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Posttranslational tyrosine geranylation in cyanobactin biosynthesis

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Abstract

Prenylation is a widespread modification widely that improves the biological activities of secondary metabolites. This reaction also represents a key modification step in biosyntheses of cyanobactins, a family of ribosomally synthesized and posttranslationally modified peptides (RiPPs) produced by cyanobacteria. In cyanobactins, amino acids are commonly isoprenylated by ABBA prenyltransferases that use C5 donors. A potential exception was the discovery of piricyclamides, from a fresh-water cyanobacterium were proposed to have a C10 geranyl group. Here we characterize a novel geranyltransferase involved in piricyclamide biosynthesis. Using the purified enzyme, we show that the enzyme PirF catalyzes Tyr *O*-geranylation, which is an unprecedented posttranslational modification. In addition, the combination of enzymology and analytical chemistry revealed the structure of the final natural product, piricyclamide 7005E1, and the regioselectivity of PirF, which has potential as a synthetic biological tool providing drug-like properties to diverse small molecules.

Graphical Abstract

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Supporting Information. A PDF file of experimental methods and additional data. The Supporting Information is available free of charge on the ACS Publications website.

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Prenylated peptides are widespread in nature. Protein prenylation is thought to improve interactions with membranes,¹⁻³ while small molecule peptide prenylation⁴⁻⁶ provides drug-like properties that may improve pharmacological properties such as low membrane permeability and chemical/physical instabilities.⁷⁻¹⁰ Among tools to catalyze broad-substrate peptide prenylation, the cyanobactin prenyltransferases are promising. Cyanobactins are ribosomally-synthesized and posttranslationally modified peptide (RiPP) natural products,¹¹ which are often modified by ABBA prenyltransferases.^{12,13} Cyanobactin prenyltransferases have the useful property of being highly chemoselective for the residue being modified, the position and orientation of modification, and isoprene donor, while also being highly flexible in terms of peptide sequence. For instance, specific examples are known that forward prenylate Trp¹⁴ or Tyr¹⁵, while others reverse prenylate Ser/Thr¹⁶ or Tyr.¹⁷ While these modifications take place natively in the context of N-C circular peptides, in vitro the enzymes accept many related substrates, including linear peptides and discrete amino acids. Additionally, one subclass is known that N-prenylates Phe at the N-terminus of short linear peptides.¹⁸ Structures of cyanobactin prenyltransferases suggest that the observed substrate selectivity is accommodated by substrate recognition focused on the residue that is modified, with a large open pocket that forms largely hydrophobic interactions with diverse substrate sequences.¹⁵

All of the characterized cyanobactin enzymes use dimethylallyl pyrophosphate (DMAPP) to generate C₅-prenylated metabolites. In 2012, the Sivonen group investigated the freshwater cyanobacterium *Microcystis aeruginosa* PCC7005, discovering a pathway that encoded cyanobactin biosynthetic machinery including a prenyltransferase, enabling the prediction of several new cyanobactin metabolites (Figure 1).¹⁹ When the *M. aeruginosa* extract was analyzed by mass spectrometry, the predicted compounds were discovered, but surprisingly their *m/z* ratio and fragmentation pattern indicated geranylation, rather than DMAPP addition. Geranylation is a biochemically uncharacterized posttranslational modification in cyanobactin pathways.

The piricyclamide pathway was of interest because the amino acid(s) being geranylated, and thus the structures of the natural products, were not known, in part due to the difficulty in obtaining sufficient material from the wild-type cyanobacteria. More importantly, while geranylation is ubiquitous, peptide geranylation is rare, being found so far in only a small number of natural products.²⁰⁻²³ A number of small molecule natural products are geranylated by ABBA-type prenyltransferases.^{12,13} However, tyrosine geranylation (C10) is potentially an unprecedented posttranslational modification, by ABBAs or by any other prenyltransferase. Here we report the biochemical characterization of PirF, a geranyltransferase involved in piricyclamide biosynthesis, and reveal the structure of the final natural product. PirF forward geranylates the phenolic hydroxyl group of Tyr in peptides and a diverse array of small phenols.

We first examined the substrate specificity of purified PirF. Considering the amino-acid sequences of piricyclamides and the substrates of other RiPPs prenyltransferases, we hypothesized that PirF might accept Ser, Thr, Trp, or Tyr as a substrate. In previous work with F-type prenyltransferases, enzymatic modification of *N*-Boc protected amino acids recapitulated the native substrate selectivity of the enzymes. Therefore, to test our

hypothesis, *N*-Boc amino acids Ser, Thr, Trp, and Tyr were incubated with PirF and the hypothetical isoprenoid donor, geranyl pyrophosphate (GPP). Liquid chromatography-mass spectrometry (LC-MS) analyses demonstrated that PirF selectively accepts *N*-Boc Tyr (**6**) as a substrate, but not the other amino acids. In cyanobactin prenyltransferases so far characterized, the isoprene group is lost when modification takes place on oxygen, but not from modification on carbon. In the LC-MS, the product of the *N*-Boc-Tyr reaction exhibited a fragment resulting from loss of 136 Da, suggesting that PirF catalyzes *O*-geranylation (Figure S17).

To understand the substrate scope, we assayed PirF with various Tyr derivatives and Tyr-containing peptides inspired from previous studies of cyanobactin prenyltransferase selectivity (Table 1).^{15,17} HPLC and LC-MS analyses demonstrated that PirF catalyzed geranylation of natural and unnatural Tyr (**7** and **8**), Tyr-Tyr-Tyr tripeptide (**9**), and cyclic and linear Tyr-containing peptides (**2** and **4**). Like PagF, a homologous enzyme involved in prenylagaramide B biosynthesis,^{15,24} PirF preferred larger peptides to small amino acids. Interestingly, small phenolic compounds including 3- or 4-hydroxybenzoic acids (**13** and **14**), 2,4- or 3,4- dihydroxybenzoic acids (**15** and **17**), and ferulic acid (**19**) were also geranylated, while Tyr-derivatives such as L-DOPA (**24**) and dopamine (**25**) were not. This broad-substrate tolerance suggests that PirF and other RiPP prenyltransferases may provide a set of synthetic-biological tools to modify many different types of compounds, especially peptide- or protein-derived compounds.

Since Tyr-Tyr-Tyr tripeptide (**9**) was the best substrate that was easily available, we used it to examine isoprenoid donor specificity, using C₅ to C₂₀ isoprenoid donors (Figure S21). No reaction was observed when PirF was incubated with DMAPP, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate, indicating that PirF selectively uses the C₁₀-carbon donor, GPP. To further verify the donor preference of PirF, we ran competition assays with GPP and C₅-C₂₀ isoprenoid donors (Figure S22). The presence of C₅-C₂₀ length donors had little effect on the end-point yields of geranylated Tyr-Tyr-Tyr, appearing not to compete significantly with GPP in the active site.

We investigated the regioselectivity of PirF on the efficient substrate Tyr-Tyr-Tyr (**9**). We previously reported a crystal structure of the homologous DMAPP prenyltransferase PagF, wherein substrate **9** was in the active site adjacent to a prenyl donor analog.¹⁵ In incubations with **9** and DMAPP, PagF was found to yield predominantly the Tyr₁ prenylated analog, with a lower amount of other products. Considering that Tyr₁ was found in proximity to the prenyl donor analog in PagF, we hypothesized that PirF would also predominantly modify the *N*-terminal Tyn. We performed a PirF reaction with Tyr-Tyr-Tyr tripeptide (**9**), purified the products, and elucidated their structures by NMR experiments (Table S6, Figure S26). As we anticipated, PirF gave an *N*-terminal Tyr₁-geranylated product (**10**, 38%) as a major product, while Tyr₂-geranylated (**11**, 14%) and doubly-geranylated products (**12**, 5%) were also detected by LC-MS and HPLC as minor products (Figures S6 and S8), implying that PirF might bind to Tyr-Tyr-Tyr (**9**) in a similar orientation to PagF. Finally, NMR data confirmed that PirF catalyzes *O*-geranylation in the forward orientation.

Based upon these results and the analysis of Leikoski *et al.*,¹⁹ piricyclamides are predicted to have an *O*-geranyl group on Tyr residue. Piricyclamides were originally discovered from a cyanobacterium *Microcystis aeruginosa* PCC7005, where four precursor peptides PirE1-E4 are encoded in the biosynthetic cluster (*pir*) (Figure 1).¹⁹ Among the four piricyclamides, piricyclamide 7005E1 and E3 were proposed to be singly geranylated; however, piricyclamide 7005E1 has two Tyr residues that can be modified. To elucidate the structure of piricyclamide 7005E1, we enzymatically synthesize it using PirF *in vitro* (Figure 2A). In cyanobactin biosyntheses, tailoring reactions such as prenylation or heterocyclization generally take place after the core-building reactions such as C- and N-terminal proteolysis of the precursor peptides.²⁵⁻²⁸ Thus, we hypothesized that cyc-MSGVDYyNP (**2**) is a natural substrate of PirF. NMR analyses of purified products revealed that enzymatic reactions with PirF provided a mixture of two regioisomers cyc-MSGVDYyNP (**3**, 60%) and cyc-MSGVDyYNP (**1**, 40%), where a lower-case y indicates the geranylated Tyr residue (Figures 2A and 2D). In addition, a trace of a doubly-geranylated product (**27**) was observed (Figure 2B).

To investigate whether *M. aeruginosa* PCC7005 also biosynthesizes cyc-MSGVDYyNP (**3**) as a major product, we compared the synthetic piricyclamides with the extract of *M. aeruginosa* PCC7005 (Figure 3). Since **1** and **3** were poorly separated by standard chromatographic methods in ultra-performance liquid chromatography (UPLC)-MS, the two synthetic piricyclamides cyc-MSGVDyYNP (**1**) and cyc-MSGVDYyNP (**3**) were chemically oxidized to methionine sulfoxides cyc-M(O)SGVDyYNP (**28**) and cyc-M(O)SGVDYyNP (**29**), respectively, providing a better separation (Figures 3B and 3C). Unexpectedly, UPLC-MS experiments demonstrated that the cyanobacterial extract contained cyc-M(O)SGVDyYNP (**28**, 87%) as a major regioisomer accompanied by small amount of cyc-M(O)SGVDYyNP (**29**, 13%). This result indicates that the cyanobacterium predominantly produces cyc-MSGVDyYNP (**1**), which is identical to the minor product from enzymatic reactions. Having this mismatch, we were further interested in the regioselectivity of PirF. Recently, the cyanobactin kawaguchipectin Trp prenyltransferase was shown to synthesize different regiochemical variants *in vitro* in comparison to *in vivo*, a finding very similar to the initial observation in our study here.¹⁴ Thus, the existing data suggested that the cyanobactin biosynthetic pathways may be more complex than indicated by current dogma.

We envisioned several possibilities: i) a different reaction preference is found in the context of the native pathway in comparison to the purified enzyme; ii) a different substrate is utilized than the expected cyclic peptide; or iii) a process such as preferential product or substrate degradation occurs *in vivo*. Because investigation of *in vivo* biosynthetic processes in cyanobacteria is anticipated to be challenging and has no precedent, to further investigate this issue, we tested the alternate substrate hypothesis ii.

To investigate a substrate preference of PirF, we used a linear peptide MSGVDYyNPFAGDDAE (**4**). Compound **4** is the putative substrate for PirG macrocyclase (Figure 1). Although for many cyanobactins the substrate is cyclic, in the case of linear cyanobactins aeruginosamides, the prenyltransferase adds DMAPP to the N-terminus prior to the action of a PatG-like protease.¹⁸ To compare the two substrates, kinetic parameters were measured with cyc-MSGVDYyNP (**2**) and MSGVDYyNPFAGDDAE (**4**) (Table 2,

Figure S2). Kinetic values were very similar for both substrates, being identical within error. To further investigate this issue, we performed competition experiments with cyc-MSGVDYYNP (**2**) and various amounts of MSGVDYYNPFAGDDAE (**4**). LC-MS analyses showed that geranylation of the cyclic substrate (**2**) was not inhibited even with a 10-fold excess of the linear substrate (**4**) (Figure S20). For several cyanobactin enzymes,^{15, 17, 29} the preferred substrates are the cyclic peptides both *in vivo* and *in vitro*, but in this case, no clear substrate preference was observed.

Considering that the linear substrate **4** was also an efficient substrate of PirF *in vitro*, we further verified the alternate substrate hypothesis ii. Using MSGVDYYNPFAGDDAE (**4**), we carried out enzymatic geranylation, purification, and NMR experiments (Figures 2C and 2E). In contrast to the reactions with cyc-MSGVDYYNP (**2**), PirF selectively gave MSGVDyNPFAGDDAE (**5**) as a single product. If active PirF behaved within the cyanobacterium as it does in this assay, a single regioisomer of piricyclamide natural product would be observed. Thus, this shows that cyc-MSGVDYYNP (**2**) is the true substrate of PirF even if the enzyme accepts the linear peptide **4** as well as **2** *in vitro*. In the kinetic analyses, double geranylation was observed both with the cyclic substrate (25%) and the linear substrate (8%); however, no double geranylation was detected in the extract of *M. aeruginosa* PCC7005 by UPLC-MS experiments. Taken together, these results indicate that the mixture of observed natural products results from a more complex process than is recapitulated in the *in vitro* assays.

In summary, we characterized PirF, a RiPP geranyltransferase catalyzing forward *O*-geranylation of Tyr, which may be a novel posttranslational modification. Using enzymatic reactions and chemical-structural analyses, we elucidated the structure of piricyclamide 7005E1 and gained information about the regioselectivity of PirF. To our knowledge, this work is the first evidence that a cyanobactin F-family enzyme recognizes a C₁₀-length isoprenoid donor instead of C₅. The biochemical basis of the geranyltransferase may guide further studies to understand the molecular mechanism of RiPPs prenyltransferases that selectively recognize carbon length of isoprenoid donors. Furthermore, the broad-substrate tolerance of PirF demonstrates the potential of cyanobactin prenyltransferases to create diverse small molecules through synthetic biology. The quality of specific chemoselectivity, within an otherwise modifiable scaffold, will be useful in building desired products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Biosynthetic gene cluster of piricyclamide (*pir*) from the cyanobacterium *M. aeruginosa* PCC7005. A precursor peptides PirE1-E4 encode the core sequences of piricyclamide 7005E1-E4.

Figure 3.

Comparison of the synthetic piricyclamides with the extract of *M. aeruginosa* PCC7005. (A) Piricyclamides produced by *M. aeruginosa* PCC7005. (B) Oxidization reaction of **1** and **3** provided the corresponding sulfoxides cyc-M(O)SGVDyYNP (**28**) and cyc-M(O)SGVDYyNP (**29**), respectively. (C) Extracted ion chromatograms from UPLC-MS analysis of the cyanobacterial extract (black), synthetic **28** (blue), and synthetic **29** (red). Extracted ion chromatograms for the product mass of the sodium form (m/z 1201.5) were shown.

Table 1.

Geranylation activity of PirF with various small molecules.

substrate ^a	yield (%) ^b
<i>N</i> -Boc tyrosine (6)	55 ± 13
L-tyrosine (7)	30 ± 8
D-tyrosine (8)	27 ± 10
Tyr-Tyr-Tyr (9)	91 ± 16
cyc-MSGVDYYNP (2)	65 ± 10
MSGVDYYNPFAGDDAE (4)	96 ± 4
2-hydroxybenzoic acid (12)	NR ^c
3-hydroxybenzoic acid (13)	25 ± 5
4-hydroxybenzoic acid (14)	81 ± 25
2,4-dihydroxybenzoic acid (15)	39 ± 4
2,6-dihydroxybenzoic acid (16)	NR
3,4-dihydroxybenzoic acid (17)	7 ± 4
3,5-dihydroxybenzoic acid (18)	NR
ferulic acid (19)	11 ± 6
sinapic acid (20)	NR
phenol (21)	NR
catechol (22)	NR
1-naphthol (23)	NR
L-DOPA (24)	NR
dopamine (25)	NR

Measurements were conducted in triplicate.

^a A concentration of 100 μM was used, except for cyc-MSGVDYYNP (50 μM); GPP was used in large excess (10-fold above phenolic substrate).^b End-point yields of geranylated products were calculated from the area ratio in HPLC trace after overnight (15 h) reaction.^c NR, no reaction: no product detected.

Table 2.

Kinetic parameters of PirF with cyclic and linear peptide substrates

substrate _a	cyc-MSGVDYYNP (2)	MSGVDYYNPFAGDDAE(4)
S (mM)	0.1	0.1
k_{cat} (min ⁻¹)	0.7 ± 0.1	1.1 ± 0.4
K_{m} (μM)	115.9 ± 0.0	118.1 ± 0.1
$k_{\text{cat}}/K_{\text{m}}$ (min ⁻¹ mM ⁻¹)	6.0 ± 0.5	10.6 ± 4.5

Measurements were conducted in triplicate. Figure S2 in SI presents progress curves used to calculate the parameters.