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N-prenylation of tryptophan by an aromatic prenyltransferase from the cyanobactin biosynthetic pathway

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ABSTRACT

Aromatic prenylation is an important step in the biosynthesis of many natural products and leads to an astonishing diversity of chemical structures. Cyanobactin pathways frequently encode aromatic prenyltransferases that catalyze the prenylation of these macrocyclic and linear peptides. Here we characterized the anacyclamide (*acy*) biosynthetic gene cluster from *Anabaena* sp. UHCC-0232. Partial reconstitution of the anacyclamide pathway, heterologous expression and *in vitro* biochemical characterization of the enzyme demonstrate that the AcyF enzyme encoded in this biosynthetic gene cluster is a Trp *N*-prenyltransferase. Bioinformatic analysis suggests the monophyletic origin and rapid diversification of the cyanobactin prenyltransferase enzymes and the multiple origins of *N*-1 Trp prenylation in prenylated natural products. The AcyF enzyme displayed high flexibility towards a range of Trp-containing substrates and represents an interesting new tool for biocatalytic applications.

Keywords: Anacyclamides • *Anabaena* • tryptophan prenylation • cyanobactins • ABBA aromatic prenyltransferase • N-prenylation

INTRODUCTION

Natural products are low molecular weight metabolites that play an important role in the development of medicine.[1,2] They are interesting targets for total synthesis as well as promising lead structures for medicinal and biological chemistry. Prenylated natural products are widely distributed in bacteria, plants and fungi and include a variety of toxins and antibiotics.[3-5] Prenylation increases affinity for membranes as well as interactions with cellular targets and is viewed as critical to the biological activity of many natural products.[3,6,7]

The aromatic prenylation of many natural products is catalyzed by prenyltransferases belonging to the ABBA superfamily.[6,8-13] ABBA aromatic prenyltransferases are named after the αββα succession of secondary structure elements.[8,14] Members of the ABBA superfamily of aromatic prenyltransferases share low primary sequence homology and are instead defined by a conserved tertiary architecture.[10,14]. This common architecture consists of a ring of solvent-exposed α-helices surrounding a central barrel of ten antiparallel β-strands.[8,14] ABBA prenyltransferases typically catalyze *C*-prenylation of aromatic substrates by electrophilic aromatic substitution similar to Friedel-Crafts alkylation although some members catalyze *O*- and *N*-prenylation reactions.[13,15] Natural products produced using ABBA aromatic prenyltransferases include indole alkaloids, peptides, flavonoids, coumarins, terpenoids, phenazines, and recently cyanobactins.[6,8-13]

Cyanobactins are macrocyclic and linear peptides produced from a precursor protein through a range of post-translational modifications including prenylation.[11,16-20] The prenylation of cyanobactins is catalyzed by prenyltransferases belonging to the ABBA superfamily.[11,16,19,21,22] Cyanobactin prenyltransferases catalyze the *O*-prenylation of Tyr, Thr, and Ser in forward or reverse orientation.[11,19,21, 22] Cyanobactins containing reverse *O*-prenylated Tyr have been shown to undergo a Claisen rearrangement to yield forward *C*-prenylated Tyr.[11,16] *N*-prenylation of the amino terminus of linear cyanobactins was also reported recently.[18] The cyanobactin kawaguchipeptin undergo C-prenylation on the *C*-3 position of the Trp indole ring.[20,21] The KgpF enzyme from the kawaguchipeptin pathway catalyzes the stereospecific forward prenylation of Trp.[21] Prenylation of the *C*-3 position of Trp has also been reported from oscillatorin[23] while the prenylation of the *N*-1 position of Trp has recently been reported for croissamide.[24]

Anacyclamides are highly diverse macrocyclic peptides belonging to the cyanobactin family of natural products.[17,18,22] Anacyclamides are typically produced in low amounts hindering their chemical characterization.[17] Here we characterize the AcyF prenyltransferase from the cyanobactin gene cluster of the cyanobacterium *Anabaena* sp. UHCC-0232. We used a combination of heterologous expression and enzymatic assays to characterize the substrate specificity of the AcyF prenyltransferase. Our results show that AcyF catalyzes the prenylation of the indole *N*-1 of Trp.

MATERIALS AND EXPERIMENTAL DETAILS

Detection of anacyclamides

Anabaena sp. UHCC-0232 (formerly *Anabaena* sp. TR-232) was grown in 40-mL culture Z8 medium lacking a source of combined nitrogen under continuous light with a photon irradiance of 5-12 µmol m^{-2} s^{-1} at 20 to 25°C for three weeks. Cells were harvested from 50 mL cultures in tubes by centrifugation at 7000 x *g* for 7 min at room temperature. Cells were freeze-dried for 24 hrs in a refrigerated chamber (Edwards High Vacuum International, Norfolk, England) and the dry weight of the cells was determined. Compounds to be analyzed were extracted with 1 mL of methanol (HiperSolv™ for HPLC, BDH Laboratory supplies, Poole, England) and mechanical disruption of the cells with 0.5 mm glass beads (Cell disruption media, 0.5 mm Glass Beads, Scientific Industries Inc. Bohemia, NY, USA) using FastPrep®-24 (Model FP120, Bio101, Thermo Electron Corporation,

Qbiogene, Inc., Carlsbad, CA, USA) at 5 m s^{-1} for 20 sec. The supernatant obtained after centrifugation of methanol extract at 20,800 x *g* for 5 min at room temperature was used for LC-MS analysis. Samples were injected in a 10 µl volume and separated by liquid chromatography using a Phenomenex Luna column C18(2) (Phenomenex, Torrance, USA) having particle size 4 µm, pore size 100 Å, length 150 mm, inner diameter 2 mm and a shorter C18 precolumn (Phenomenex, Torrance, USA) having an inner diameter of 2.0 mm and 4.0 mm long. The mobile phase consisted of 0.1% aqueous formic acid (Fluka, Sigma-Aldrich, Steinheim, Germany) as solution A and 0.1% formic acid in isopropanol (Sigma-Aldrich, Steinheim, Germany) as solution B. Linear gradient constant ratio was changed as follows: 2.5 min development in solvent A, decreased up to 95% (i.e. 5% solvent B), 75% solvent B after 35 min followed by elution in 100% solvent B after 35.01 min. The flow rate was 0.15 mL min^{-1} with separation columns running at a temperature of 40°C. Samples were ionized by electronic spray ionization (ESI) method for mass spectrometry (Trap XCT Plus, Agilent, USA). Nitrogen was used as spraying gas operated at 50 psi pressure. The drying gas was flown at 350° C at the rate of 8 l min^{-1}. The ion source (ESI) and the first vacuum chamber separating the glass hair pipe ion source end voltage was 3270V and glass hair tube output voltage was 300V. The ion driver voltage was 41.5 V with a trap drive value of 82.2. Positive ions generated by the ionization method were analyzed by MS 200-1100 (*m/z*) mass-charge ratio region for anacyclamides and MS 200-1500 (*m/z*). Accurate mass and UV-Vis data was recorded with UPLC-QTOF (Waters Acquity I-Class-Synapt G2-Si) equipped with diode array UV detector.

Identification of anacyclamide gene cluster

Genomic DNA was extracted from *Anabaena* sp. UHCC-0232 as previously described.[42] The *acy* gene cluster was amplified by PCR using the primers acyCF (5'-ATTAACACCAAATACGACCACGACGG-3') and acyGR (5'-TTATGCTGATTTTGCCACAGACCAAGAACG-3') and cloned into the pCR2.1-TOPO vector as previously described.[17] Transformants were grown overnight with shaking at 120 rpm at 37 ºC in 50 mL of LB medium containing 100 µg mL^{-1} of ampicillin for LC-MS analysis. A set of ten transformants were analyzed for the heterologous expression of prenylated anacylamide.[17] The cells were harvested by centrifugation at 7,000 g for 7 min (Eppendorf Centrifuge 5840R, Eppendorf), extracted with MeOH and analyzed by LC-MS. Plasmid was purified from the strain producing highest amount of anacyclamide and named pTR232. The pTR232 plasmid was sequenced by primer walking using the BigDye® Terminator Cycle Sequencing Ready Reaction kit (version 3.1) (PE Applied Biosystems) and analyzed on an ABI 3730xl automated sequencer (Tables S1-S3). The *acy* gene cluster was visualized using Glimmer as implemented in Artemis (Sanger Institute) and manually annotated using a combination of BLASTp, searches and the CDD and using InterProScan searches (Table S3). The predictions for start sites of the genes were checked and refined manually. The annotated sequence of the *acy* gene cluster from *Anabaena* sp. UHCC-0232 was deposited in GenBank under the accession number MH559352.

Heterologous expression of kawaguchipeptins

The *acy* gene cluster was reorganized by a combination PCR and restriction digests to form a monocistronic gene cluster composed of *acyA*, *acyB*, *acyC*, *acyF* and *acyG* genes organized behind a T7/lacO promoter to create the plasmid pABCFG. In order to compare the AcyF enzyme to the previously characterized KgpF enzyme from the kawaguchipeptin pathway a synthetic gene encoding the AcyE precursor peptide was designed to encode a kawaguchipeptin core sequence.[20] The codon-optimized synthetic anacyclamide *acyE* precursor gene encoding a kawaguchipeptin WLNGDNNWSTP core (Figure S1) was placed behind a T7 promoter and lac operator to allow inducible expression of the precursor gene and synthesized in pUC57 at Genscript Inc. The promoter and synthetic *acyE* gene were subcloned to pACYC184 with a p15A origin of replication and chloramphenicol resistance gene to create the plasmid pSYNE (Figure S1). The pSYNE and

pABCFG plasmids were used to co-transform *E. coli* BL21 (DE3) (Figure S1). Selection of desired clones was done by using 30 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin. Transformants were grown to an optical density of 0.6 with shaking at 120 rpm at 37 ºC induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in 50 mL of LB medium containing selective antibiotics, harvested and freeze-dried. Twenty milligrams of freeze dried transformants were extracted in 100% methanol for LC-MS analysis as earlier described.

Expression and purification of AcyF

Codon optimized full-length *acyF* gene was synthesized at Genscript Inc. and cloned in pJexpress 411 plasmid (ATUM, USA) using Clontech In-Fusion® cloning kit to allow expression of AcyF as a fusion product with an *N*-terminal Tobacco etch virus (TEV) protease-cleavable His_6 tag (Figure S2). The 35.6 kDa AcyF protein was expressed in *E. coli* BL21 (DE3) cells grown on LB Lennox medium and incubated at 37°C with shaking at 200 rpm until OD_{600} is 0.4-0.6. An aliquot of this primary culture was used to inoculate a secondary culture grown on terrific broth containing trace elements autoinduction medium (ForMedium™, UK) supplemented with kanamycin (30 μg mL^{-1}) and incubated at 20°C with shaking at 200 rpm for 48 h.

A batch of cell culture in 1L bioreactor (Applikon Biotechnology BV, The Netherlands) was prepared in order to produce of large amounts of the enzyme, following the fed-batch fermentation protocol for high cell density cultivation and using glucose as a carbon source.[25] Expression was induced using 1 mM IPTG and temperature was maintained at 20°C using a cooling jacket. Cells were harvested by centrifugation at 4,000g, 4 °C for 15 min and resuspended in lysis buffer containing 300 mM NaCl, 20 mM Tris pH 8.0, 20 mM imidazole pH 7.5, 3 mM β-mercaptoethanol (BME), EDTA-free protease inhibitor tablets (Thermo Scientific) and 0.4 mg DNase (Sigma Aldrich) per gram pellet. Cells were disrupted using Stansted pressure cell homogeniser EP FPG 12805 (2 cycles, 4 °C at 30 kPsi). The cell lysate was cleared by centrifugation at 40,000 g, 45 min, 4 °C, and loaded onto a 5 mL Ni-Sepharose 6 FF (GE Healthcare) previously equilibrated with wash buffer (same composition as lysis buffer but without DNAase). The column was washed with 20 CV wash buffer and recombinant AcyF eluted by increasing the imidazole concentration to 125 mM imidazole in the same buffer. The elution peak was loaded onto a HiPrep 26/10 Desalting column (GE, Healthcare), previously equilibrated in gel filtration buffer containing 300 mM NaCl, 10 mM HEPES (pH 8.0) and 1 mM tris(2-carboxyethyl)phosphine (TCEP). The protein was concentrated using Vivaspin concentrators (Sartorius), 30 k molecular-weight cut-off at 4 °C and injected into a HiLoad™ 16/600 Superdex™ 75 pg SEC column (GE Healthcare) equilibrated in gel filtration buffer. The protein was concentrated and loaded on a HiTrap DEAD FF IEX column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris-HCl, pH 7.4, eluted with a linear gradient 0 % to 80 % elution buffer containing 20 mM Tris-HCl, 500 mM NaCl, pH 7.4 and concentrated using Vivaspin concentrators. The purity of the protein was monitored by SDS-PAGE and its identity was confirmed by the MS of its tryptic digest. We have tested the thermal stability of the enzyme over a range of pH values and in presence of different salt concentrations by carrying out a thermal shift assay using qPCR and SYPRO orange dye as previously described.[43] Our results showed that the optimum pH is 8.0 and that the enzyme prefers high salt concentration (300mM-800mM).

Prenylation assays

Assays were set up to assess the potential prenylation of AcyF. Each reaction contains 100 μM substrate, 1 % DMSO, 1 mM DMAPP, 12 mM $MgCl_2$, 10 - 20 μM enzyme in buffer containing 300 mM NaCl, 10 mM HEPES pH 7.5 and 3 mM TCEP. All reactions were incubated at 37 °C for 72 hours and analyzed by LC-MS. Reactions to be analyzed were diluted 1:1 (v/v) with methanol (ChromasolvTM for HPLC, >99.9 %, Honeywell), and cleared by centrifugation. The supernatant was then analyzed by Liquid Chromatography (LC) coupled with a diode array detector and

connected to a mass spectrometer (MS). LC-MS system (Agilent 1100 series HPLC) consisted of thermoregulated autosampler, quaternary pump and column thermostat was used for the separation of the samples. Samples were injected in a 10 µl volume and were first separated by liquid chromatography using an ACE5 C18-HL (5 µm, 90Å, 250 x 4.6 mm) column. The mobile phase consisted of H_2O with 0.1 % trifluoroacetic acid (TFA, Sigma-Aldrich) as solution A and acetonitrile (Honeywell) with 0.1 % TFA as solution B. A linear gradient of 2 to 100 % of solution B was used over 35 min, followed by 5 min of wash. The flow rate was 1 mL min^{-1} and samples were ionized by electronic spray ionization (ESI) using LTQ Orbitrap XL (Thermo). Prenylation assays were analyzed also with Waters UPLC-QTOF Acquity I-Class-Synapt G2-Si system equipped with diode array UV detector (UV-Vis spectral records).

NMR analysis of prenylated *cyclo*[TSQIWGSPVP]

A 5 mL reaction was incubated at 37 °C for 72 h and concentrated to complete dryness with rotary evaporator under reduced pressure at temperature of 37 °C. The organic residue was dissolved in 5 mL methanol and purified using an Agilent Technologies 1260 Infinity high-pressure liquid chromatography system and ACE 5 C18 – HL column (5 µm, 90Å, 250 × 10 mm) connected to a diode array detector. Injection volume was 100-250 µL and the mobile phase consisted of degassed mQ water as solution A and acetonitrile (Honeywell) as solution B. Linear gradient of 2 % to 100 % of solution B over 34 min was used. The gradient started with 4 min equilibration in 2 % solvent B in A, elution after 4.01 min until 38 min as mentioned before and finally the column is re-equilibrated for 4 min for the next injection. The flow rate was set at 4.73 mL min^{-1} and the column temperature at 30 °C. Fractions containing prenylated *cyclo*[TSQIWGSPVP] were collected manually, pooled together, dried under a stream of nitrogen (Noblegen Nitrogen Gas Generator N_2) and stored at -20 °C. NMR data were recorded on a Bruker Ascend^{TM} 600 spectrometer. NMR spectra were referenced with residual solvent ^1H (δ_H 2.50) and ^{13}C (δ_C 39.51) signals for DMSO-d_6. NMR spectra were referenced with residual solvent ^1H (δ_H 2.50) and ^{13}C (δ_C 39.51) signals for DMSO-d_6.

Phylogenetic analysis and network analysis

A sequence similarity network of the 932 ABBA prenyltransferases was generated using the EFI-Enzyme Similarity Tool[44] in order to illustrate the diversity of this enzyme. The sequence similarity network contained 932 nodes with 83,071 edges constructed by pairwise BLAST alignments with an E-value better than 10^{-20} and an alignment score of 25. Cytoscape 3.2.1 was used to visualize the sequence similarity network using the organic layout.[45] A phylogeny of the 34 ABBA prenyltransferases with from a variety of natural product pathways was constructed from this data set (Table S4). The ABBA protein sequences were aligned using MUSCLE[46] to produce an alignment of 280 positions. were retained and used for the construction of a phylogenetic tree using PHYML.[47] Phylogenetic trees were constructed using the JTT amino acid substitution model, four substitution rate categories, an estimated proportion of invariable sites, an estimated gamma-distribution shape parameter, and using a BIONJ starting tree. The stability of the in-group relations was assessed with 1000 bootstrap replicates. The resulting phylogenetic tree was rooted using midpoint rooting using the RETREE and visualized using TREEVIEW.

RESULTS AND DISCUSSION

The 8.3 kb *acy* gene cluster from *Anabaena* sp. UHCC-0232 encoded AcyA, AcyB, AcyC, AcyE, AcyF and AcyG as well as 2 open reading frames with unclear roles in the biosynthesis of anacyclamide (Figure 1). The *acyE* gene encodes a 47 amino acid precursor protein with a HQPWHAAP core, which is excised, macrocyclized and prenylated to form the mature anacyclamide A8P.[17] LC-MS analysis of methanol extracts confirmed that *Anabaena* sp. UHCC-0232 produced the prenylated anacyclamide A8P (*m/z* 993.5063 $[M+H]^+$, Δ 0.9 ppm, Figure S3, Table S5) as previously

reported.[17] Heterologous expression of the *acy* gene cluster from the pTR232 plasmid in *Escherichia coli* TOP10 resulted in the successful production of the prenylated anacyclamide A8P with an identical mass, product ion spectrum from ion *m/z* 993.5 $[M+H]^+$ and retention time (Figure 1). A detailed analysis of the product ion spectrum of protonated anacyclamide A8P (*m/z* 993.6) produced by HPLC-ESI-ITMS clearly assigned the prenyl group to Trp (Figure S3, Table S5). The prenyl group from Trp in anacyclamide A8P was more readily lost in the ion source (+MS) of the ion trap compared to kawaguchipeptin A (Figure S4). However, this analysis could not unambiguously assign the position or orientation of the prenyl group.

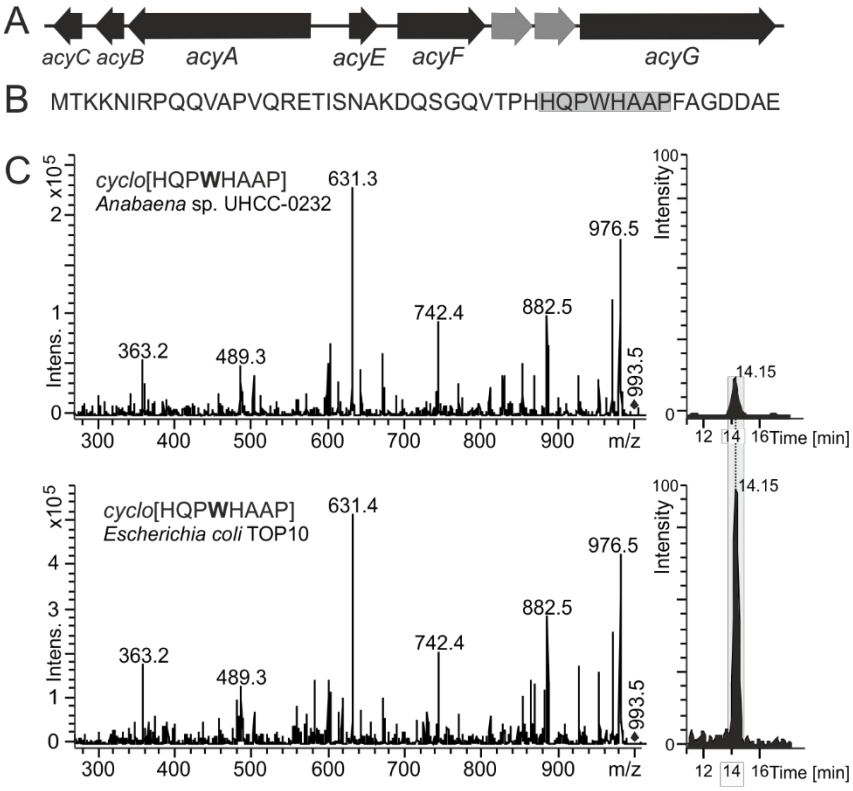

Figure 1 The organization and heterologous expression of the anacyclamide (*acy*) biosynthetic gene cluster from *Anabaena* sp. UHCC-0232. (A) The *Anabaena* sp. UHCC-0232 *acy* biosynthetic gene cluster encodes 6 cyanobactin biosynthetic enzymes organized in a bidirectional operon as well as two hypothetical open reading frames. (B) The *acyE* gene encodes a 47-aa precursor protein with a core HQPWHAAP sequence that is excised, macrocyclized and prenylated to form the mature anacyclamide. (C) The *Anabaena* sp. UHCC-0232 *acy* gene cluster was cloned to produce the pTR232 plasmid and expressed in *Escherichia coli* TOP10 resulting in the production of a peptide with the same mass, product ion spectrum from *m/z* 993.5 $[M+H]^+$ and retention time as the anacyclamide A8P produced by *Anabaena* sp. UHCC-0232. The putative prenylated Trp residue is marked in bold.

We constructed a two-plasmid expression system based on the *acy* gene cluster from *Anabaena* sp. UHCC-0232 in order to compare AcyF to the previously characterized KgpF Trp prenyltransferase from the kawaguchipeptin (*kgp*) biosynthetic pathway.[20,21] We constructed a minimal *acy* gene cluster encoding AcyA-AcyG to produce the pABCFG plasmid (Figure S1). A synthetic *acyE* gene, encoding an anacyclamide precursor protein with a kawaguchipeptin WLNGDNNWSTP core sequence replacing the native HQPWHAAP core, was constructed and subcloned to produce the plasmid pSYNE (Figure S1). Co-expression of these two compatible plasmids in *E. coli* BL21 (DE3) resulted in the production of a bisprenylated peptide with the same mass as kawaguchipeptin A using the *acy*

biosynthetic machinery (Figure 2, Figure S1). Expression of the pMQ123i plasmid encoding the *kgp* biosynthetic gene cluster[20] in *E. coli* BL21(DE3) resulted in the production of kawaguchipeptin A. Surprisingly, kawaguchipeptins produced using the *kgp* biosynthetic gene cluster and the bisprenylated kawaguchipeptin produced using the *acy* biosynthetic gene cluster had different retention times (Figure 2). We also observed clear differences in UV absorption spectra of the two peptides (Figure S5). The clear differences in retention time and UV absorption spectra suggested a possible regiospecific difference in the Trp prenylated cyanobactins produced in *E. coli* using the *acy* and *kgp* biosynthetic pathways.

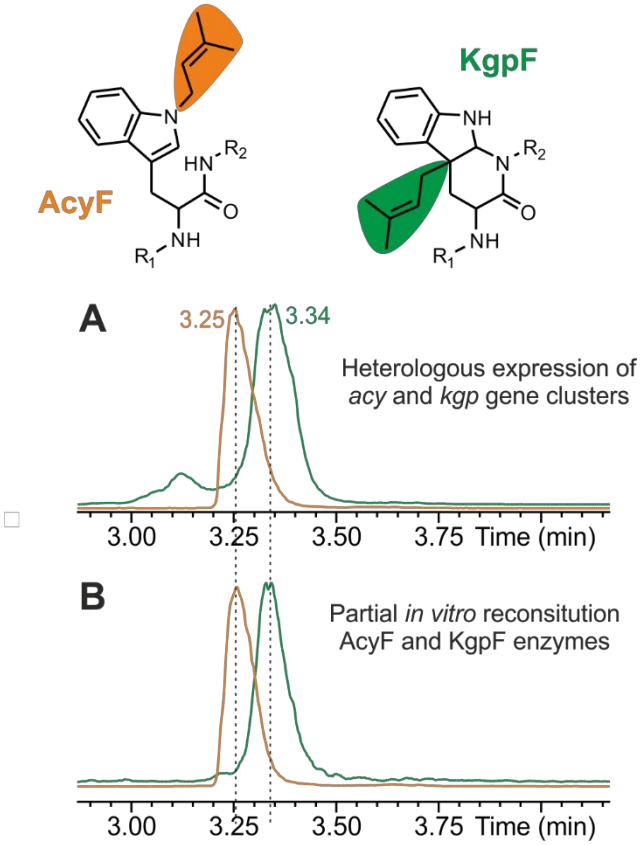

Figure 2 Extracted ion chromatograms (EIC) with protonated molecule showing the retention time differences between kawaguchipeptin chemical variants produced by heterologous expression of acy and kgp biosynthetic gene clusters in vivo and produced by prenylation of cyclo[WLNGDNNWSTP] by AcyF and KgpF in in vitro biochemical assays (A) Kawaguchipeptins produced by heterologous expression of the acy and kgp biosynthetic gene clusters had identical masses (m/z 1421.68, [M+H]+) but different retention times (B) Prenylation of synthetic cyclo[WLNGDNNWSTP] catalyzed by AcyF and KgpF prenyltransferases also resulted in the production of kawaguchipeptins with identical masses (m/z 1421.68) but different retention times.

We overexpressed recombinant AcyF in *E. coli* BL21 (DE3) as a fusion protein with an *N*-terminal TEV-cleavable Histag in order to characterize the prenylation reaction (Figure S2). The enzyme was purified using affinity chromatography on a nickel column followed by size exclusion and ion exchange to yield ~0.32 mg protein /g cell pellet. The enzyme purity and identity were checked by SDS PAGE (Figure S2) and LC-MS analysis of a tryptic digest, respectively. Large scale production of AcyF was achieved by growing the bacterial cells in 1L fermentor, following the fed-batch fermentation protocol method[25] using glucose as a carbon source. We obtained ~120 g of dry cell

pellet per 1 L culture using this protocol compared to a yield of ~22 g dry cell pellet from 1 L culture on terrific broth containing trace elements autoinduction medium (ForMedium™, UK) in baffled shake flasks. Initial assays suggested that synthetic *cyclo*[TSQIWGSPVP] was a suitable substrate for AcyF enzyme. We scaled up an enzymatic reaction, using *cyclo*[TSQIWGSPVP] as an acceptor and DMAPP as a donor, and monitored the reaction using LC-MS. The reaction product was purified with HPLC and analyzed by UV, MS and NMR. The protonated mass of *cyclo*[TSQIWGSPVP] increased from *m/z* 1053.5387 (Δ 2.2 ppm) to *m/z* 1121.6016 (Δ 2.3 ppm) in the enzymatic reaction (Figure S6) consistent with the incorporation of a 68.06 Da prenyl unit into the substrate. No changes to the substrate mass were observed in control reactions lacking DMAPP and/or AcyF. The indole ring of Trp is the only chromophore in *cyclo*[TSQIWGSPVP] absorbing at 260-280 nm and the red shift in the UV spectrum (Figure S5) yielding immediate evidence that the prenyl group had been incorporated into the indole ring. There were clear differences in the UV spectra of *cyclo*[TSQIWGSPVP] prenylated using AcyF or KgpF (Figure S5). Members of the ABBA superfamily of prenyltransferases catalyze the regioselective prenylation of all positions in the indole ring of Trp.[5,13,26] NMR analysis was therefore performed to determine the position of the prenyl group in the indole ring in prenylated *cyclo*[TSQIWGSPVP].

The substrate *cyclo*[TSQIWGSPVP] and the purified prenylated *cyclo*[TSQIWGSPVP], were analyzed with ^{1}H, ^{13}C, ^{1}H - ^{1}H COSY, ^{1}H - ^{1}H TOCSY, ^{1}H - ^{13}C HSQC, ^{1}H - ^{13}C HMBC, ^{1}H – ^{15}N HSQC, ^{1}H – ^{15}N HMBC and ^{1}H - ^{1}H ROESY (Table S6, Figures S7-S8). NMR analyses clearly showed that the prenyl group was located on the heterocyclic nitrogen of the Trp indole ring side chain. ^{1}H and ^{15}N-HSQC spectra of *cyclo*[TSQIWGSPVP] in DMSO-d_6 showed 11 NH signals, 2 from primary and 8 from secondary carboxamide groups and a δ_H 10.86 ppm doublet signal (Figure S9A) which is typical for the proton in the indole ring heterocyclic nitrogen.[27-29] This proton showed correlation signals with carbons δ_C 109.4, 123.4, 126.9 and 135.9 ppm in ^{1}H ^{13}C-HMBC which in case of *cyclo*[TSQIWGSPVP] could originate only from the sp2 carbons of the indole ring (Figure S10A). Furthermore, the proton in NH-29 splits the CH-28 proton signal to a doublet in the substrate while in prenylated *cyclo*[TSQIWGSPVP] H-28 is a singlet (Figure S9). δ_H 10.86 ppm signal (NH-29) and aforementioned ^{13}C-HMBC correlation signals were absent from the corresponding spectra of prenylated *cyclo*[TSQIWGSPVP] (Figures S9B and S10B). There were also ^{13}C-HMBC signals between prenyl group and the indole ring (Figure S10B). The ready loss of the prenyl group from Trp in anacyclamide A8P in the ion source of the ion trap compared to kawaguchipeptin A is compatible with this result. Together, these results demonstrate that the prenyl group was bound to the indole ring nitrogen (Figure 3). Prenyl unit signals δ_C, δ_H = 43.7, 4.69 (CH_2), 120.8, 5.32 (=CH), 135.7 (=C<), 18.1, 1.80, 25.7, 1.70 (CH_3) (Figure S10B, Figure 3) showed that the prenyl unit had a forward prenyl structure.[27]

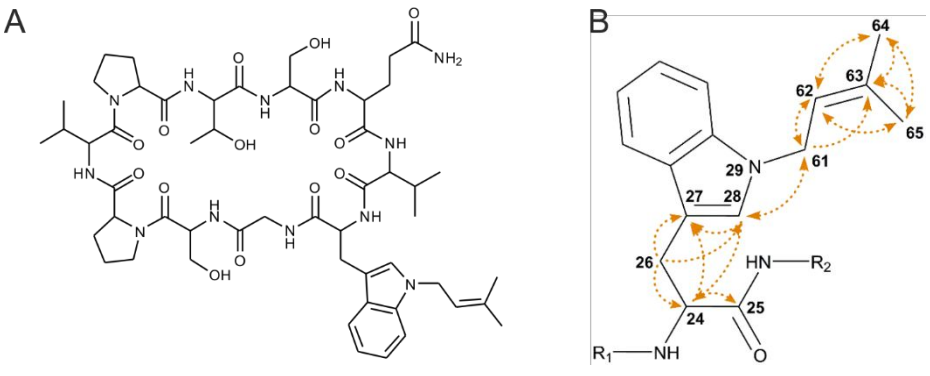

Figure 3. NMR analysis of prenylated synthetic *cyclo*[TSQIWGSPVP] demonstrated that the AcyF enzyme catalyzes the prenylation of the indole nitrogen of Trp. Key HMBC correlations which

show the attachment of prenyl group to the indole ring nitrogen and that the prenyl group is in forward orientation (Table S6).

Prenylation increases the structural diversity of natural products.[2-5,10] Aromatic prenyltransferases belonging to the ABBA superfamily are frequently involved in the transfer of prenyl groups in a wide range of natural products include indole alkaloids, peptides, coumarins, terpenoids and phenazines amongst others.[6,8-12,14] Indole alkaloid biosynthesis involves the transfer of prenyl group to tryptophan from DMAPP donor catalyzed by dimethylallyl tryptophan synthase.[14,30-32] These indole alkaloid prenyltransferases catalyze the regiospecific prenylation at different positions of the indole ring of Trp. FtmPT2 from *Aspergillus fumigatus* catalyzes the *N*-prenylation of the indole alkaloid fumitremorgin B.[33] Representative members of the indole alkaloid ABBA prenyltransferases catalyze the regioselective normal or reverse prenylation of *N*-1, *C*-2, *C*-3, *C*-5, *C*-4, *C*-6, and *C*-7 of the indole ring.[14,30,31,33-36] ABBA prenyltransferases encoded in the cyanobactin natural product biosynthetic pathways are reported to catalyze the forward and reverse prenylation of Ser, Thr, and Tyr[11,16] and more recently the forward prenylation of *C*-3 of Trp.[20,21] Our results show that the AcyF enzyme from *Anabaena* sp. UHCC-0232 *acy* biosynthetic pathway is a *N*-prenyltransferase acting on *N*-1 in the indole ring of Trp. There striking parallels between the regioselectivity of the Trp prenyltransferase from indole alkaloid and cyanobactin pathways despite the low overall sequence homology suggesting that other positions of the indole ring might be prenylated by cyanobactin Trp prenyltransferases.

Members of the ABBA family of aromatic prenyltransferases are promiscuous and can work on a variety of aromatic acceptors to produce novel compounds.[8,13,19,34,37] Furthermore they catalyze the transfer of a C5, C10 or C15 prenyl group derived from the corresponding isoprenyl diphosphate metabolites onto a variety of electron-rich aromatic acceptors.[7] ABBA prenyltransferases characterized typically exhibit a strict specificity for their prenyl donors in contrast to the diversity of their aromatic acceptors.[37] Some ABBA prenyltransferase have been identified that accept a range of prenyl donors *in vitro* or have been engineered to accept new donors through mutagenesis of active sites in the substrate-binding pocket.[37-39] Our heterologous expressions and biochemical assays demonstrate here that AcyF can accept macrocyclic peptide substrates sharing little sequence homology. We detected prenylation by AcyF of *cyclo*[HQPWHAAP], *cyclo*[WLNGDNNWSTP], and *cyclo*[TSQIWGSPVP] during the course of this study either through heterologous expression or *in vitro* biochemical assays. The kinetics of the enzyme reaction with the latter substrate was determined and showed (Km (mM) = 3.32, k_{cat}/K_m ($s^{-1}M^{-1}$) = 47.69). We investigated the *in vitro* prenylation of several protected Trp derivatives and macrocyclic peptides. We found that AcyF can readily prenylate *cyclo*[WLNGDNNWSTP] and *cyclo*[TSQIWGSPVP] as well as the protected Trp derivatives, boc-Trp-OH, and z-Trp-OH (Figure 4, Figures S11-S15). In addition, we tested the ability of AcyF to use the C_{10}-geranyl donor (GPP) instead of DMAPP using *cyclo*[WLNGDNNWSTP] as substrate and our results showed that the enzyme could not use this cofactor. KgpF is reported to prenylate Fmoc-Trp when using DMAPP as a donor[21] but not boc-Trp-OH.[20,21] ABBA prenyltransferases catalyze the *N*-prenylation of the indole ring of Trp in a variety of peptide substrates including ilamycin,[40] cyclomarin/cyclomarazine,[15,41] and fumitremorgin B.[33] However, in contrast to the AcyF enzyme, IlaA and CymD are reported to act on free Trp rather than the macrocyclic substrates[40,41]. ABBA prenyltransferases are amenable to enzyme engineering to alter selection of substrates and their preference for prenyl chain length as well as the regioselectivity and stereoselectivity of the prenyl reaction.[38,39] This promiscuity is a general feature of ABBA prenyltransferases and has been reported for a variety of natural products from other pathways.[19, 37,38] Their promiscuity for different aromatic substrates, stability, solubility and amenability to protein

engineering makes ABBA prenyltransferases attractive tools for chemoenzymatic synthesis of new prenylated derivatives with drug discovery applications.

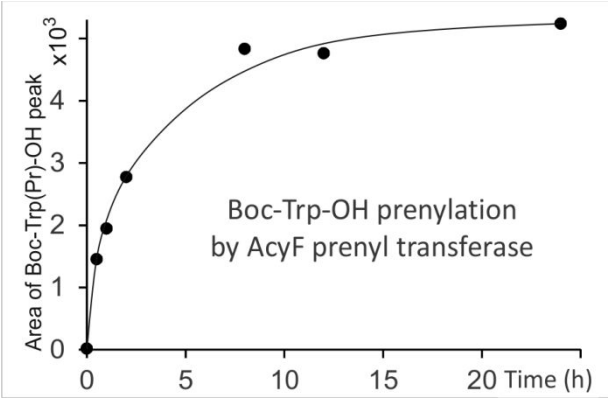

Figure 4. The AcyF enzyme catalyzed the prenylation of protected Trp derviatives such as Boc-Trp-OH. The area of the prenylated Boc-Trp-OH was measured from the sodium adduct ion *m/z* 395.19 peak.

We constructed a sequence similarity network of all proteins sharing the ABBA aromatic prenyltransferase fold from the non-redundant database at NCBI (Figure 5). The vast majority of the ABBA aromatic prenyltransferases fell into five clusters containing cryptic biosynthetic gene clusters encoding mainly unknown natural products (Figure 5). The cyanobactin prenyltransferases formed a distinct cluster separate to other ABBA aromatic prenyltransferases (Figure 5). We compared 34 characterized ABBA aromatic prenyltransferases from different natural product pathways including cyanobactins, indole alkaloids, and aminocoumarins (Figure 5). The overall sequence homology was low with some proteins sharing as little sequence homology as 7% in pairwise comparisons. A PhyML phylogenetic tree was constructed using an alignment guided by tertiary structure and excluded ambiguous regions. Cyanobactins formed a well-supported monophyletic clade in this analysis (Figure 5). The prenyltransferases grouped according to natural product class and phylogenetic lineage rather than substrate specificity (Figure 5). The proteins shared a conserved tertiary sequence with a poorly conserved primary sequence. The AcyF and KgpF enzymes formed a cluster together with other cyanobactin prenyltransferases distinct from prenyltransferases of indole alkaloid pathways (Figure 5). ABBA prenyltransferases catalyze the *N*-prenylation of the indole ring of Trp in a variety of peptide substrates including ilamycin,[40] cyclomarin/cyclomarazine[15,41] fumitremorgin B.[33] Prenyltransferases catalyzing the *N*-prenylation of Trp did not form separate cluster suggesting the rapid evolution of substrate specificity and multiple origins of Trp *N*-1 prenylation (Figure 5). The inherent promiscuity ABBA prenyltransferases display towards aromatic acceptors could in part explain their broad distribution in natural product biosynthesis and acquisition by different natural product families.

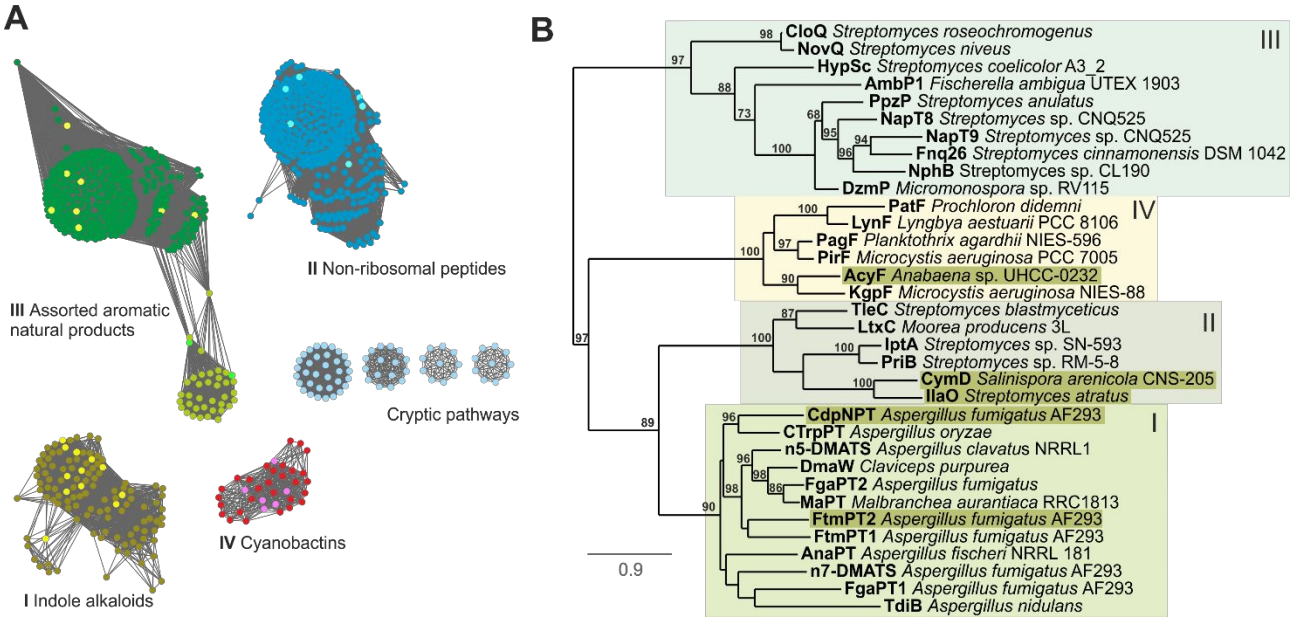

Figure 5 Sequence similarity network and phylogenetic analysis of relationships between members of the ABBA family of aromatic prenyltransferases (A) A sequence similarity network based on 932 members of the ABBA family of aromatic prenyltransferases (Alignment score ≥25, ≥21% sequence identity). Clusters with more than five representatives shown and characterized prenyltransferases are indicated with a bright color (B) Phylogeny of 34 ABBA aromatic prenyltransferases from a variety of natural product families showing the monophyly of cyanobactin prenyltransferases. *N*-1 Trp prenyltransferases do not form a single monophyletic cluster (highlighted in olive).

In summary, we report here that the AcyF enzyme from the anacyclamide pathway of *Anabaena* sp. UHCC-0232 catalyzes the *N*-prenylation of Trp in a series of macrocyclic peptide and protected Trp substrates. Our results suggest that the regioselectivity of Trp prenylation by ABBA on indole alkaloids may be mirrored in prenyltransferases of the cyanobactin pathway. The promiscuity of the ABBA family of aromatic prenyltransferases is likely to be an intrinsic property of cyanobactin prenyltransferases. The relaxed substrate specificity exhibited by AcyF towards diverse Trp residues suggesting some potential as a promising enzyme for the synthesis of Trp derivatives or libraries of macrocyclic peptides containing *N*-prenylated Trp residues.

ASSOCIATED CONTENT

Supporting information
Supplementary Tables S1-S6 and Figures S1-S15 (PDF)

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Author Contributions

W.E.H. and D.P.F. designed the research, carry out experiments, analyzed the data and supervised the work done at University of Aberdeen and University of Helsinki respectively. J.J and M.W described and characterized cyanobactin chemical variants by HR-LCMS. N.L and A.M. obtained the *acy* gene cluster sequence. N.L., A.P., A.M. and D.P.F analyzed the acy gene cluster sequence. N.L and A.P. expressed the *acy* gene cluster in *Escherichia coli*. A.P. engineered the *acy* gene cluster to produce kawaguchipeptin analogs. The *acyF* gene was cloned in pJexpress 411 vector by W.E.H., the AcyF enzyme expression and purification was done by L.D., the preparation of high cell density culture in fermenter was done by E.Y., and determination of the enzyme kinetics was done by S.A.J. The *in vitro* prenylation assays and the NMR analysis of the prenylated product by L.D. Annotation of the NMR data was carried out by L.D., W.E.H., and J.J. The manuscript was written by L.D., A.P., A.M., J.J., K.S., W.E.H., and D.P.F.

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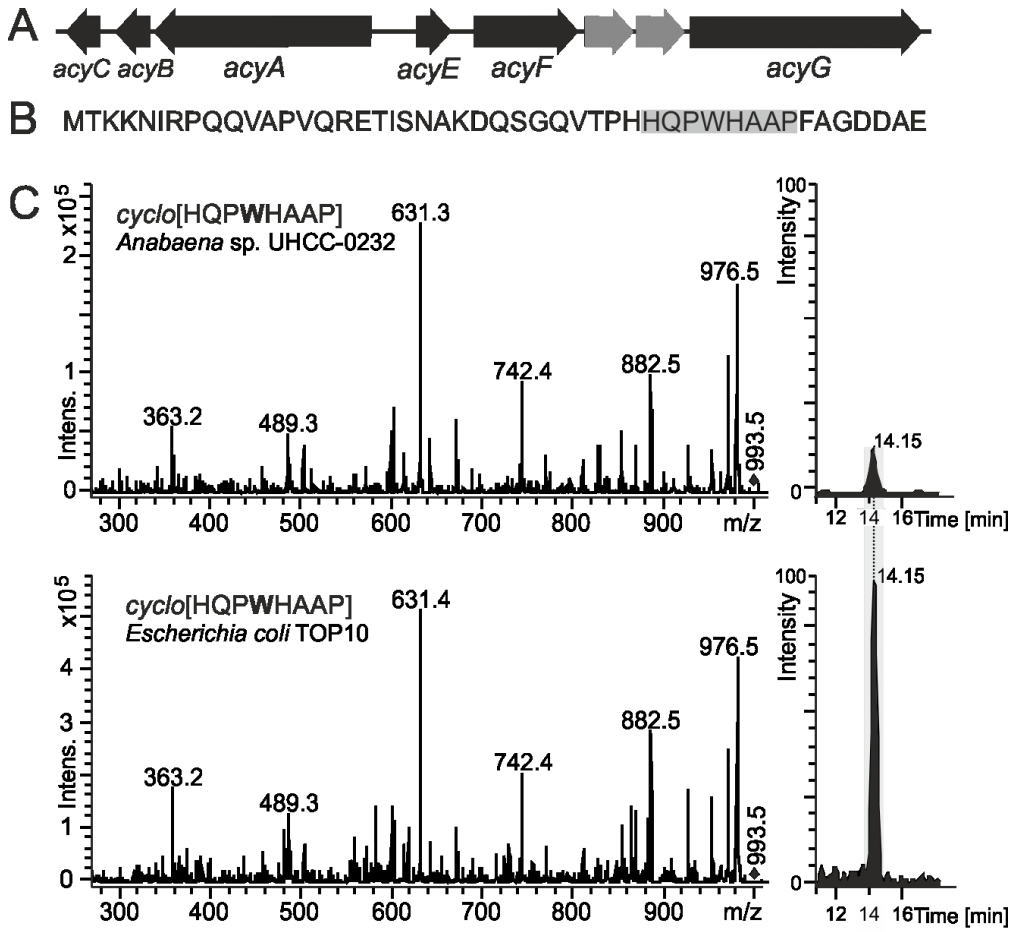
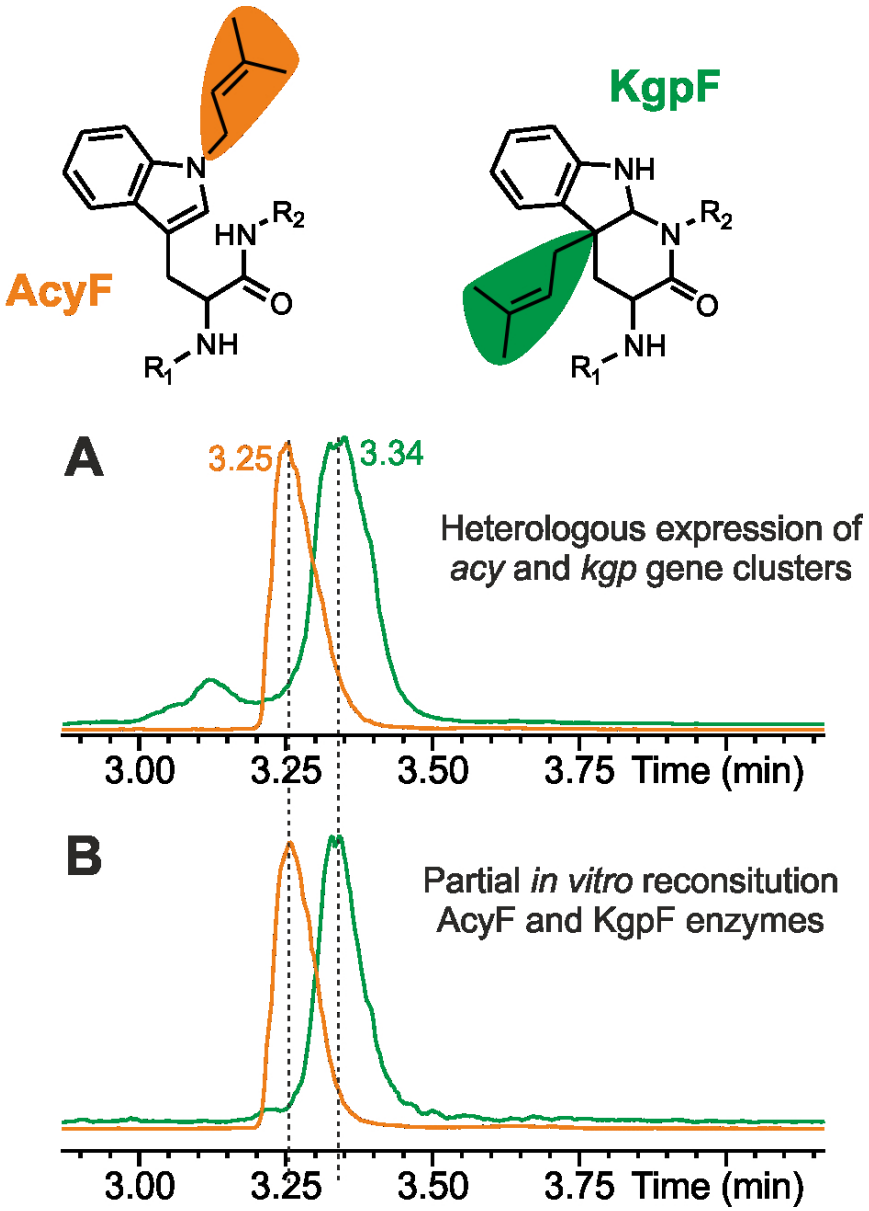


Figure 1 The organization and heterologous expression of the anacyclamide (acy) biosynthetic gene cluster from Anabaena sp. UHCC-0232. (A) The Anabaena sp. UHCC-0232 acy biosynthetic gene cluster encodes 6 cyanobactin biosynthetic enzymes organized in a bidirectional operon as well as two hypothetical open reading frames. (B) The acyE gene encodes a 47-aa precursor protein with a core HQPWHAAP sequence that is excised, macrocyclized and prenylated to form the mature anacyclamide. (C) The Anabaena sp. UHCC-0232 acy gene cluster was cloned to produce the pTR232 plasmid and expressed in Escherichia coli TOP10 resulting in the production of a peptide with the same mass, product ion spectrum from m/z 993.5 [M+H]+ and retention time as the anacyclamide A8P produced by Anabaena sp. UHCC-0232. The putative prenylated Trp residue is marked in bold.



Extracted ion chromatograms (EIC) with protonated molecule representingshowing the rRetention time differences between kawaguchipeptin chemical variants produced by heterologous expression of acy and kgp biosynthetic gene clusters in vivo and produced by prenylation of cyclo[WLNGDNNWSTP] by AcyF and KgpF in in vitro biochemical assays (A) Kawaguchipeptins produced by heterologous expression of the acy and kgp biosynthetic gene clusters had identical masses (m/z 1421.68, [M+H]+) but different retention times (B) Prenylation of synthetic cyclo[WLNGDNNWSTP] catalyzed by AcyF and KgpF prenyltransferases also resulted in the production of kawaguchipeptins with identical masses (m/z 1421.68) but different retention times.

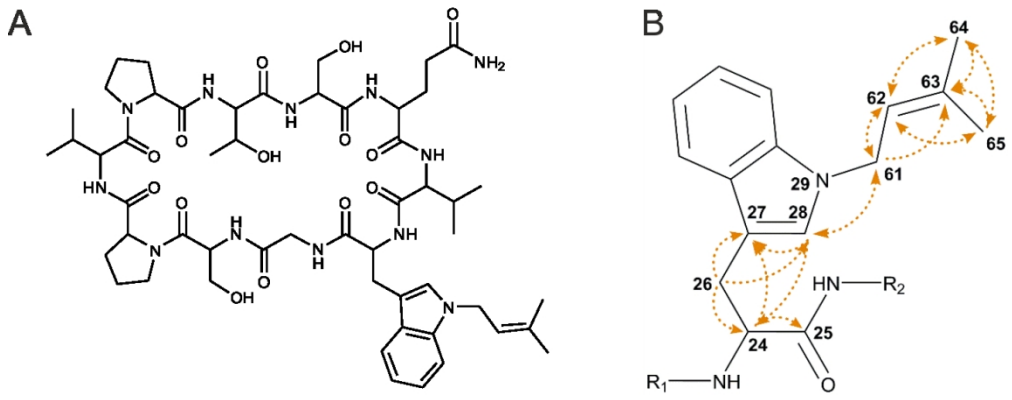
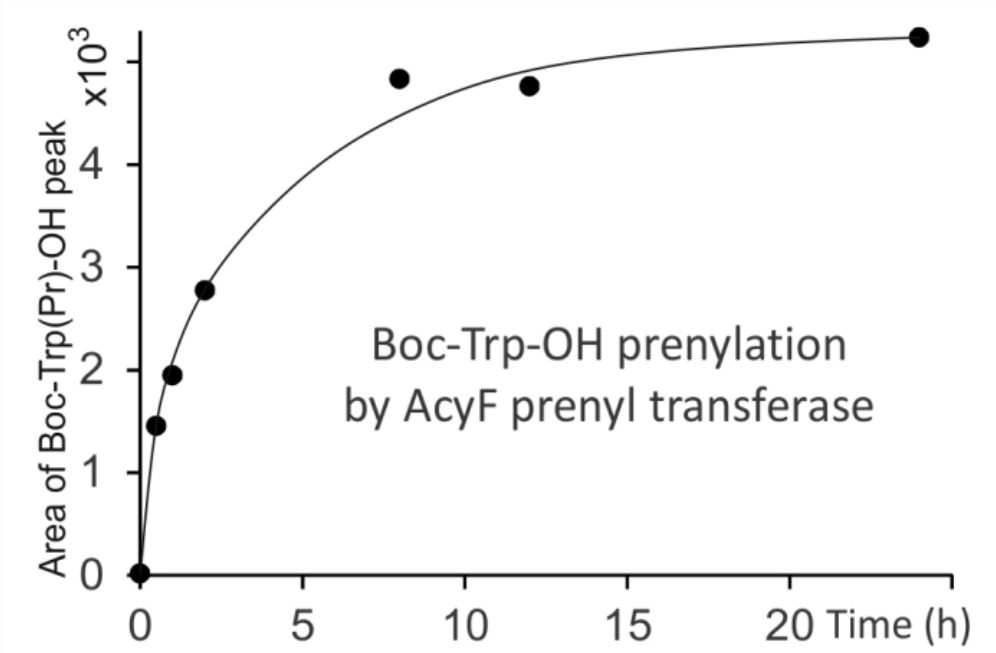


Figure 3. NMR analysis of prenylated synthetic cyclo[TSQIWGSPVP] demonstrated that the AcyF enzyme catalyzes the prenylation of the indole nitrogen of Trp. Key HMBC correlations which show the attachment of prenyl group to the indole ring nitrogen and that the prenyl group is in forward orientation (Table S2).



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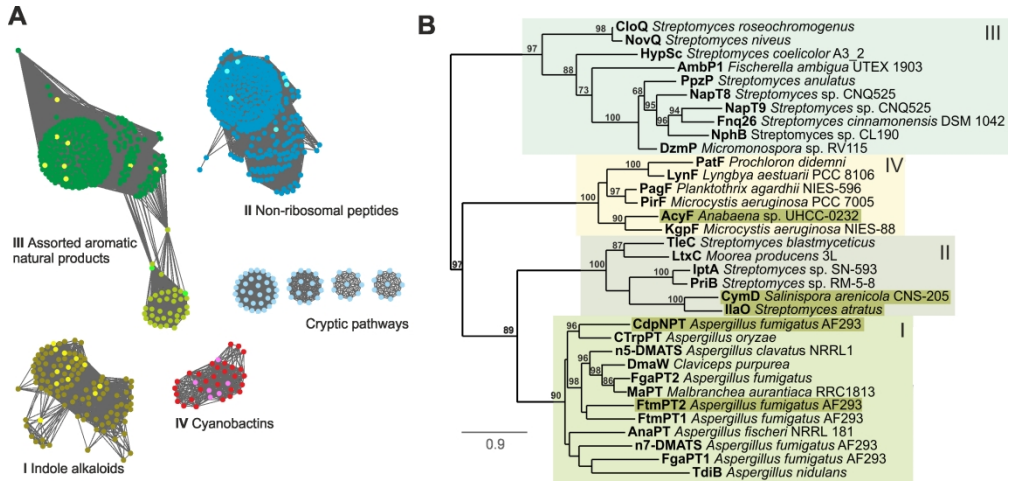

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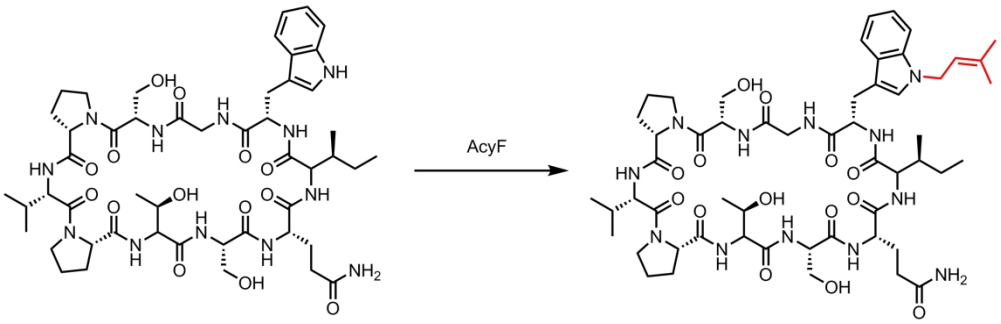

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