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COMMUNICATION

Genome mining based discovery of the cyclic peptide tolypamide and ToIF, a Ser/Thr forward O-prenyltransferase

Mugilarsi Purushothaman[a], Snigdha Sarkar[b], Maho Morita[c], Muriel Gugger[d] and Eric W. Schmidt *[b], Brandon I. Morinaka*[a]

[a] M. Purushothaman, Prof. Dr. B. I. Morinaka
Department of Pharmacy
National University of Singapore
18 Science Dr 4, Singapore 117543 (Singapore)
E-mail: phambi@nus.edu.sg
[b] S. Sarkar, Prof. Dr. E. W. Schmidt
Department of Medicinal Chemistry
University of Utah
Salt Lake City, Utah 84112 (USA)
E-mail: ew1@utah.edu
[c] M. Morita
Laboratory of Chemical Biology of Natural Products, Graduate School of Bioagricultural Sciences
Nagoya University
Furo-cho, Chikusa, Nagoya 464-8601 (Japan)
[d] M. Gugger
Institut Pasteur, Collection des Cyanobactéries
Département de Microbiologie, Paris 75015 (France)

*These authors contributed equally.

To whom correspondence should be addressed. ews1@utah.edu, phambi@nus.edu.sg

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Abstract: Cyanobactins comprise a widespread group of peptide metabolites produced by cyanobacteria that are often diversified by post-translational prenylation. Several enzymes have been identified in cyanobactin biosynthetic pathways that carry out chemically diverse prenylation reactions, representing a resource for the discovery of post-translational alkylating agents. Here, genome mining was used to identify orphan cyanobactin prenyltransferases, leading to isolation of tolypamide from the freshwater cyanobacterium, *Tolyphothrix* sp. The structure of tolypamide was confirmed by spectroscopic methods, degradation, and enzymatic total synthesis. Tolypamide is forward prenylated on a threonine residue, representing an unprecedented post-translational modification. Biochemical characterization of cognate enzyme ToIF revealed a prenyltransferase with strict selectivity for forward O-prenylation of serine or threonine, but with relaxed substrate selectivity for flank peptide sequences. Since cyanobactin pathways often exhibit exceptionally broad substrate tolerance, these enzymes represent robust tools for synthetic biology.

Over the past decade, peptides have secured a stronghold in the pharmaceutical market due to numerous advantages over traditional small molecule drugs.[1] Bioactive peptides are effective in targeting protein-protein interactions, have low incidences of off-target effects and cytotoxicity. However, these properties are sometimes compromised by their poor bioavailability caused by proteolytic degradation, rapid renal clearance and failure to cross cellular membranes. The bare peptides can be chemically modified in multiple ways to boost their therapeutic efficacy.[2] One means to improve membrane permeability is lipidation or prenylation, but the current methods for selective enzymatic peptide alkylation are relatively limited.[3]

Prenyltransferase enzymes catalyze the intermolecular transfer of prenyl groups from isoprene donors to a variety of acceptors.[4] On peptides and proteins, these acceptors include electron-rich positions on aromatic rings and heteroatoms. The reaction is initiated by the generation of the allylic carbocation from prenyl diphosphate precursor, followed by nucleophilic attack by an electron rich group to produce the final prenylated product. A prenylation reaction can have multiple outcomes depending on isomerization of the intermediate carbocation, regiospecificity, chemoselectivity, and rearrangement of the preliminary product. As a result, the prenyltransferase enzyme family is capable of generating structurally diverse products from a limited number of precursors.

Cyanobactins are a chemically diverse class of ribosomally synthesized and posttranslationally modified peptide natural products (RIPPs)[5] isolated from cyanobacteria and have demonstrated anticancer and antimarial activities.[6] Characteristic posttranslational modifications of this class are macrocyclization, heterocyclization (to form thiazole, oxazole, thiazoline, and oxazoline), and prenylation. Cyanobactin pathways show promise in biotechnology because many of the tailoring enzymes exhibit exceptionally relaxed substrate selectivity.[6, 7] A significant subset of cyanobactins are tailored by the F-type prenyltransferases, which append an isoprene unit to Ser, Thr, Tyr and Trp residues or at the N- or C-terminus of linear peptides.[6b, 6c, 9] These prenyltransferases have the same secondary structural motifs as ABBA type enzymes with a bundle of 10 anti-parallel β-sheets surrounded by a ring of 10 solvent exposed α-helices.[9] The interior of the active site is similar to other prenyltransferases, exhibiting strict selectivity for the prenyl donor, amino acid acceptor, and the direction of prenylation at either C-1 (forward) or C-3 (reverse) position of the dimethylallyl cation.[9b, 10] Although the prenyl donor is usually derived from dimethylallylpyrophosphate, one enzyme using geranyl pyrophosphate has been characterized.[11] In comparison to other prenyltransferases with capped active sites that sequester small substrates, cyanobactin F enzymes have a large, open hydrophobic cavity adjacent to the active site, which allows them to accommodate larger peptide substrates.[12] This peculiarity also
gives the cyanobactin F enzymes strict selectivity for the prenylation donor and acceptor amino acid, but often

extremely relaxed selectivity for the remainder of the peptide. This has proven to be useful in the biotechnological generation of diverse, alkylated peptides.\textsuperscript{[8f, 13]} In addition to these enzymes, a major fraction of F enzyme family exemplified by PatF appear to be essential for biosynthesis, but are inactive and may serve a non-prenylating role in the pathways.\textsuperscript{[12]} Prenylation is usually the last step of the cyanobactin biosynthetic pathway and in presence of inactive prenyltransferase genes, non-prenylated cyclic peptides are observed as mature natural products.\textsuperscript{[13]}

TruF1 was the first cyanobactin prenyltransferase to be unambiguously characterized both \textit{in vivo} and \textit{in vitro}.\textsuperscript{[8c, 13]} However, the poor solubility and low \textit{in vitro} activity of TruF1 prevented extensive structural characterization. TruF1 is the only identified prenyltransferase that modifies Ser and Thr hydroxyl groups, prenylating them with DMAPP in the reverse orientation. As such, there remains a significant gap in the cyanobactin chemical space, where there is no well-behaved, promiscuous catalyst for prenylation of Ser and Thr residues. Here, we analyzed the prenyltransferase sequence-function space to identify an orphan F enzyme bearing sequence similarity to TruF1.

The pathway was de-orphanized through the identification of a novel cyanobactin natural product, tolypamide (1). The cyclic peptide was forward-prenylated on Thr, representing a previously unknown post-translational modification. The TolF prenyltransferase was characterized \textit{in vitro}, demonstrating its selectivity for Ser or Thr forward prenylation in the context of several peptide sequences. TolF represents a robustly expressed and stable enzyme useful in the modification of Ser and Thr residues.

To investigate the sequence-function and chemical space of cyanobactin prenylation, a sequence similarity network (SSN) was generated for cyanobactin prenyltransferase InterPro family (IPR031037, TIGR04445) (Figure 1A).\textsuperscript{[14]} This resulted in two large clusters, one containing inactive enzymes (PatF, TruF2, and TenF) and the other with enzymes catalyzing forward O-prenylation on Tyr (PagF and PirF).\textsuperscript{[8b, 11, 13, 15]} The SSN also contained smaller clusters representing other regiospecific transformations such as N-prenylation on tryptophan (AcyF), prenylation on the terminal amino acid (AgeMTPT, MusF1, MusF2), C-prenylation on tryptophan (KgpF) and reverse O-prenylation on Tyr (LynF) (Figure 1C).\textsuperscript{[8b, 8a, 8c-11]} Often, clustering is diagnostic for the function of uncharacterized enzymes, and indeed all characterized enzymes fell into clusters predictive of their chemistry. However, prenyltransferase TolF from \textit{Tolypothrix} sp. clustered with LynF but only shared ~53% amino acid sequence identity, suggesting a possibly divergent function. TolF was compared with other characterized cyanobactin prenyltransferases in a Bayesian phylogenetic tree (Figure 1B), revealing that TolF is evolutionarily more closely related to TruF1, despite the proteins sharing only ~48% identity. We therefore predicted that TolF might produce new chemistry. Because of this possibility, and because no cyanobactins or cyanobactin biosynthetic genes from \textit{Tolypothrix} had previously been described, we chose to investigate this pathway further.

The gene \textit{tolF} was identified in \textit{Tolypothrix} sp. PCC 7601, a freshwater filamentous cyanobacterium isolated from a lake in the state of Connecticut, USA. We identified the 10.4 kb long \textit{tol} gene cluster from a 9.96 Mbp draft genome (Figure 2A), containing the signature genes involved in cyanobactin biosynthesis.\textsuperscript{[16]} \textit{tolE} encodes the precursor peptide, which is the substrate for enzymatic modification. TolE contains recognition sequences

Figure 1. Sequence-function and chemical space of cyanobactin prenyltransferases. A) Sequence similarity network showing the clustering of cyanobactin prenyltransferases according to their regiospecificity using an alignment score threshold of 86. B) Bayesian phylogenetic tree of TolF with previously characterized prenyltransferases show TolF falls in the same clade as TruF1, showing similar substrate preference. C) Regioselective and chemoselective modes of prenylation carried out natively by characterized cyanobactin prenyltransferases. Reverse and forward prenylation patterns are represented within and outside the dashed box, respectively. The type of transformation is indicated by the atom (italicized) followed by the residue, Nterm or Cterm. The enzyme is shown in parenthesis. The R-group = Me and H for Thr and Ser, respectively.
To test our predictions, we initiated efforts to detect the cyanobactin product from the tol cluster in *Tolyphothrix* sp. PCC 7601. The LC/MS chromatogram of the extracted culture medium showed a peak with two major ions at m/z 671.2668 Da and m/z 603.2047 Da (Figure 2B). The neutral mass difference of m/z 68 Da between the two ions is typical of the loss of isoprene.\[18\] Moreover, the mass of the parent ion precisely matches the structure predicted from the biosynthetic gene cluster. Large scale fermentation was carried out to isolate the compound, tolypamide (1), for spectroscopic studies.

Extensive 1D and 2D NMR analysis, including COSY, HSQC, HMBC and NOESY, was carried out to elucidate the structure of 1 (Table S1 and Figures S2-S6). The 'H NMR spectrum showed characteristic features of a peptide, such as α-methylene multiplets (δH = 5.5-3.5 ppm) and clear NH proton signals (δH = 8.18-7.23 ppm). Examination of the 2D NMR spectra led to the assignment of seven spin systems consisting of Thr, Ala, Pro, Tyr, a prenyl group and two Cys, which collectively matched our initial predicted core sequence. The NH peak at δH 7.23 ppm in the Thr spin system indicated that it was not modified by heterocyclization. Each of the Cys spin systems showed a methylene carbon (δC 37.3–38.8 ppm) correlated to a methyl carbon (δC 77.9–78.4 ppm) both resonating further downfield than usual. In addition, the absence of NH peaks in the Cys spin systems along with 13C chemical shifts that were consistent with literature data to thiazoline-containing peptides, supported heterocyclization of both Cys residues to thiazoline rings.\[20\] The two methyl singlets (δC 1.47 and 1.26 ppm) in the 'H NMR were assigned to a terminal gem-dimethyl of a prenyl spin system indicating forward prenylation in 1. Further support for this assignment was obtained by the strong HMBC correlation between the β-proton (δH 3.95 ppm) of Thr and the methylene carbon (δC 65.3 ppm) of the prenyl group. Both these observations suggested that the prenyl group was attached to the Thr through an ether linkage, thus revealing the identification of the structure of forward-prenylated Thr (PrThr) unit, which to the best of our knowledge has not been previously observed.

The absolute configuration of (1) was assigned using a Marfey’s type method, confirming the presence of L-Ala, L-Thr, and L-Cys.\[21\] During the hydrolysis, both Pro and Tyr racemized because of the adjacent azoline double bond.\[22\] Subjecting 1 to ozonolysis prior to derivatization revealed the presence of L-Pro. The L-Pro in 1 was assigned as the trans rotamer based on the difference in 13C chemical shifts of β and γ carbons of Pro (ΔδC = 4.5 ppm).\[22\] Hydrolysis in deuterium chloride led to a selective deuterium labelling of the D-isomers, demonstrating that the D-Pro and D-Tyr were hydrolysis artefacts and revealing that all amino acids in 1 are in the L-configuration.\[22\]

Tolympamide is structurally unique among characterized cyanobactins in that the cyclic heptapeptide contains two thiazoline rings and a forward O-prenylated threonine. The latter is unprecedented among this class of natural products. Under the growth conditions tested, tolypamide (1) is the only detectable product by LC/MS which can be connected with the tol pathway. Although a number of cyanobactins have shown cytotoxic activity,\[19b\] tolypamide (1) did not show significant cytotoxic or antimicrobial activity against a panel of six cancer cell lines (DU145, A549, HeLa CCL2, HepG2, and MDA-MB 231) or bacterial strains (*E. coli* ATCC 25922, *S. aureus* ATCC 29737 and *P. aeruginosa* ATCC 9027), respectively.

**Figure 2.** Isolation of the cyanobactin tolypamide (1) from *Tolyphotrix* sp. A) The tol gene cluster and partial sequence of precursor peptide (ToIE) with the core regions in bold. RSI, RSII and RSIII are annotated. B) Total ion chromatogram of the crude extract of *Tolyphotrix* sp. PCC 7601. The compound of interest, tolypamide (1) eluted at t = 13.80 mins. Inlay is the extracted mass spectrum of the peak at t = 13.80 mins with m/z = 671.26 Da and m/z = 603.20 Da (loss of the prenyl group). The other peaks observed did not show m/z values which could be correlated to a tol pathway product. C) 2D NMR correlations establishing the planar structure of 1. D) Structure of tolypamide (1) (RSs) I, II, and III, which should be responsible for docking with TolD, TolA, and TolG, respectively.\[17\]

Collectively, if similar to other cyanobactin pathways, the four proteins (ToIE, TolD, TolA and TolG) would produce an N-C cyclic peptide in which all Cys/Thr residues would be heterocyclized to thiazoline/ oxazoline residues.\[16\] ToIE contained three copies of the core peptide sequence, TAPCVC, encoding the final cyclic natural product. Each core peptide was flanked by RSI (GVDAS) and RSII (SYD), so that one equivalent of ToIE would produce three equivalents of the cyclic peptide product. The role of ToEF was unknown, but its evolutionary relationship to TruF1 suggested that it might prenylate the non-heterocyclized Thr residue present in the core peptide sequence.

To test our predictions, we initiated efforts to detect the cyanobactin product from the tol cluster in *Tolyphotrix* sp. PCC 7601. The LC/MS chromatogram of the extracted culture medium showed a peak with two major ions at m/z 671.2668 Da and m/z 603.2047 Da (Figure 2B).
We further used this subset of related substrates to answer the major scientific question posed by this study: what governs the forward versus reverse prenylation of Ser and Thr residues? Extremely sequence-diverse natural and synthetic peptides are known to be reverse-prenylated on Ser/Thr residues by TruF1, which is unfortunately challenging to characterize biochemically. Therefore, ToIF offered us the opportunity to characterize the serine/threonine-prenylating class of enzymes for the first time and to obtain deeper insight into the regioselectivity of DMAPP addition. We hypothesized that ToIF would be intrinsically forward-prenylating, regardless of the sequence of peptide substrate. To test this hypothesis, we used the most synthetically accessible robust substrate, the disulfide dimer 6. When 6 was treated with ToIF and DMAPP at scale, both mono-prenylated and di-prenylated products were identified (Figure S14F). To obtain sufficient compound for purification, peptide dimer 6 was reduced using tris (2-carboxyethyl)phosphine (TCEP) and treated with iodoacetamide (IAA) to produce 7, which when treated with ToIF and DMAPP afforded mono-prenylated 8 in good yield (Figure 3B). The reduced form of 6 and compounds 7 and 8 were purified and used for NMR experiments (Figure S8).

The $^1$H NMR and HSQC spectra were consistent with the proposed structures, and they were assigned using COSY and TOCSY experiments (Figure S9-S12 and Tables S3-S5). The peptide sequences were further supported by ROESY correlations and tandem mass spectrometry. Comparison of $^1$H NMR spectra of 6 (reduced form), 7 and 8 shows the disappearance of the Thr hydroxyl group ($\delta_1 5.51$ ppm) and emergence of $^1$H chemical shifts from forward prenyl group in compound 8, indicating that the prenyl group is installed on the Thr residue (Figure S9). The $^1$H NMR spectrum of 8 showed the signature chemical shifts associated with a forward prenyl moiety, with diastereotopic $\alpha$-methylene protons ($\delta_1 4.01, 3.87$ ppm), $\alpha$-methylene proton ($\delta_1 5.24$ ppm) and sharp gem-dimethyl singlets ($\delta_1 1.60, 1.69$ ppm). The isoprene $\alpha$-methylene protons ($\delta_1 4.01, 3.87$ ppm) showed ROESY correlations with Thr via the Thr proton ($\delta_1 3.63$ ppm) and $\gamma$-proton ($\delta_1 1.13$ ppm) (Figure S10). Taken together with the Thr, Ser and Tyr chemical shifts in 6, these data unambiguously demonstrate that ToIF is forward prenylating on Thr.

Previous studies indicated that TruF1 is likely strictly Ser/Thr reverse prenylating, while here we demonstrate that ToIF is rigorously forward prenylating, preferring Thr. Both TruF1 and ToIF are more broadly accommodating of other residues flanking the essential Ser/Thr. Previous crystal structures of prenyltransferases complexed with substrates and prenyl donors allow some general conclusions about this class to be drawn. In the PagF (forward Tyr prenylating) structure, phenolic oxygen of the substrate is 1 Å closer to the C-1 carbon of dimethylallyl group than the C-3 carbon, resulting in favourable O-prenylation of Tyr. A recent structure of a non-cyanobactin enzyme, the Trp N$prenyltransferase$, CymD showed that the C-3 carbon of the
dimethylallyl group is 2.5 Å closer to the substrate than the C-1 carbon, leading to facile reverse prenylation.\(^{[31]}\) We expect similar structural nuances give rise to different regiospecificities of TolF and TruF, although their relatively distant sequence similarity makes direct comparison challenging (Figure S16).

| Substrate\(^{[4]}\) | % Conversion\(^{[4]}\) | Significance |
|------------------|------------------|--------------|
| Cyclic-[\(\text{IAPC}^{\text{YC}}\)] (2) \(^{[4]}\) | 100% (1) | Native TolF substrate |
| IAPCYC (3) | 6% | TolE core |
| Cyclic-[\(\text{TSAIPPFP}^\text{P}\)] (4) | 82% | TruF substrate analog |
| TSAIPFPPSYD (5) | 5% | TolE core analog |
| TVPALSYD dimer (6) | 55% (mono-prenylated), 25% (di-prenylated) | TolE core analog |
| TVPALSYD (7) \(^{[4]}\) | 72% (8) | TolE core analog |
| IPPYLPFAGDDE (9) | 15% | PagE core analog |
| TAGRY (10) | 4% | Most distant |
| Fmoc-Thr-OH (11) | NR\(^{[4]}\) | Single amino acid |
| H-Thr-Thr-OH (12) | NR\(^{[4]}\) | Linear tripeptide |
| SVPALICYD (13) \(^{[4]}\) | 6% | Nterminal Ser |
| SVPALIYD (14) \(^{[4]}\) | 4% | Nterminal Ser |

[a] The site of prenylation is underlined in the substrate on the basis of NMR or MS/MS data. In some cases, it cannot be determined by MS.\(^{[b]}\) C\(^{-}\) indicates thiazoline generated using RSI-TruD.\(^{[c]}\) C\(^{\text{H}}\) indicates Cys treated with iodoacetamide.\(^{[d]}\) % conversion were calculated from MS traces and do not represent isolated yield.\(^{[e]}\) Conversion = (\(A_{\text{product}}/A_{\text{product}} + A_{\text{starting material}}\)) x 100.\(^{[f]}\) NR = No reaction

ToF was selective for DMAPP and could not accept isopentenyl pyrophosphate (IPP) or longer C\(_{10}-\text{C}_{20}\) isoprenoids including geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). A closer examination of ToF sequence alignment with other prenyltransferase revealed that the residues involved in sequestering the pyrophosphate moiety of the isoprene donor are conserved in ToF, including Arg66, Lys135, His137, Tyr191 and Arg298 (Figure S16).\(^{[46]}\) The carboxylation intermediate generated from isoprene pyrophosphate donor is also shielded from solvent at the active site cavity by several hydrophobic residues. In 2018, Estrada et al. showed that a single Phe to Gly mutation at the hydrophobic active site of PagF, shifts the donor preference to GPP from DMAPP.\(^{[15]}\) This is a direct consequence of active site expansion in presence of smaller glycine residue. In ToF, the equivalent residue is the larger Met232, which might result in a more constricted active site incapable of accommodating C\(_{10}-\text{C}_{20}\) isoprenoid cofactors.

In conclusion, we show how focused genome mining leads to the discovery of a novel cyanobactin, tolpyamide (1) and a new post-translational modification with applications in peptide and pharmaceutical chemistry. ToF catalyzes a new regio- and chemo-selective isoprenylation reaction capable of modifying diverse peptides. To our knowledge, ToF is the first example of a cyanobactin prenyltransferase that catalyzes the forward prenylation of Ser/Thr in peptides. With the discovery of ToF, nearly every chemically possible combination of peptide O-prenylation has now been observed, including hydroxyl forward and reverse prenylation, phenolic OH forward and reverse prenylation, and carboxylate prenylation. Coupled with the known C- and N-modifying F proteins, there is now a broad toolset for alkylating diverse peptide sequences. Cyanobactin pathways remain a rich source novel natural products and biosynthetic machinery that is useful in biotechnology. It is notable that there are many far other orphan F proteins and other unknown proteins revealed in SNN diagrams, implying an even broader diversity awaiting discovery. Finally, the discovery ToF as a robust and stable Ser/Thr O-prenyltransferase creates opportunities to study the structural biology and evolution of this enzyme family.

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TolF is a novel O-prenyltransferase which catalyzes forward O-prenylation on threonine and serine residues. A combination of genome mining, natural products chemistry, and biochemical assays were used to identify the biosynthetic gene cluster, natural product, and functionally validate this enzyme. This study fills a gap in the sequence-function space of prenyltransferases and introduces TolF as a promising tool in synthetic biology.

Institute and/or researcher Twitter usernames: @NUSingapore, @nuspharmacydept