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## Investigating the Initial Steps in the Biosynthesis of Cyanobacterial Sunscreen Scytonemin

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Photosynthetic cyanobacteria have evolved a variety of strategies for coping with exposure to damaging solar UV radiation,<sup>1</sup> including DNA repair processes,<sup>2</sup> UV avoidance behavior,<sup>3</sup> and the synthesis of radiation-absorbing pigments.<sup>4</sup> Scytonemin (**1**) is the most widespread and extensively characterized cyanobacterial sunscreen.<sup>5</sup> This lipid soluble alkaloid accumulates in the extracellular sheaths of cyanobacteria upon exposure to UV-A light, where it absorbs further incident radiation  $\lambda_{\text{max}} = 384 \text{ nm}$ .<sup>6</sup> The natural product also possesses interesting biological activity; as a micromolar inhibitor of *polo*-like kinase 1, it exhibits anti-proliferative and anti-inflammatory properties.<sup>7</sup>

A gene cluster responsible for the production of scytonemin was recently identified in *Nostoc punctiforme* ATCC 29133; however, no functions were assigned to specific gene products.<sup>8</sup> In this communication, we outline a possible biosynthetic route to the natural product (Scheme 1a). We also report the characterization of two enzymes involved in the initial steps of this pathway and identify a remarkably selective acyloin reaction as a key step in constructing the carbon framework of the pigment.

The scytonemin gene cluster consists of 18 open reading frames (ORFs), including a total of eight genes involved in the biosynthesis of probable precursors tryptophan and tyrosine and a number of ORFs bearing no significant homology to characterized proteins (for the complete annotation, see Supporting Information). Among the remaining genes, we identified two potential candidates for enzymes utilized in the early stages of scytonemin assembly: NpR1275, which resembles leucine dehydrogenase,<sup>9</sup> and NpR1276, homologous to the thiamin diphosphate (ThDP)-dependent enzyme acetolactate synthase.<sup>10</sup> We also noted the possible involvement of a putative tyrosinase, NpR1263, in later oxidation steps, potentially paralleling the well-established role of these enzymes in melanin biosynthesis.<sup>11</sup>

We hypothesized that NpR1275 could oxidize tryptophan and/or tyrosine to the corresponding pyruvic acid derivative (**4** and **5**). A subsequent acyloin reaction involving these two substrates, catalyzed by NpR1276, would assemble the precursor (**3**) to one half of the scytonemin skeleton. Decarboxylation, cyclization, and oxidation steps, either enzyme-catalyzed or spontaneous, could provide monomer **2**. Notably, the structure of this proposed intermediate is closely related to a known cyanobacterial metabolite, nostodione A.<sup>12</sup> A final oxidative dimerization, either spontaneous or promoted by tyrosinase NpR1263, would afford scytonemin.

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Supporting Information Available: Experimental details and characterization data for new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

To explore the validity of this hypothesis, NpR1275 and NpR1276 were amplified from *N. punctiforme* ATCC 29133 genomic DNA and their products overexpressed in *E. coli* as C-His-tagged fusions. The tryptophan dehydrogenase activity of NpR1275 was confirmed using a spectrophotometric assay measuring the formation of NADH.<sup>13</sup> Although this enzyme readily catalyzed the oxidative deamination of tryptophan, the analogous reaction with tyrosine was extremely slow. It is possible that *p*-hydroxyphenylpyruvic acid (**5**) is generated directly by putative prephenate dehydrogenase NpR1269 and consumed without prior conversion to the amino acid, as scytonemin cluster lacks the transaminase used to convert **5** to tyrosine in the final step of the tyrosine biosynthetic pathway.

With the role of NpR1275 in generating putative substrates for NpR1276 established, we explored the reactivity of this ThDP-dependent enzyme. Treating a mixture of indole-3-pyruvic (**4**) and *p*-hydroxyphenylpyruvic acids (**5**) (500  $\mu$ M each) with NpR1276 (400 nM) in the presence of MgCl<sub>2</sub> (5 mM) and ThDP (1 mM) in pH 7.5 Tris-HCl buffer resulted in the formation of a single new peak by HPLC analysis (Figure 1a). This reactivity was dependent on the presence of NpR1276, as well as both Mg<sup>2+</sup> and ThDP cofactors.

Isolation and characterization of the new product peak from a large-scale enzymatic preparation afforded isomeric acyloins **6** and **7** in a 1.4:1 ratio (Scheme 1b). However, the HPLC retention time of this material did not match that of the original product as confirmed by co-injection of a synthetic standard of **7** with the NpR1276 reaction mixture. Further experiments demonstrated that the acyloin products **6** and **7** formed during incubation of quenched reaction mixtures at room temperature (Figure 1b).

Suspecting that **6** and **7** arose from an initial  $\beta$ -ketoacid adduct (**3**), a reductive quench was attempted in hopes of preventing the presumed decarboxylation event. Treatment of the NpR1276 reaction mixture with sodium borohydride (100 mM) afforded two major products (**8** and **9**) that were not cofactor-derived (Figure 1c). NMR characterization (<sup>1</sup>H, <sup>13</sup>C, HMBC) revealed these compounds to be diastereomeric diols generated from the reduction of a single  $\beta$ -ketoacid regioisomer (**3**) (Scheme 1b).<sup>14</sup> A mixture of **8** and **9** was also obtained by treating **3** with sodium borohydride immediately upon its collection from an analytical HPLC run. Approximately 10% of the material underwent decarboxylation, as indicated by the presence of additional diol products.

The structure of  $\beta$ -ketoacid **3** provides important information about the timing of key bond-forming events in the NpR1276-catalyzed transformation. Isolation of a single regioisomer is indicative of a highly selective reaction of the ThDP cofactor with *p*-hydroxyphenylpyruvic acid (**5**), followed by nucleophilic attack of cofactor-bound **5** onto indole-3-pyruvic acid (**4**) (for mechanism, see Scheme S4, Supporting Information). The lack of products arising from coupling between two identical pyruvic acid derivatives (Figure 1b) indicates an exquisite level of enzymatic control over the binding and activation of both substrates.<sup>15</sup> Although ThDP-dependent enzymes have been engineered to perform selective C-C bond-forming reactions,<sup>16</sup> this degree of specificity is largely unprecedented in natural systems.<sup>17,18</sup> Elucidation of the factors responsible for this selectivity may provide valuable information for future engineering efforts.

Finally, a number of biologically active acyloin- and diol-containing natural products from various sources appear to be assembled using related biosynthetic logic.<sup>19</sup> Each of these molecules could conceivably arise from the action of a ThDP-dependent enzyme on pyruvic acid-containing substrates diverted from primary metabolism. Such enzymes may represent a rich, untapped source of biocatalysts capable of selective C-C bond formation.

In summary, we have proposed a biosynthesis for the cyanobacterial pigment scytonemin and characterized two enzymes utilized in the early stages of this pathway. Further study of the

NpR1276-catalyzed reaction, as well as the other enzymatic transformations involved in the assembly of scytonemin, will be the focus of future investigations.

## Supplementary Material

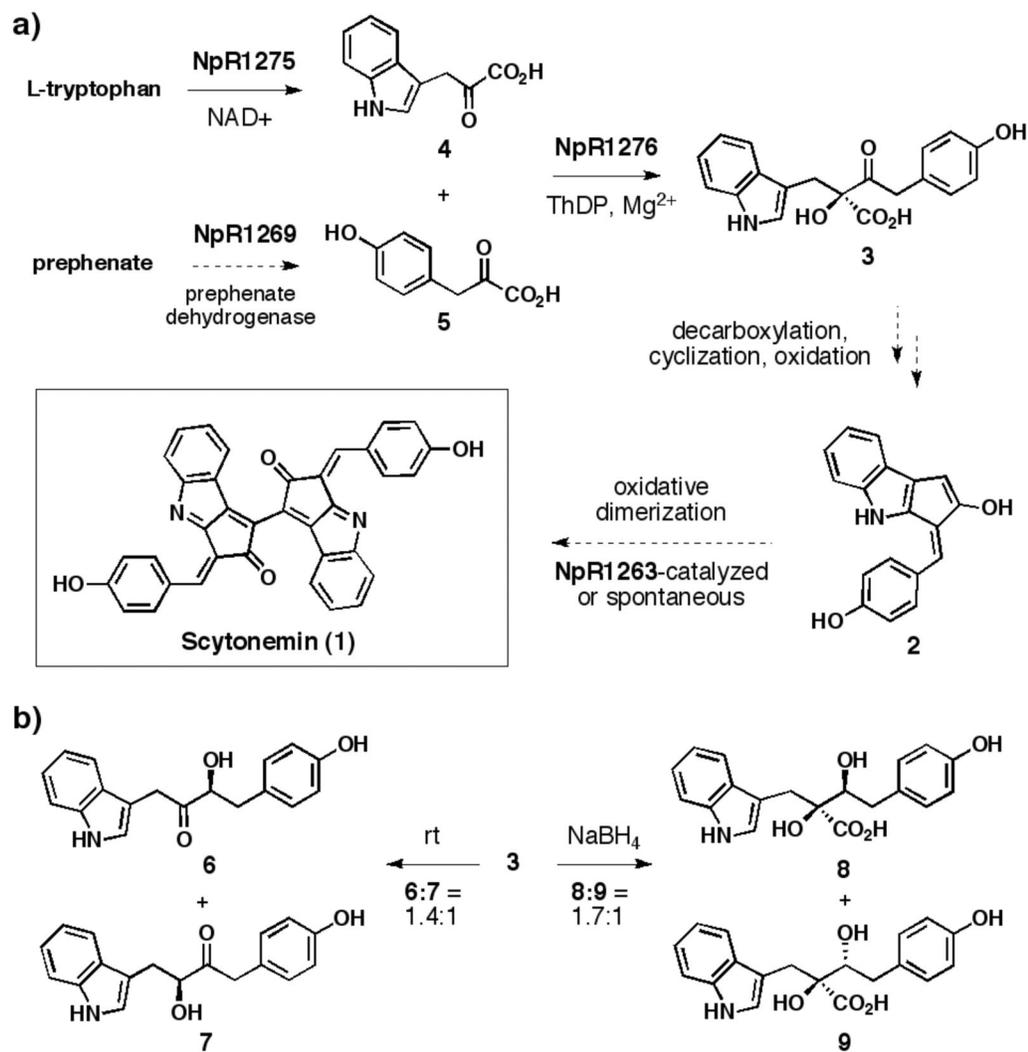
Refer to Web version on PubMed Central for supplementary material.

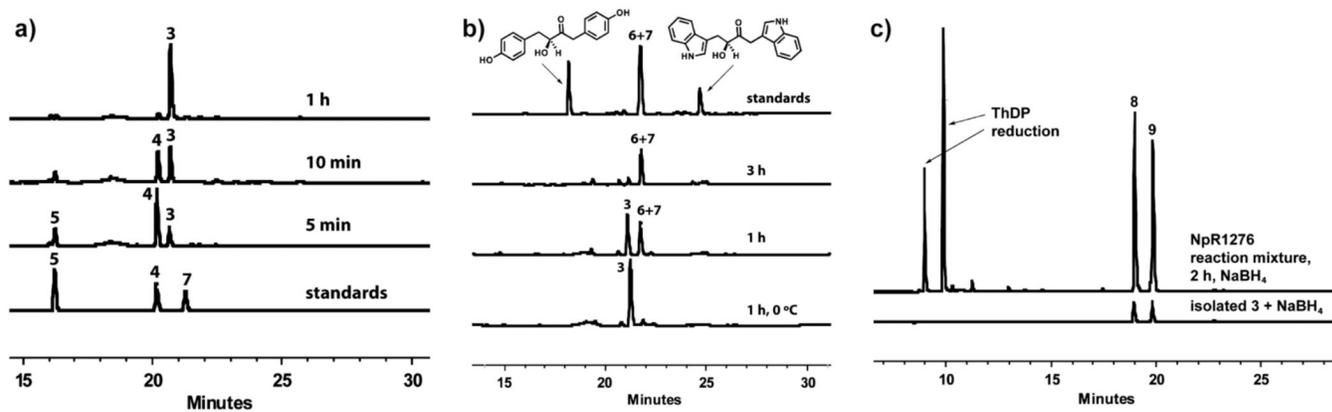
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- (14). The absolute and relative stereochemistry of **8** and **9** have not yet been assigned.
- (15). When treated with **4** or **5** individually, NpR1276 will catalyze homocoupling at a greatly reduced rate (see Supporting Information)
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- (18). The instability and poor aqueous solubility of pyruvic acids **4** and **5** have prevented quantitative kinetic analysis and comparison of NpR1276 with related ThDP-dependent enzymes.
- (19). For the structures and isolation references of these related natural products, see Supporting Information.





**Figure 1.**

a) HPLC time course for NpR1276-catalyzed formation of  $\beta$ -ketoacid 3 (280 nm, MeOH quench). b) HPLC time course of  $\beta$ -ketoacid decarboxylation during rt incubation of acidified reaction mixtures (220 nm). c) HPLC traces of  $\text{NaBH}_4$  reduction products (280 nm).