impressive structural motif capable of curtailing Methicillin Resistant *S. aureus* (MRSA) virulence. Together, these results offer promising avenues to develop new antibiotic strategies for the future.

2. Results and discussion

Indole vinyl isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase were expressed in *E. coli* and were purified to homogeneity (figure 2). We streamlined biosynthesizing analogues such as 2-methyl-L-tryptophan (**7b**) using an additional *in vitro* enzymatic reaction, by TrpB1 (recombinant tryptophan synthase) from the hyperthermophilic bacterium *Thermotoga maritima*. (Hettwer and Sterner 2002) In the presence of L-serine, an indole derivative, and PLP, *Tm*TrpB1 catalyzes the formation of a tryptophan

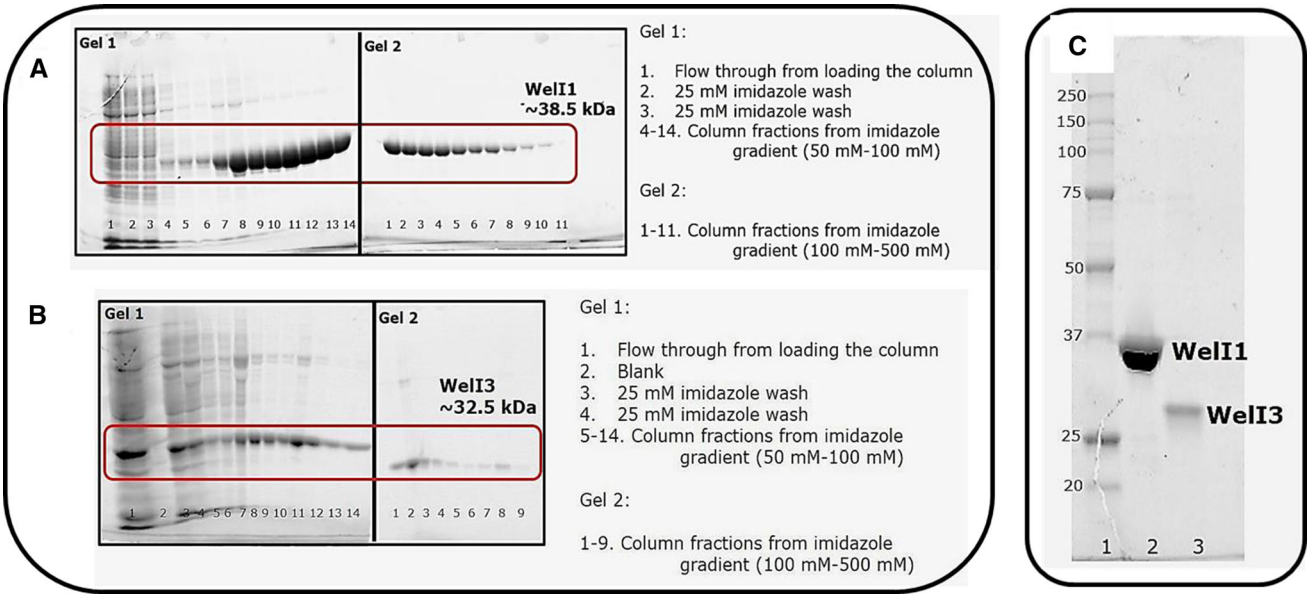


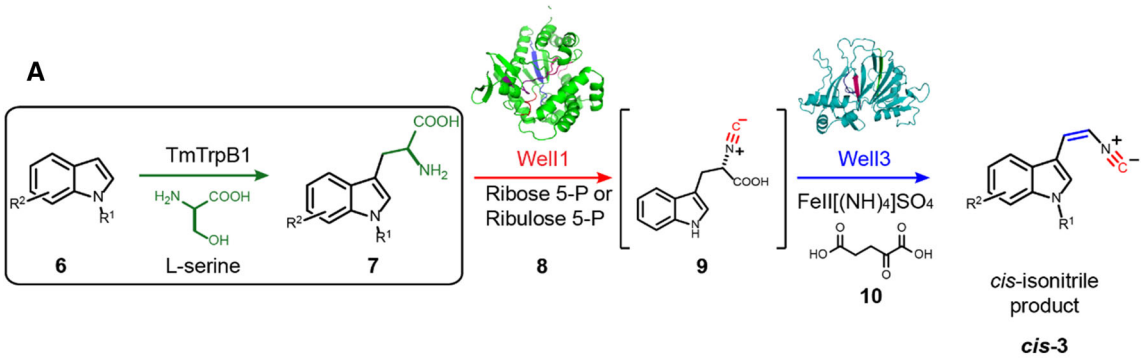
Figure 2. (**A**) SDS-PAGE analysis of the Ni-NTA purification of WelI1. (**B**) SDS-PAGE analysis of the Ni-NTA purification of WelI3. (**C**) SDS-PAGE analysis of the Ni-NTA purificed of WelI1 and WelI3. WelI1 is 37205.6 Da and 31239.3 Da

derivative and accepts a variety of indole precursors as substrates. Therefore, *E. coli* BL21-(DE3)-RIPL cells transformed with a plasmid encoding *Tm*TrpB1 (pET28a-*tm*trpB1) were cultured and induced to overexpress *Tm*TrpB1, which was subsequently purified by Ni-NTA chromatography (figure S1). An assay was set up containing 2-methyl indole and allowed to incubate at 80°C for approximately 48 hours. The reaction was monitored by thin layer chromatography and the product was purified by silica gel flash chromatography.

A single-pot enzymatic assay was set up as outlined in figure 3A. This tandem assay was based on previous experiments with L-Tryptophan as the natural substrate (Micallef *et al*. 2014). In this study, we probed seven distinct L-tryptophan derivatives as possible analogs of the natural substrate. Assay components included the enzymes (21–28 µM isonitrile synthase and 11-19 µM Fe(II)-α-ketoglutarate-dependent dioxygenase) in addition to 2 mg of L-tryptophan or its respective analog. The assay buffer consisted of 2.5 mM ribulose-5-phosphate (exchangeable with its keto-enol tautomer ribose-5-phosphate), equimolar amount of α-ketoglutaric acid disodium salt dehydrate, 100 µM $(NH4)_2$-$Fe(SO_4)_2$), and 5% (v/v) glycerol. After incubation at 37°C for 18 h , the assays were extracted with ethyl acetate (v:v: 1:1) and analog production was evaluated by LC-HRMS, MS-MS fingerprinting and NMR analyses. Figure 3A displays the biocatalytic sequence employed in this investigation, including the use of TmTrpB1 for generation of **7b**.

For each assay, HPLC analysis of the organic extracts from the isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase catalyzed reaction mixtures showed the formation of corresponding products (figure 3B). The extracts were simultaneously analyzed by high resolution mass spectrometry (figure 3B, entries 1–8). Following chromatographic separation, we obtained high % yields in the range of 60-90% depending on each substitution on the indole ring. We were able to scale up the synthesis of **3a, 3c, 3e and 3g** to 850-920 µmol levels without losing biocatalytic efficiency (table S1). By increasing L-Trp (or its analog **7c**, **7e** or **7g**) quantities and correspondingly increasing the amount of ribose-5-phosphate and that of α-KG, the scaled-up synthetic assays occurred with similar efficiencies. Product structures of **3a, 3c, 3e and 3g** as the major product in each case as were verified through NMR data analyses presented in the supporting information.

Overall, as revealed in figure 3B, each of the seven distinct substrate analogs was recruited successfully resulting in their corresponding indole-isonitrile analogs efficiently. HPLC analysis indicated the presence of both *cis* and *trans* isomers of the natural product analogs, and their ratios were quantified through HPLC analyses. A C2-methyl substituent (**7b**) upon turnovers



B

Entry	Structure of L-Trp / Analog	HPLC chromatogram (product peak) abs @ 310 nm	HRMS (M^+ or $[M+H]^+$) Expected	HRMS (M^+ or $[M+H]^+$) Found	Ratio of *cis:trans* isomers	% yield	Structure of Isonitrile (major) Product
1	7a	int / Rt	169.0766	169.0769	> 100 : 1	85%	*cis*-3a
2	7b	int / Rt	183.0922	183.0935	10 : 1	90%	*cis*-3b
3	7c	int / Rt	183.0922	183.0913	3.5 : 1	90%	*cis*-3c
4	7d	int / Rt	187.0672	187.0681	6 : 1	85%	*cis*-3d
5	7e	int / Rt	183.0922	183.0923	1 : 1	75%	*cis*-3e
6	7f	int / Rt	183.0922	183.0930	8 : 1	85%	*cis*-3f
7	7g	int / Rt	199.0871	199.0862	10 : 1	65%	*cis*-3g
8	7h	int / Rt	185.0709	185.0730	1 : 1	60%	*cis*-3h

Figure 3. (**A**) Sequential biocatalysis involving three enzymatic steps. (**B**) List of substrate analogs incorporated into the WelI1 and WelI3 catalysis, resulting in isonitriles **3 a-h**. Ratios of *cis:trans* isomers measured from HPLC.

by isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase caused smooth production of indole vinyl isonitrile with a methyl substituent, as shown in **3b**. With the exception of 5-methoxy-substituted **7g**, all other L-Trp derivatives (**7c**, **d**, **e**, **f** and **h**) produced product isonitrile analogs that favored the *cis* isomer marginally (< 10:1) (figure 2B). Interestingly, substitutions at C5 position with a methoxy group, or at C2 position with a methyl group or having no substitution at all, produces a >10:1 ratio favoring the *cis* isonitrile. All other analogs produced corresponding products in about a ∼5:1 ratio, or even an equimolar mixture as in **3h**.

Elemental composition of major products and MS^2 fragmentation patterns (table S2) further supported the production of these seven analogs of indole-isonitriles (**7b-h**). Unambiguous characterization of major products from each of the scaled-up reactions are carried out with NMR of the respective ^{1}H and ^{13}C nuclei as shown in the supporting information. Furthermore, the LC retention times, followed by MS information fingerprinting their fragmentation patterns are also corroborating and are provided in the supporting information (figures MS1-MS16, SI). Based on these results, it is clear that isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase tolerate a broad range of substitutions on the indole ring portion of L-Trp. As the efficiency of these transfomations was good enough for access to diverse bioactive molecules, we setup our next phase of study to probe their antivirulence activity.

3. Novel indole vinyl isonitriles show antivirulence property against MRSA

We next addressed the possibility of these novel isonitrile-containing analogues possessing valuable pharmacological property. As shown in figure 4, we measured the MRSA growth and hemolysis inhibitory effects. We followed methods that were reported earlier (Yu *et al*. 2014) and also tested a series of new indole vinyl isonitriles produced through our earlier investigations. In figure 4, the green bar refers to MRSA growth, and for antivirulent property measurement, one would ideally like to see this bar at near 100% growth, to ensure that it is not pressurizing resistance development. The red bar indicates extent to which hemolysis, caused by the virulence of the pathogen, is still observed in the presence of the antivirulent drug like compound. Therefore, we like to ideally see a low % for its intensity, referring to highly antivirulent compound activity.

DMSO served as a vehicle with no detectable growth inhibition, and no inhibition of hemolysis. Diflunisal, a well-established control served as a standard. None of the indole vinyl isonitriles tested at concentrations up to 50 μg/mL had any detectable bactericidal effect. They allowed MRSA to grow sustainably, thereby posing less of a chance for the pathogen to develop resistance. Interestingly, C2-methyl substituted isonitrile (**3b**) possessed a detectable inhibition of α-toxin production while displaying favorable combination of growth and hemolysis inhibitory effects. C5- methyl substituted isonitrile (**3e**) had a similar effect. The parent isonitrile **3a** was slightly less effective than **3b** and **3e** suggesting that the methyl substitution favorably increases the antivirulence property of these compounds. We compared these positive results to a series of rigorous controls. Analogs where all structural motifs were identical except for the isonitrile functionality were tested. A nitrile in place of the isonitrile, an ester in place of the isonitrile and its halogenated analogs, and aldehyde functionality in place of the isonitrile were tested as controls (figure 4, pairs of bars 6–9 from left). Significantly these functional groups offered no antivirulent property evident by the lack of hemolysis inhibitory effect. The C3-aldehyde containing indole also did not possess any antivirulence to the degree as the isonitrile functional group indicating clearly that the isonitrile functionality renders bioactivity. The N1-methyl substituted analogs (**3c)** gave impressive hemolysis inhibitory activity and in this case alone, we were able to independently measure this activity for each geometrical isomer. Upon evaluating these individually, we found the *trans* isomer was slightly more efficacious than its *cis* isomer. Overall, while indole isonitriles were previously investigated for their ability to serve as antibiotics, their antivirulent property were not investigated before. This study illustrates that they possess the potential and therefore future medicinal chemistry is worthy to delineate their mode of efficacy.

4. Mechanistic details of indole vinyl isonitrile biosynthesis

In order to rationalize the broad substrate tolerance of isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase, we turned to homology-based modelling and *in silico* docking studies, given that

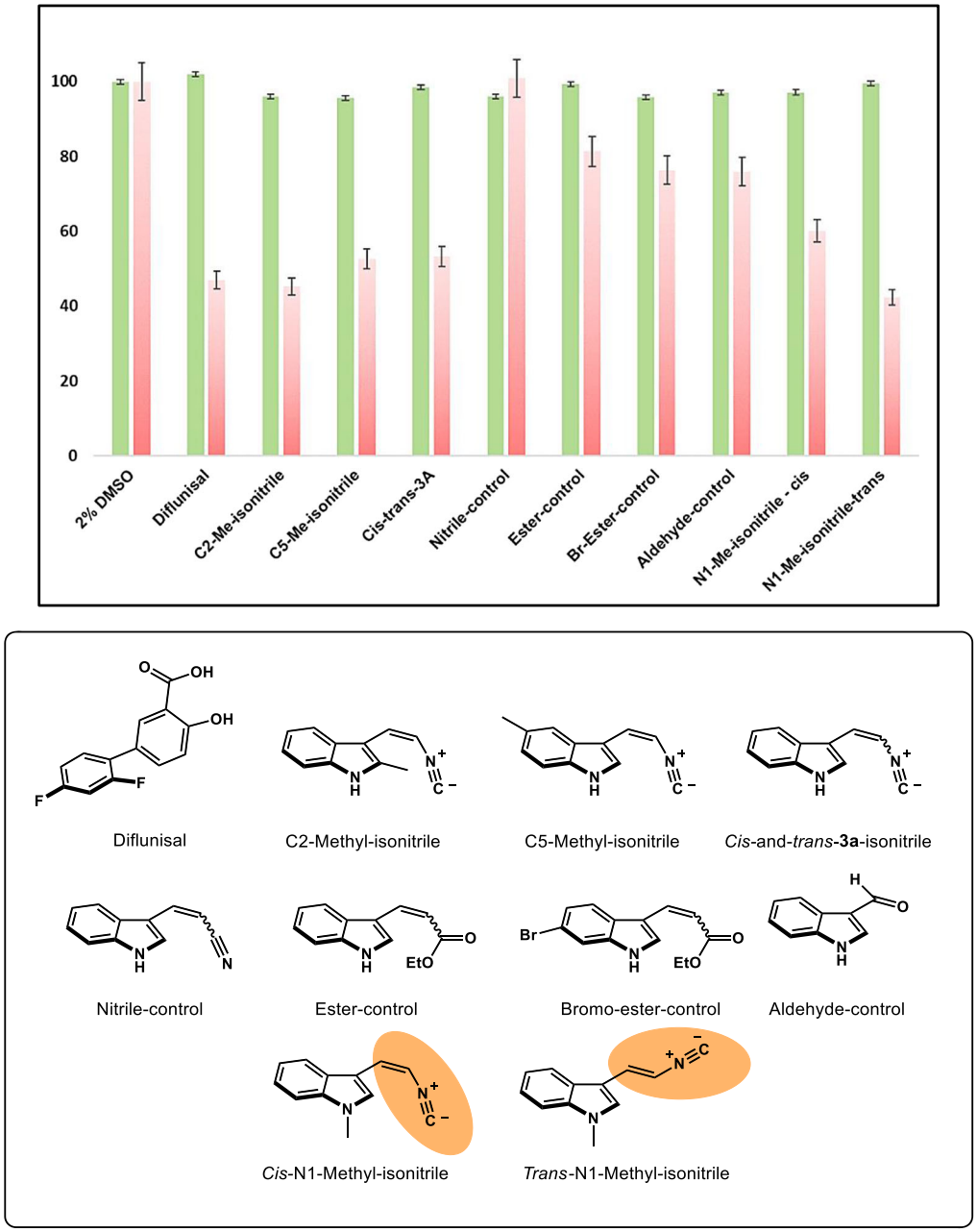
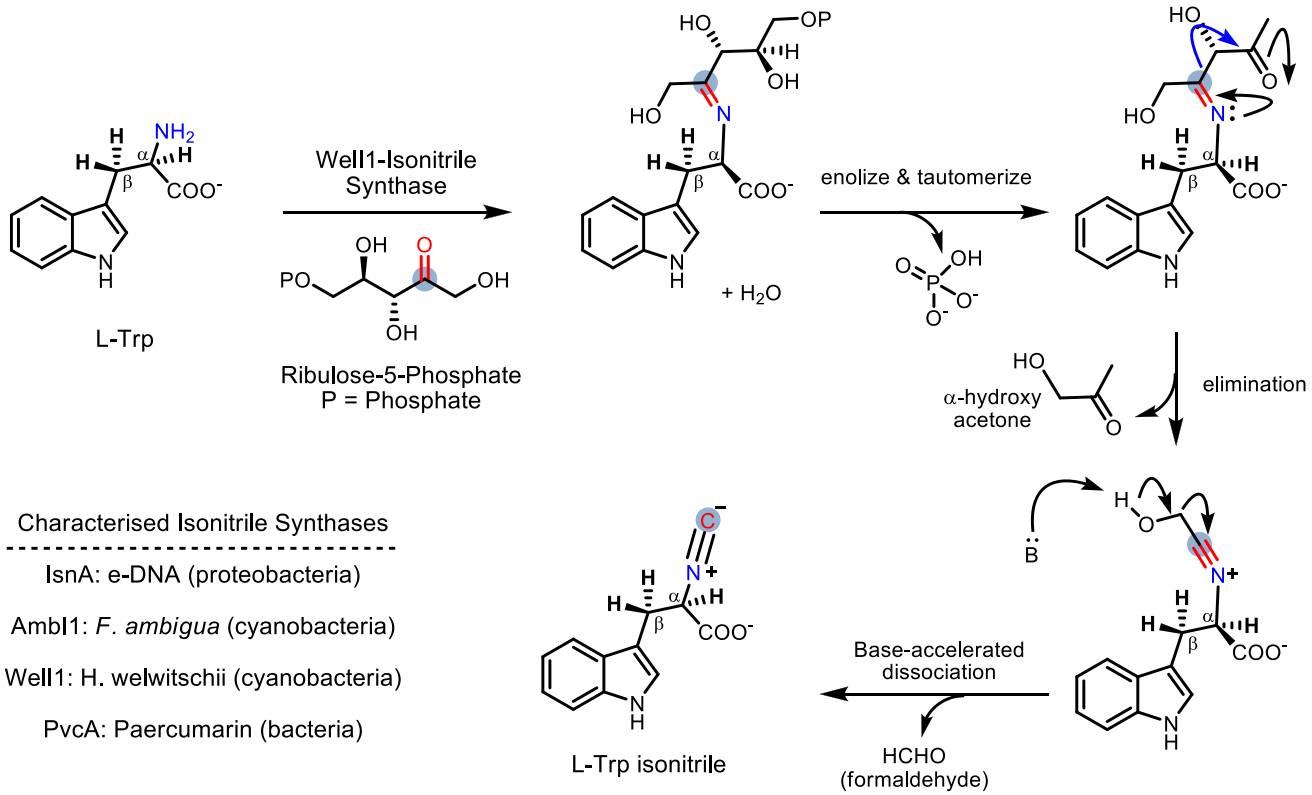


Figure 4. Evaluation of antivirulence potential for indole-isonitriles produced in this study. Green bars in plot above refer to % MRSA bacterial growth and red bars indicate % of hemolysis inhibited in the presence of the indole vinyl isonitrile or a control agent.

neither WelI1 nor WelI3 have been crystallized yet. The pathway to indole isonitrile **3** was characterized from eDNA-derived metagenomes by Brady and Clardy (2005). Expression of the isocyanide (*isn*) biosynthetic cluster from eDNA led to identification of the roles of IsnA and IsnB (homologs of isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase respectively).

The study by Brady *et al*. about the eDNA-derived isonitrile synthase (IsnA) utilized a C13-labeled system and it unambiguously revealed that the 1C source for isonitrile incorporation was a C_5-sugar-phosphate: Ribulose-5-P. At the time, these were intriguing results wherein a C_5-sugar-phosphate provided the source for isonitrile carbon, as opposed to glycine as originally interpreted (from isotope feeding experiments) by Moore *et al.* (Bornemann *et al*. 1988). Subsequent studies have revealed a generally accepted mechanism for the the formation of Trp-isonitrile from L-Trp through the formation of a Schiff's base with Ribulose-



Scheme 2. Currently accepted mechanism of isonitrile biosynthesis from α-amino acid and a 1C donor (Ribulose-5-phosphate). Ref: EC: 4-1-99-25 (*https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC4/1/99/25.html*).

5-P followed by degradative elimination of P*i*, H_2O, α-hydroxyacetone and formaldehyde as shown in Scheme 2 (Chang *et al*. 2017). The structure of paercumarin, an *isn*-derived metabolite was identified in *Pseudomonas aureginosa* and the mechanistically related IsnA-B homologs PaPvcA and PaPvcB were then biochemically characterized. (Clarke-Pearson and Brady 2008) Three dimensional structures of PaPvcA and PaPvcB were reported later by Drake and Gulick (Drake and Gulick 2008). Recent mechanistic investigations and an improved crystallographic structure of PaPvcB discovered additional homologs XnPvcB and EaPvcB with divergent biochemical pathways to distinct products. (Zhu *et al*. 2015)

Isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase display a high degree of sequence conservation with its respective homologs (> 90%, BLASTP), among Group V strains, indicating their shared biogenesis to the hapalindole core assembly. Furthermore, isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase share significant active site similarity to PaPvcA and PaPvcB respectively (table S3, SI). This observation allowed us to question whether Fe(II)-α-ketoglutarate-dependent dioxygenase and PaPvcB have structural similarity, because if so, we could use structural similarities and differences to explain the relaxed substrate specificities observed in this study.

Therefore, we first built a homology model of Fe(II)-α-ketoglutarate-dependent dioxygenase (based on PaPvcB) and docked its substrate to create a three dimensional model to explain the mode of substrate binding to the active site (figure 5). Similar to Tipton and Gulick's study, we used clavaminate synthase (Zhang *et al*. 2000) as a model for extracting coordinates of Fe(II) and we reconstructed a homology model of Fe(II)-α-ketoglutarate-dependent dioxygenase, based on PaPvcB's 2.1 Å structure. The model built for Fe(II)-α-ketoglutarate-dependent dioxygenase significantly superimposed with the structure of PaPvcB with a Phyre TM-score of 0.9 (figure S2, SI). This combined with the pairwise multiple sequence alignment offered a high degree of confidence in predicting molecular level interactions. Figure 5 shows the docked poses generated through the Schrodinger suite, after the model generated for Fe(II)-α-ketoglutarate-dependent dioxygenase was reconstructed with active site Fe(II) and α-KG. Residues that help define substrate binding

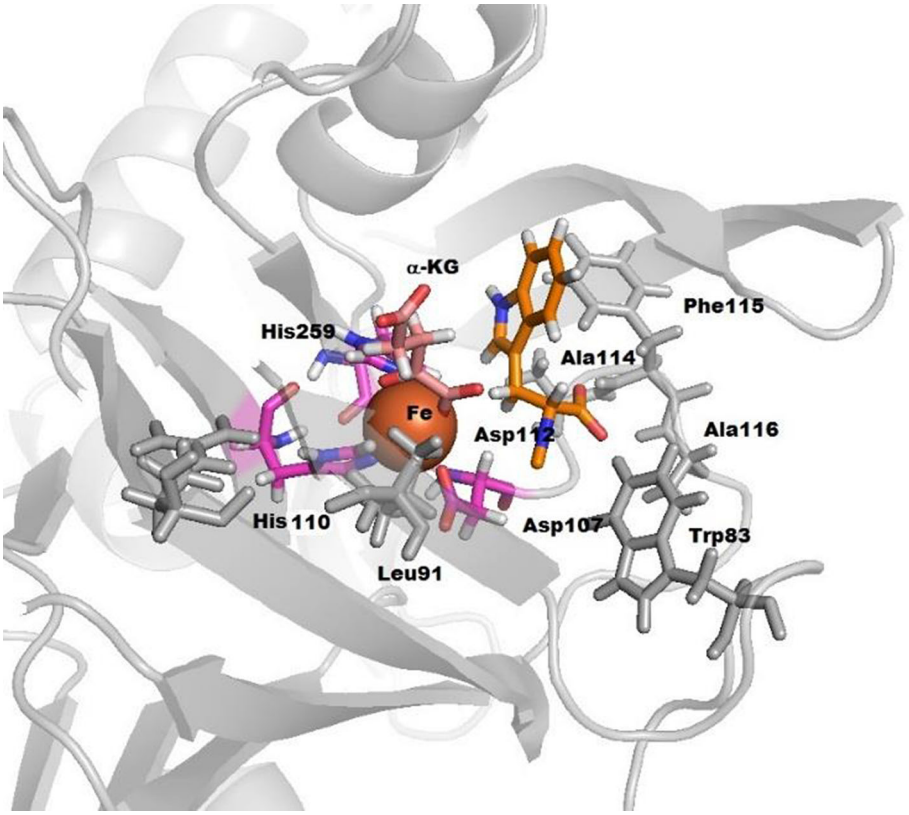


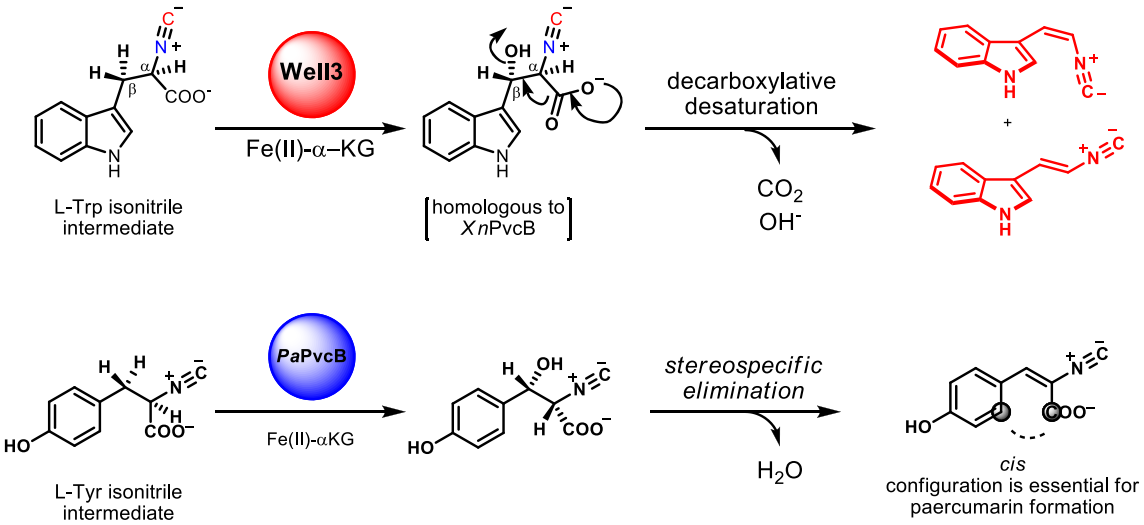
Figure 5. Side view of WelI3 bound to L-tryptophan isonitrile (**9**) with benzylic methane proximal to Fe(II), shown in brown; α-KG, His110; His259 and Asp112 shown in magenta; Substrate isonitrile **2** is shown in orange. Non-binding residues that impact the size of the active site pocket are labelled.

and channeling, as defined for PaPvcB were highly conserved. These included: Trp83, Arg274, Leu89, Leu91, Arg270, His110, His259 and Asp112 bind to Fe(II) and are conserved in WelI3. The distinct and significant difference between PaPvcB and WelI3 based on Clustalω alignments is absence of the hydrophobic pocket comprising of Met114-Tyr115-Leu-116 (PaPvcB numbering) in WelI3. Instead, in Fe(II)-α-ketoglutarate-dependent dioxygenase we find a larger pocket filled with relatively smaller residues such as Ala114-Phe115-Ala116 as a triad. We then docked L-tryptophan isonitrile (**2**) and identified key poses wherein the substrate **2** presents its pro-*S*-hydrogen on β-carbon within 3.0 Å from Fe(II).

This result derived from bioinformatic alignment and docking, allows us to reason that the expanded substrate profile displayed by WelI3 could be seen as an effect of having three (114–116) relatively smaller residues that open up the site for indole-derived, sterically bulkier groups; and consequently, that the mechanism of Fe(II)-α-ketoglutarate-dependent dioxygenase resembles those observed for PvcB homolog *Xn*PvcB. Based on this reasoning, we present our proposed pathway to formation of *cis and trans* isomers of indole isonitrile **3,** to be caused by constraints of stereospecifity imposed by Fe(II)-α-ketoglutarate-dependent dioxygenase during the decarboxylative, desaturation step (indicated by elimination of the β-OH group) as represented in Scheme 3.

Overall, in the presence of ribose-5-phosphate (or its tautomer, ribulose-5-phosphate), the isonitrile synthase catalyzes the incorporation of the isonitrile carbon to the L-tryptophan N-terminus, resulting in a (relatively unstable) tryptophan-isonitrile intermediate (**2**). This intermediate is then presumably hydroxylated *in situ* at Cβ-position, by Fe(II)-α-ketoglutarate-dependent dioxygenase, a non-heme oxygenase, and subsequently, decarboxylated and desaturated (concomitantly) resulting in indole-isonitrile products (**3**).

Indole vinyl isonitriles are antibacterial as were established long before. Their antivirulence property is revealed here for the first time. Also, as a relevant aspect, these isonitiriles are the first stable intermediates in the biosynthetic pathway leading to the hapalindoles. Natural products biogenetically arising from L-Tryptophan derived metabolic pathways



Scheme 3. Putative mechanism of Fe(II)-α-ketoglutarate-dependent dioxygenase (WelI3) based on its analogy to *Pa*PvcB.

continue to spur novel concepts related to enzymatic mechanism. (Alkhalaf and Ryan 2015) In that regard, the hapalindole pathway has offered a suite of mechanistically novel enzymes. The fact that both isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase from Hapalindole pathway, allow a broader substrate diversity opens doors for mechanistic investigations in the future. Milligram quantities of commercially available tryptophan derivatives can be quite costly, in addition, the prospect of a green method employing three biocatalysts to produce hapalindole-type analogs from indole derivatives (much less expensive compared to tryptophan derivatives), sparked our interest. This investigation communicates one of the early reports toward biosynthetically producing hapalindole-type analogs in addition to providing the first support towards utilizing a tri-catalytic biosynthetic methodology to produce such analogs in a more environmentally friendly, cost-effective, time efficient manner. Since the isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase system has already been shown to work under cell lysate conditions (Micallef *et al*. 2014), the *Tm*TrpB1 catalyzed reaction could be attempted at the lysate stage as well. Side-stepping the purification of all three proteins would only cause this biosynthetic methodology to become even more time and cost effective. Modifications made to this early biosynthetic intermediate could eventually serve as a template for novel hapalindole natural product analogs with promising therapeutic value.

5. Conclusions

Biocatalysts in general are green and cost-effective options to recruit in synthesis. Enzymes performing unique transformations could serve as alternatives and could complement transition metal-based chemistries, if only the robustness of newer enzymes with unique bond-forming repertoires are firmly established. Plasmid-induced production of enzymes and their catalysis employed strategically to solve synthetic problems hold promise toward development of sustainable synthesis of complex molecules. In this study, we obtained indole isonitriles with distinct substitution patterns on the heterocyclic ring in high efficiency by employing both isonitrile synthase and Fe(II)-α-ketoglutarate-dependent dioxygenase as sequential biocatalysts. The convenience of scaled up reactions provide quantitative access to indole isonitriles that are accessed otherwise through multi-step operations. Through a sequential three-step biocatalytic variant, catalytic action of a hyperthermophilic *Tm*TrpB1 was employed for synthesis of C2-methyl indole-isonitrile in 80% yield. Multiple members of this product class offer a mechanistically unique probe to curtail antibiotic resistance, against MRSA. Using homology modeling and substrate docking, based on a 2.1 Å crystal structure of *Pa*PvcB, the relaxed substrate specificity is rationalized. Given that hapalindole-type of alkaloid natural products are extremely diverse and pharmacologically promising, studies on establishing substrate promiscuity for enzymes involved in hapalindole biosynthesis

have barely started to emerge. We predict that this study will pave ways for combinatorial biosynthesis of hapalindole alkaloids, a biosynthetically diverse collection of natural products with significant bioactivity.

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References

Alkhalaf LM and Ryan KS 2015 Biosynthetic manipulation of tryptophan in bacteria: pathways and mechanisms. *Chem. Biol.* **22** 317–328

Bhat V, Dave A, MacKay JA, and Rawal VH 2014 The chemistry of hapalindoles, fischerindoles, ambiguines, and welwitindolinones; in *The alkaloids: chemistry and biology* (ed) H-J Knölker (Academic Press) pp 65–160

Bornemann V, Patterson GML and Moore RE 1988 Isonitrile biosynthesis in the cyanophyte *Hapalosiphon fontinalis*. *J. Am. Chem. Soc.* **110** 2339–2340

Brady SF, Bauer JD, Clarke-Pearson MF and Daniels R 2007 Natural products from isnA-Containing biosynthetic gene clusters recovered from the genomes of cultured and uncultured bacteria. *J. Am. Chem. Soc.* **129** 12102–12103

Brady SF and Clardy J 2005 Cloning and heterologous expression of isocyanide biosynthetic genes from environmental DNA. *Angew. Chemie Int. Ed.* **44** 7063–7065

Bunn MB 2016 Unraveling genetically encoded pathways leading to bioactive metabolites in group V cyanobacteia. Ph. D. Thesis, Case Western Reserve University, Ohio

Chang W-C, Liu X, *et al*. 2017 In vitro stepwise reconstitution of amino acid derived vinyl isocyanide biosynthesis: detection of an elusive intermediate. *Org. Lett.* **19** 1208–1211

Clarke-Pearson MF and Brady SF 2008 Paerucumarin, a new metabolite produced by the *pvc* gene cluster from *Pseudomonas aeruginosa*. *J. Bacteriol.* **190** 6927

Drake EJ and Gulick AM 2008 Three-dimensional structures of *Pseudomonas aeruginosa* PvcA and PvcB, two proteins involved in the synthesis of 2-Isocyano-6,7-dihydroxycoumarin. *J. Mol. Biol.* **384** 193–205

Gordon RJ and Lowy FD 2008 Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.* **46** S350–S359

Hettwer S and Sterner R 2002 A novel tryptophan synthase β-Subunit from the hyperthermophile *Thermotoga maritima*. *J. Biol. Chem.* **277** 8194–8201

Hillwig ML and Liu X 2014 A new family of iron-dependent halogenases acts on freestanding substrates. *Nat. Chem. Biol.* **10** 921–923

Hillwig ML, Zhu Q, Ittiamornkul K and Liu X 2016 Discovery of a promiscuous non-heme iron halogenase in ambiguine alkaloid biogenesis: implication for an evolvable enzyme family for late-stage halogenation of aliphatic carbons in small molecules. *Angew. Chemie Int. Ed.* **55** 5780–5784

Hillwig ML, Zhu Q and Liu X 2014 Biosynthesis of ambiguine indole alkaloids in cyanobacterium fischerella ambigua. *ACS Chem. Biol.* **9** 372–377

Hoppe I and Schöllkopf U 1984 Synthesis and biological activities of the antibiotic B 371 and its analogs. *Liebigs Ann. der Chemie* **1984** 600–607

Ittiamornkul K, Zhu Q, Gkotsi DS, Smith DRM, Hillwig ML, Nightingale N, Goss RJM and Liu X 2015 Promiscuous indolyl vinyl isonitrile synthases in the biogenesis and diversification of hapalindole-type alkaloids. *Chem. Sci.* **6** 6836–6840

Keasling JD 2010 Manufacturing molecules through metabolic engineering. *Science* **330** 1355

Keasling JD, Mendoza A and Baran PS 2012 A constructive debate. *Nature* **492** 188–189

Kuo D, *et al*. 2015 Novel quorum-quenching agents promote methicillin-resistant *Staphylococcus aureus* (MRSA) wound healing and sensitize MRSA to β-lactam antibiotics. *Antimicrob. Agents Chemother.* **59** 1512

Kwon SJ, Mora-Pale M, Lee M-Y and Dordick JS 2012 Expanding nature's small molecule diversity via in vitro biosynthetic pathway engineering. *Curr. Opin. Chem. Biol.* **16** 186–195

Li S, *et al*. 2015 Hapalindole/Ambiguine biogenesis is mediated by a cope rearrangement, C-C bond-forming cascade. *J. Am. Chem. Soc.* **137** 15366–15369

Liu X, Hillwig ML, Koharudin LMI and Gronenborn AM 2016 Unified biogenesis of ambiguine, fischerindole, hapalindole and welwitindolinone: identification of a monogeranylated indolenine as a cryptic common biosynthetic intermediate by an unusual magnesium-dependent aromatic prenyltransferase. *Chem. Commun.* **52** 1737–1740

Lu Z, Yang M, Chen P, Xiong X and Li A 2014 Total synthesis of hapalindole-type natural products. *Angew. Chemie Int. Ed.* **53** 13840–13844

Maimone TJ, Ishihara Y and Baran PS 2015 Scalable total syntheses of (-)-Hapalindole U and (+)-Ambiguine H. *Tetrahedron* **71** 3652–3665

Micallef ML, Sharma D, Bunn BM, Gerwick L, Viswanathan R and Moffitt MC 2014 Comparative analysis of hapalindole, ambiguine and welwitindolinone gene clusters and reconstitution of indole-isonitrile biosynthesis from cyanobacteria. *BMC Microbiol.* **14** 213

Moore RE, *et al*. 1987 Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *J. Org. Chem.* **52** 1036–1043

Moore RE, Cheuk C and Patterson GML 1984 Hapalindoles: new alkaloids from the blue-green alga *Hapalosiphon fontinalis*. *J. Am. Chem. Soc.* **106** 6456–6457

Ongley SE, Bian X, Zhang Y, Chau R, Gerwick WH, Müller R and Neilan BA 2013 High-Titer heterologous production in E. coli of lyngbyatoxin, a protein kinase c activator from an uncultured marine cyanobacterium. *ACS Chem. Biol.* **8** 1888–1893

Otto M 2010 Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus Annu*. *Rev. Microbiol.* **64** 143–162

Park A, Moore RE and Patterson GML 1992 Fischerindole L, a new isonitrile from the terrestrial blue-green alga *Fischerella muscicola*. *Tetrahedron Lett.* **33** 3257–3260

Richter JM, Ishihara Y, Masuda T, Whitefield BW, Llamas T, Pohjakallio A and Baran PS 2008 Enantiospecific total synthesis of the hapalindoles, fischerindoles, and welwitindolinones via a redox economic approach. *J. Am. Chem. Soc.* **130** 17938–17954

Savile CK, *et al*. 2010 Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science* **329** 305

Scheuer PJ 1992 Isocyanides and cyanides as natural products. *Acc. Chem. Res.* **25** 433–439

Simpson JS and Garson MJ 2004 Biosynthetic pathways to isocyanides and isothiocyanates; precursor incorporation studies on terpene metabolites in the tropical marine sponges *Amphimedon terpenensis* and *Axinyssa n.* sp. *Org. Biomol. Chem.* **2** 939–948

Spallarossa M, Wang Q, Riva R and Zhu J 2016 Synthesis of vinyl isocyanides and development of a convertible isonitrile. *Org. Lett.* **18** 1622–1625

Tommasi R, Brown DG, Walkup GK, Manchester JI and Miller AA 2015 ESKAPEing the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discov.* **14** 529–542

Wainman YA, Neves AA, Stairs S, Stöckmann H, Ireland-Zecchini H, Brindle KM and Leeper FJ 2013 Dual-sugar imaging using isonitrile and azido-based click chemistries. *Org. Biomol. Chem.* **11** 7297–7300

Walton K and Berry J 2016 Indole alkaloids of the stigonematales (Cyanophyta): chemical diversity, biosynthesis and biological activity. *Mar. Drugs* **14** 73

Wilson RM, Stockdill JL, Wu X, Li X, Vadola PA, Park PK, Wang P and Danishefsky SJ 2012 A fascinating journey into history: exploration of the world of isonitriles en route to complex amides. *Angew. Chem. Int. Ed. Engl.* **51** 2834–2848

Yu G, Kuo D, Shoham M and Viswanathan R 2014 Combinatorial synthesis and in vitro evaluation of a biaryl hydroxyketone library as antivirulence agents against MRSA. *ACS Comb. Sci.* **16** 85–91

Zhang Z, Ren J, Stammers DK, Baldwin JE, Harlos K and Schofield CJ 2000 Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. *Nat. Struct. Biol.* **7** 127–133

Zhu J, Lippa GM, Gulick AM and Tipton PA 2015 Examining reaction specificity in PvcB, a source of diversity in isonitrile-containing natural products. *Biochemistry* **54** 2659–2669

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