Argicyclamides A-C unveil enzymatic basis for guanidine bis-prenylation.

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ABSTRACT: Guanidine prenylation is an outstanding modification in alkaloid and peptide biosynthesis, but its enzymatic basis has remained elusive. We report the isolation of argicyclamides, a new class of cyanobactins with unique mono- and bis-prenylations on guanidine moieties, from *Microcystis aeruginosa* NIES-88. The genetic basis of argicyclamide biosynthesis was established by the heterologous expression and *in vitro* characterization of biosynthetic enzymes including AgcF, a new guanidine prenyltransferase. This study provides important insight into the biosynthesis of prenylated guanidines and offers a new toolkit for peptide modification.

Cyanobactins are a class of ribosomally-synthesized and posttranslationally modified peptides (RiPPs) produced by cyanobacteria.[1-3] Although their chemical structures are hypervariable in terms of amino acid sequences, cyanobactins are biosynthesized through a common pathway.[4,5] Cyanobactins are directly encoded in *C*-terminal region of precursor peptide (IPR031036) as core peptide. Core peptide is flanked by recognition sequences (RSs) that facilitate proper recognition from biosynthetic enzymes modifying core peptide. Universal proteolytic step that remove region preceding core peptide (leader peptide) is mediated by subtilisin-like S8A protease (IPR023830) represented by PatA.[6] Subsequently, second subtilisin-like S8A protease represented by PatG removes region following core peptide (follower peptide) to yield head-to-tail cyclic cyanobactins or linear cyanobactins.[6,7] In many cases, core peptide undergoes divergent modification steps including heterocyclization to generate oxazol(in)e/thiazol(in)e,[8-11] methylesterification on *C*-terminal carboxylic acid,[7] and forward or reverse prenylation and geranylation.[12-17] The prenylation is catalyzed by ABBA-type prenyltransferases (PTases, IPR031037) that are selective for the isoprenyl donor (dimethylallyl diphosphate: DMAPP or geranyl diphosphate: GPP) and the residue that is prenylated.[12-17] On the other hand, they exhibit extremely relaxed specificity against the remainder of the prenyl acceptor, and thus cyanobactin PTases could be versatile biochemical tools for late stage peptide modifications.[12,18] The catalytic variations of cyanobactin PTases revealed that the residues prenylated by these PTases are not only Tyr, Ser, Thr, and Trp,[12-17] but also *N*- and *C*-termini of linear cyanobactins.[7,19] However, the prenylation sites are limited to these residues, and the prenylation of a charged side chain has not been observed.

During the course of our efforts toward the discovery of natural products, by targeting cyanobacteria from the NIES collection, we found the unreported compounds **1**-**3** from the well-studied strain *M. aeruginosa* NIES-88, a producer of micropeptins and kawaguchipeptins (**4**, **5**).[20-22] The LCMS profile of compounds **1**-**3** showed molecular ion peaks at *m/z* 1058, 990 and 922, respectively (Figure S1). This is reminiscent of a homolo-

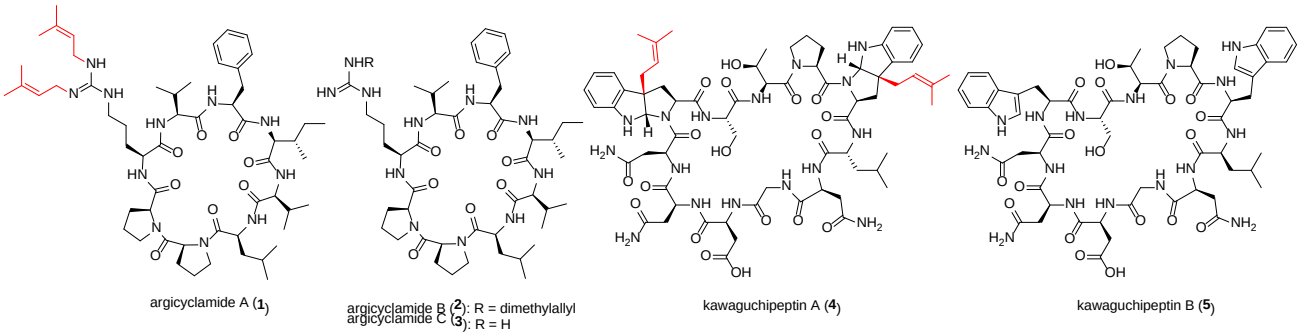

Figure 1. Structures of cyanobactins from *M. aeruginosa* NIES-88. Argicyclamides A-C (**1**-**3**) are newly reported in this study.

gous series that differs by 68 atomic mass units, and this difference corresponds to a prenyl group. The HRESI(+)MS analysis of purified **1** yielded a molecular ion $[M + H]^+$ at *m/z* 1058.7128, indicative of the molecular formula $C_{57}H_{91}N_{11}O_8$ (Δ0.3 mDa). The ^1H and ^{13}C NMR spectra of **1** in CD_3OD (Table S1) showed eight signals for the Cα methines of α-amino acids (δ_C 66.5, 63.5, 62.4, 61.1, 59.8, 58.4, 57.2, 51.7; δ_H 4.66, 4.55, 4.24, 4.23, 4.23, 4.09, 3.78, 2.99), and six signals for amide NH protons (Figure S37), probably due to slow exchange to deuterium through inter-residual hydrogen bonding. In addition, signals for an *N*-prenyl unit were also observed, and two sets of them are overlapped according to the integral intensity (δ_C 138.6, 119.8, 40.7, 25.8, 18.1; δ_H 5.22, 3.82, 1.76, 1.71). A detailed analysis of the 2D NMR data established that **1** is the octapeptide composed of Phe, Ile, Val (x 2), Leu, Pro (x 2), and Arg residues (Figure 2). The sequence of these residues was determined by interpretations of the HMBC and ROESY spectra in CD_3OD, which allowed the assignment of two partial structures: the dipeptide Arg-Val(1), and the hexapeptide Phe-Ile-Val(2)-Leu-Pro(1)-Pro(2) (Figure 2a). It was difficult to connect these two fragments, due to the overlapped signals for the α-methine protons of Arg and Val(1) at δ_H 4.23 in CD_3OD (Figure 2a). These two fragments were connected based on the ROESY correlations in DMSO-d_6 (Figure 2b), which allowed us to close the linear fragments into a head-to-tail cyclic octapeptide. The two prenyl units are attached to the two N^{ω}s of guanidine in Arg, as deduced from the DQF ^1H-^1H COSY (in DMSO-d_6) correlations between $N^{\omega}H$ (δ_H 7.42) and CH_2 (δ_H 3.75), and $N^{\delta}H$ (δ_H 7.37) and CH_2 (δ_H 3.11), as well as the HMBC correlation between C^{ω} and CH_2 (δ_H 3.75) (Figure 2b). The symmetrical pattern of bis-prenylation on N^{ω} agrees with the overlapped signals of two prenyl groups, thus excluding the possibility of an N^{ω}, N^{δ}-asymmetric bis-prenylated guanidine. The

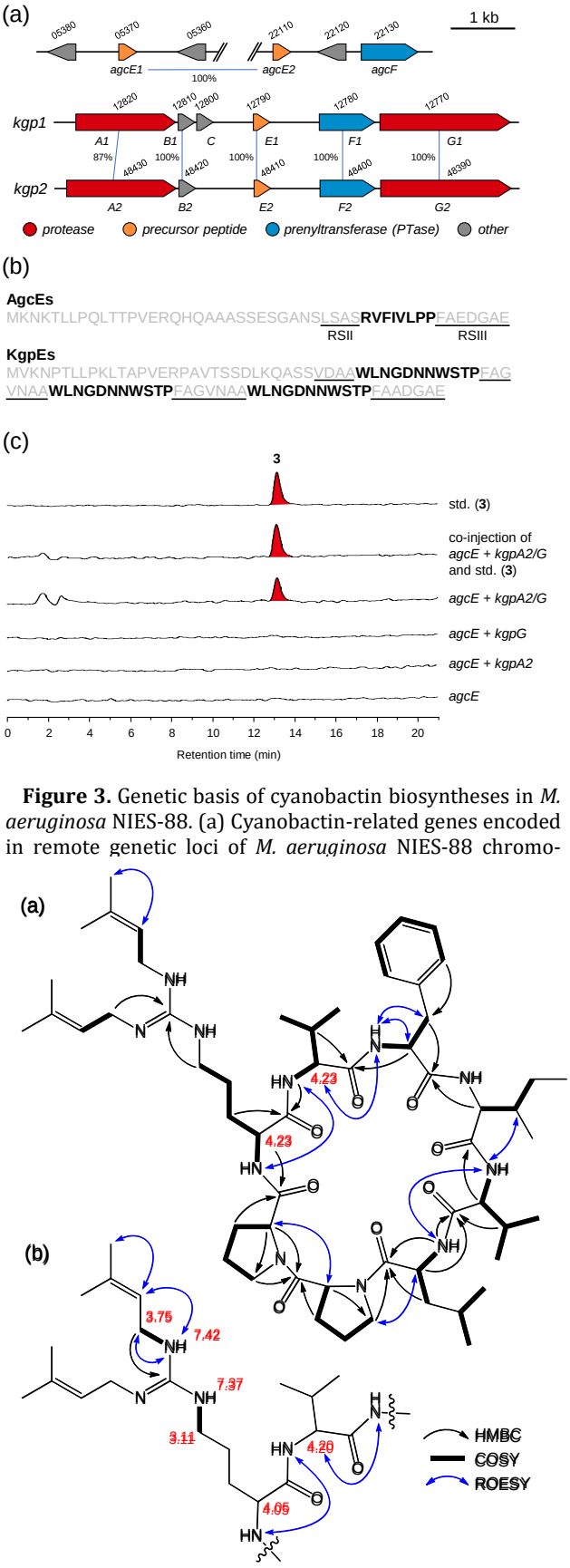

Figure 3. Genetic basis of cyanobactin biosyntheses in *M. aeruginosa* NIES-88. (a) Cyanobactin-related genes encoded in remote genetic loci of *M. aeruginosa* NIES-88 chromo-

Figure 2. Key 2D NMR correlations of **1** in CD_3OD (a) and DMSO-d_6 (b).

amide bond between two Pro residues was assigned to be *cis* conformation based on ROESY correlations and diagnostic carbon chemical shifts ($\Delta\delta_{C\beta} - \delta_{C\gamma}$).[23]

Compounds **2** and **3** have the molecular formulas of $C_{52}H_{83}N_{11}O_8$ (Δ0.4 mDa) with $[M + H]^+$ at *m/z* 990.6495, and $C_{47}H_{75}N_{11}O_8$ (Δ0.0 mDa) with $[M + H]^+$ at *m/z* 922.5873, respectively. Detailed analysis of the 2D NMR spectra revealed that **2** and **3** possess the same macrocyclic scaffold as **1**, except that **2** has only one prenyl unit at Arg, while **3** lacks prenylation at Arg (Figure S2, Table S2). The Marfey's analysis of **3** confirmed that the macrocyclic scaffold exclusively consists of L-amino acids (Figure S3). Structure of cyclic scaffold was further confirmed by total synthesis of **3**, using conventional solid phase peptide synthesis, followed by ring closure with a coupling regent (Figure S4).[24-26] While **1**-**3** were not cytotoxic, **1** inhibited growth of *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Bacillus subtilis* with MIC of 3.12 - 6.25 μM. Notably, as the number of prenyl group increases from one to two, the antimicrobial activity was significantly enhanced. (Table S3, Figures S5-S10).

The unique *N*-prenylation of **1** prompted us to investigate its biosynthetic mechanism. As the cyclic scaffolds of argicyclamides exclusively consist of L-amino acids, we hypothesized that these are ribosomally synthesized peptides. Based on this assumption, we searched for a putative precursor peptide of argicyclamides in the genome of *M. aeruginosa* NIES-88 by tBLASTn with their possible peptide backbone sequence (RVFIVLPP) as a query, however, the corresponding gene was not found in the public genome data. Therefore, we re-sequenced *M. aeruginosa* NIES-88 by using both long and short read sequencers. *De novo* hybrid assembly generated complete sequence of 5.5 Mb chromosome and two additional small plasmids. Scanning whole genome sequence identified two copies of putative precursor peptide genes of argicyclamide, *agcE1* and *agcE2*, which are encoded in the remote genetic loci (Figure 3a, Supplementary results and discussion). While no cyanobactin-related genes were encoded in the flanking region of *agcE1*, a putative enzyme AgcF, which shares homology with cyanobactin PTases such as Trp *C*-prenyltransferase KgpF (AAid, 41%),[14] Trp *N*-prenyltransferase AcyF (AAid, 41%),[15] and *N*α-prenyltransferase MusF2 (AAid, 35%),[19] was found in the flanking region of *agcE2*. However, although the cyclic scaffolds of argicyclamides should be constructed by a set of PatA/G-like proteases, no such proteases were encoded in the vicinity of the *agcE1* nor *agcE2*. This led us to hypothesize that ArgEs are processed by putative PatA/G-like proteases that are encoded in remote genetic loci. To search them, complete genome of *M. aeruginosa* NIES-88 was analyzed with HMM-based annotation.[27] This validated that KgpA and KgpG, proteases in kawaguchipeptin biosynthesis, are the only PatA/G-like proteases (IPR023830) in *M. aeruginosa* NIES-88. Notably, we detected two sets of near-identical kawaguchipeptin biosynthetic gene clusters (*kgp1* and *kgp2*, Figure 3a) that reside in remote loci in *M. aeruginosa* NIES-88 chromosome (Supplementary results and discussion). Genes coding for B, E, F, G proteins are identical between *kgp1* and *kgp2*. On the other hand, while *kgpA2* encodes full-length of PatA-like protease, *N*-terminal region of *kgpA1* encoding catalytic triad is truncated, thus KgpA1 is likely to be inactive. Based on these observations and the fact that the RSII (recognition sequence of PatA-lile protease) and RSIII (recognition sequence of PatG-like protease) of AgcEs are similar to those of KgpE, kawaguchipeptin precursor peptide (Figure 3b), we hypothesized that AgcEs could be matured by KgpA2 and KgpG, proteases for kawaguchipeptin biosynthesis.

To test this hypothesis, we co-expressed the precursor peptides *agcE* and *kgpA2*/*kgpG* in the heterologous host *E. coli* BL21 (DE3). LC-MS analyses of the transformant's metabolites revealed the production of **3**, the nonprenylated cyclic peptide (Figure 3c). Omitting *kgpA2* or *kgpG* gene resulted in the loss of **3** production. To further access the production of **3** *in vitro*, recombinant KgpG was incubated with synthetic AgcE-core with follower peptide (RVFIVLPPFAEDGAE) (Figure S11). This resulted in KgpG-dependent production of **3** (Figure S12). These results demonstrated substantial promiscuity of kawaguchipeptin proteases and suggest that these are employed for constructing the cyclic scaffolds of both argicyclamides and kawaguchipeptins, which are structurally distinct.

Next, we investigated the biosynthetic origin of the bis-prenylated guanidine moiety in **1**. To this end, the putative prenyltransferase AgcF was expressed in *E. coli* and characterized *in vitro*, using dimethylallyl diphosphate (DMAPP) as a prenyl donor and synthetic **3** as an acceptor. A time-course analysis revealed the initial generation of **2**, the mono-prenyl-

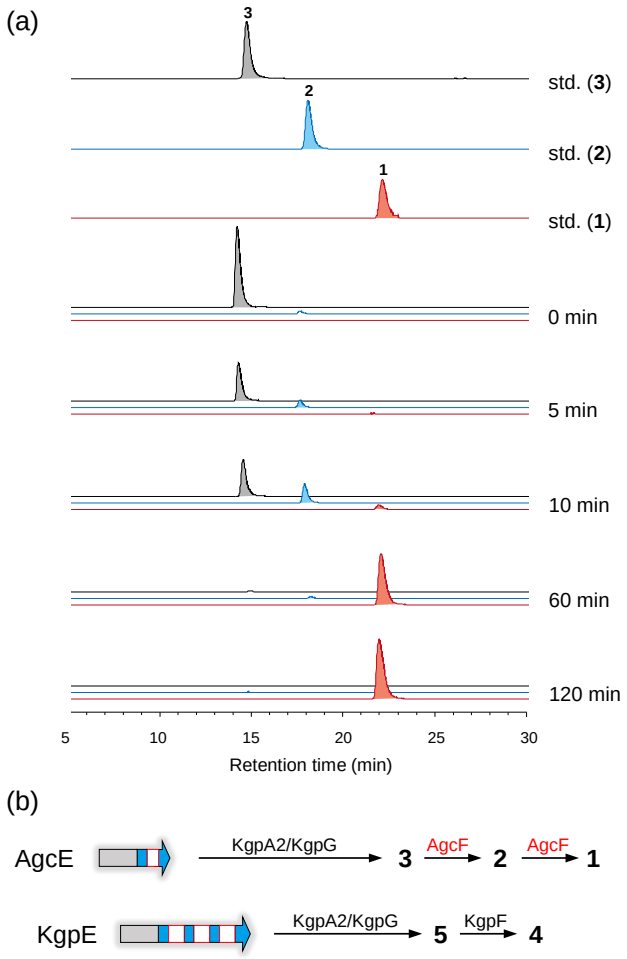

Figure 4. *In vitro* characterization of AgcF and proposed biosynthetic schemes of argicyclamides (**1**-**3**) and kawaguchipeptins (**4**, **5**). (a) AgcF catalyzed sequential prenylations to convert **3** to **1**, with the intermediacy of **2**. Extracted ion chromatograms for **1** (*m/z* 1058.5000, red), **2** (*m/z* 990.5000, blue), and **3** (*m/z* 922.5000, black) are shown. (b) KgpA/G are shared with the *agc* and *kgp* pathways to afford distinct cyclic scaffolds (**3**, **5**), which are tailored by specialized PTases.

ated product, followed by the accumulation of **1**, the bis-prenylated product (Figure 4a). This demonstrated that AgcF catalyzes two rounds of prenylation on the guanidine moiety. This conversion is Mg^{2+}-dependent, and is optimal at 37°C, pH 8.0 (Figure S13–S14). AgcF showed no prenylation activity on synthetic analogs of **3** with Arg substituted to Trp, Tyr, Ser, Thr, or Lys (**6**-**10**, Figure S15). No prenylation was observed for linear analogs (**11** and **12**, Figure S15). These results shows that AgcF is highly selective for **3** as prenyl acceptor. Next, specificity of AgcF on isoprenyl donor was assessed by using geranyl diphosphate (GPP) and farnesyl diphosphate (FPP). Contrary to other cyanobactin PTases that are highly selective for the isoprenyl donor,[12-16,28] AgcF was capable of catalyzing the mono-geranylation of **3** (Figures S16-S17), highlighting its unusual tolerance. FPP was not accepted as an isoprenyl donor.

To gain structural insights into AgcF catalysis, we generated a computational model of AgcF, using the crystal structure of PagF, an *O*-Tyr PTase in prenylagaramide biosynthesis, as the template (AAid, 42%) (Figure S18).[13] The Mg^{2+} and diphosphate binding site, as well as the proposed catalytic residue (Glu49 in AgcF and Glu51 in PagF) that activates the prenyl acceptor, are well conserved between AgcF and PagF. The substitution of Glu49 to Ala in AgcF abolished its prenylating activity, showing the general importance of this residue in the catalysis of cyanobactin PTases (Figure S19). Stark differences between AgcF and PagF were observed in the residues forming the active site entrance, where the bulky residues in PagF are substituted with substantially smaller residues in AgcF; for example, F69/G67, H138/G133, W271/C267, and Y292/L289, respectively (Figure S18). The enlarged active site should facilitate the accommodation of a bulky substrate and enable the sequential bis-prenylation of guanidine.

A phylogenetic analysis revealed that cyanobactin PTases form clades according to their chemo-selectivities (Figure S20). Notably, AgcF composes a small but distinct clade together with its close homologs, and all share the aforementioned substitutions at the active site entrance (Figure S21). The putative Arg-containing precursor peptides encoded in the neighboring regions of AgcF-like PTases suggest the presence of a new class of cyanobactins, with a bis-prenylated Arg residue yet to be identified (Figure S22).

In this study, we discovered argicyclamides (**1-3**), a new group of cyanobactins with a unique bis-prenylated Arg residue. Based on the complete genome sequence and series of biochemical analyses, we proposed unique biosynthetic route of **1-3**, in which the precursor peptide is processed by distantly encoded maturation proteases participating in a distinct cyanobactin biosynthetic pathway (Figure 4b). In general, cyanobactin maturation proteases act on single or multiple precursor peptides encoded in neighboring genetic loci.[4,5,9,16,29,30] However, to our knowledge, cyanobactin proteases processing distantly encoded precursor peptides have not been previously reported.

Notably, Pancrace et al. recently reported first biosynthetic investigation of prenylated guanidines on aeruginoguanidines/microguanidines, a group of cytotoxic non-ribosomal peptides produced by *Microcystis*.[31] Although not validated experimentally, guanidine prenylation is proposed to be catalyzed by AgdJ that belongs to decaprenyl diphosphate synthase-like family (IPR001441), which shares no sequence homology with AgcF, suggesting that several enzyme families have evolved convergently to achieve guanidine prenylation. AgcF, a newly identified PTase in this study is, to our knowledge, the first guanidine PTase with biochemical validation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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