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Heterologous and *in vitro* reconstitution of fuscanodin, a lasso peptide from *Thermobifida fusca*

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Abstract

Lasso peptides are a class of ribosomally-derived natural products typified by their threaded rotaxane structure. The conversion of a linear precursor peptide into a lasso peptide structure requires two enzymatic activities: cleavage of the precursor via a cysteine protease and cyclization via isopeptide bond formation. *In vitro* studies of lasso peptide enzymology have been hampered by difficulties in obtaining pure, soluble enzymes. We reasoned that thermophilic bacteria would be a good source for well-behaved lasso peptide biosynthetic enzymes. The genome of the thermophilic actinobacterium *Thermobifida fusca* encodes for a lasso peptide with an unprecedented Trp residue at its N-terminus, a peptide we have named fuscanodin. Here we reconstitute fuscanodin biosynthesis *in vitro* with purified components, establishing a minimal fuscanodin synthetase. These experiments have allowed us to probe the kinetics of lasso peptide biosynthesis for the first time, and we report initial rates of fuscanodin biosynthesis. The fuscanodin biosynthetic enzymes are insensitive to substrate concentration and operate in a near single-turnover regime *in vitro*. While lasso peptides are often touted for their stability to both chaotropic and thermal challenges, fuscanodin is found to undergo a conformational change consistent with lasso peptide unthreading in organic solvents at room temperature.

Graphical Abstract

Introduction

Ribosomally-synthesized and posttranslationally modified peptides (RiPPs) are a diverse class of molecules characterized by a genetically encoded precursor which undergoes a series of modifications by associated processing enzymes to form the final product.¹ One member of this class is lasso peptides, which are characterized by their unique three dimensional structure in which a 7–9 amino acid (aa) ring is formed by an isopeptide bond

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between the N-terminus and a Asp or Glu sidechain, with the remaining C-terminal thread passing through the ring to form a rotaxane structure.^{2, 3} Early lasso peptides were separated into two classes: class I lasso peptides contain two disulfide bonds while class II lasso peptides are devoid of disulfide bonds. Class II lasso peptides often include steric locks, large amino acids within the thread that maintain the rotaxane structure.^{2, 3} With the advent of genome mining approaches for lasso peptide discovery,^{4, 5, 6, 7} it is now appreciated that the vast majority of lasso peptides belong to class II. Gene clusters encoding lasso peptides consist of the precursor peptide, termed A, a leader peptide protease B, and the isopeptide bond forming enzyme C (Figure 1A, 1B).⁶ In a subset of these identified clusters, the B gene is split between two ORFs, denoted B1 and B2⁸ (Figure 1A). Subsequently, it has been determined that the B1 gene product functions as a leader peptide binding protein common across several families of RiPPs.⁹ This “split-B” gene cluster architecture is especially prevalent in actinobacterial gene clusters. In addition to these core proteins, there are often other proteins also associated with the clusters, such as ABC transporters which serve as immunity factors,³ isopeptidases which cleave the mature peptides,^{10, 11} and other proteins which perform additional post-translational modifications.^{6, 12, 13, 14}

To date, the predominant method for studying lasso peptide biosynthesis has been through heterologous expression, in which the gene cluster is modified and the effect on expression is examined.^{4, 5, 15, 16} In addition, there have been studies investigating leader peptide binding and cleavage *in vitro*.^{9, 17, 18} However, the only lasso peptide biosynthesis to be reconstituted *in vitro* is that of microcin J25, a lasso peptide produced by *E. coli*.^{19, 20} In these studies, only endpoint analysis of product formation was carried out with some product quantification via mass spectrometry. Moreover, the B and C enzymes were difficult to express and purify solubly.²⁰ In order to overcome these limitations, we searched for lasso peptide gene clusters from thermophilic bacteria, as these proteins are often more stable than those from bacteria found at lower temperatures.²¹

We identified a gene cluster from the bacterium *Thermobifida fusca*, which is found in elevated temperatures of compost heaps and related areas.²² The cluster has a typical “split-B” architecture for *Actinobacteria* (Figure 1A). A lasso peptide gene cluster in *T. fusca* was predicted by Tomoda and colleagues,²³ but their predicted precursor peptide was incorrect. We were drawn to this particular peptide because it includes an unprecedented Trp residue at the N-terminus of the core peptide (Figure 1C). All early examples of class II lasso peptides have Gly at the N-terminus of the core peptide.^{4, 10, 15, 24} In 2014, Marahiel and co-workers established that amino acids other than Gly could be accommodated at this position.²⁵ Lasso peptides with 19 of the 20 amino acids in the N-terminal core peptide position have been predicted,⁶ and several additional examples have been experimentally confirmed.^{13, 26, 27, 28} Here we show that the *T. fusca* lasso peptide gene cluster can be successfully expressed in *E. coli*, and we have named the resulting peptide fuscanodin. We further studied the stability of fuscanodin, providing evidence that this peptide unthreads in the presence of organic solvents. In addition, we were able to purify the processing enzymes from the gene cluster, enabling the first *in vitro* studies of “split-B” lasso peptide biosynthetic enzymes. Using this system, we were able to examine for the first time the reaction rates of lasso peptide biosynthesis.

Results

Heterologous expression of a novel lasso peptide from *Thermobifida fusca*

Heterologous expression has been used extensively for the production of lasso peptides from a range of bacteria, as determining the conditions under which the native producer begins expression is often very challenging.¹⁰ In order to confirm the functionality of the lasso peptide gene cluster from *T. fusca*, we began with heterologous expression in *E. coli*. The native gene cluster of *T. fusca* contains three GTG start codons (*tfuC*, *tfuB1*, *tfuD*). In order to improve expression in *E. coli*, each of these codons was changed to ATG using overlap PCR and the final cluster was inserted into the pASK75 vector. This plasmid was then used for production in minimal medium at several different temperatures and expression times. Through these experiments, the maximum production was found from induction of expression at OD₆₀₀=0.2–0.3, followed by growth at 20 °C for 70 hours. Both the cell pellet and the medium were extracted and analyzed by MALDI-MS (Figure S1). Examination of the resulting spectra showed a peak corresponding to a single dehydration of the predicted fuscanodin core peptide. This species was found in both the cells and medium, with a larger amount in the cells. Heterologous expression of actinobacterial lasso peptides in *E. coli* has been difficult,¹⁴ with only one other example recently published.²⁹

The expression was then scaled up to obtain peptide for NMR analysis. For these larger scale expressions, the cell extract was resuspended in 50:50 acetonitrile (ACN)/water and injected on a semiprep column on HPLC. On the typical gradient used for our purification of lasso peptides, the product eluted at close to 25 minutes, indicating high hydrophobicity consistent with the peptide's primary sequence. We developed a modified gradient (see Methods for details) to increase separation of the highly hydrophobic fuscanodin. Using this gradient, the peptide eluted at two different retention times: 12.5 minutes and 12.7 minutes. When the extract was allowed to incubate at room temperature in 50:50 ACN/water, the 12.7 minute peak disappeared, and the 12.5 minute peak increased in area (Figure 2A, Figure S2). The identity of fuscanodin was further confirmed using tandem mass spectrometry (Figure 2B).

This 12.5 minute peak was collected and samples from a series of expressions were pooled and resuspended in 90:10 CD₃OH/H₂O + 0.1% TFA for NMR studies (1.36 mg/mL). Upon examination of the initial 1D NMR spectrum, it was found that the NH resonances were too broad for useful 2D experiments (Figure S3). For a second set of NMR experiments, the peptide was resuspended in DMSO-d₆ at 1.8 mg/mL, which has been used as a solvent for several lasso peptide structures.^{12, 30, 31, 32} In this case there was sufficient signal for correlation experiments and TOCSY and NOESY experiments were performed (Figures S4 and S5). When the spectra were analyzed and all residues were assigned (Table S1), it was noted that there were very few NOE signals between ring residues and thread residues, indicating an unthreaded lasso peptide. In order to further characterize this purified product, a branched cyclic form of the peptide corresponding to the putative unthreaded form was synthesized. This peptide was then run on HPLC and found to have the same retention time as the purified natural product, confirming the identity of the 12.5 minute peak as an unthreaded lasso peptide (Figure S6). Having established that the 12.5 minute peak

corresponds to an unthreaded species, we propose that the 12.7 minute peak corresponds to a threaded lasso structure. As discussed above, this species converts to the unthreaded form upon incubation in ACN/water (Figure 2A). Thus the organic solvents, like ACN, used to aid in the solubility and purification of fuscanodin may also be leading to its unthreading. One previous structure of an unthreaded lasso peptide, lassomycin,¹² is reported in the PDB. In this case, DMSO was also used as a solvent, and the NMR experiments were carried out at 40 °C.

Purification of maturation enzymes for *in vitro* studies

Previous *in vitro* studies of lasso peptide biosynthesis have been hindered by the instability of the maturation enzymes.^{18, 20, 33} Since proteins from thermophilic bacteria are often more stable, we decided to test the expression level of the three biosynthetic proteins, named TfuB1, TfuB2, and TfuC. Previous work in our lab and others has shown that the RiPP recognition element (RRE)-containing B1 protein can be expressed and purified^{9, 17, 18}, but the C and B2 proteins are more unstable or co-purify with chaperones.^{18, 20} We began by expressing each of the proteins individually and were able to purify 6xHis-tagged versions of TfuB1 and TfuC (Figure 3A, Figure S7), but TfuB2 co-eluted with other proteins and could not be readily purified. Based upon previous work in which the B and C enzymes were suggested to form a complex,^{20, 34, 35} we decided to try a strategy of co-expressing TfuB2 with TfuB1 or TfuC. While co-expression of TfuB1 and TfuB2 was not fruitful, we found that co-expression of TfuB2 and TfuC with N-terminal 6xHis tags from pRSF Duet followed by purification with Ni-NTA resin led to a decent yield of soluble TfuB2 and TfuC (Figure 3B). The purified TfuB2 and TfuC proteins were analyzed using a gel filtration column and found to elute as a single peak, using a Western blot to confirm their identity (Figure 3C). This observation strongly suggests that TfuB2 and TfuC form a 1:1 complex.

Evaluation of *in vitro* fuscanodin biosynthesis

After purification of the enzymes, the next step was to verify their activity. A reaction was set up using the purified maturation proteins (TfuB1, TfuB2, and TfuC) along with linear precursor peptide (TfuA) prepared by solid-phase peptide synthesis. Given the homology of TfuC to asparagine synthetases, which adenylate aspartate to convert it to asparagine,³⁶ excess ATP was added to the reaction as well. The reaction was run for 30 minutes at 37 °C before it was stopped by the addition of an equal volume of ACN. The reactions were then analyzed using LC-MS and the cyclized peptide was detected at the same retention time as seen for the purified peptide from heterologous expression (Figure 4A). When the reaction was injected onto LC-MS without ACN addition, there were once again two peaks (Figure 4B), both with the expected masses for the $M+2H^+$ and $M+3H^+$ charge states (Figure 4C). This *in vitro* result was analogous to what had been seen for heterologous expression of fuscanodin (Figure S2). In this case, either the methanol necessary for precursor solubility or, more likely, the ACN present in the HPLC or LC-MS is leading to the formation of two peaks. To simplify analysis and enable quantification, all subsequent samples were allowed to incubate in the ACN/buffer mix overnight prior to LC-MS.

To verify that we had a minimal fuscanodin synthetase, a set of reactions were set up in which each of the components (proteins, ATP, Mg^{+2}) was removed and the results of each

reaction were again analyzed using LC-MS. Through these experiments we confirmed that each of the three proteins (TfuB1, TfuB2, and TfuC) and ATP/Mg²⁺ was necessary for maturation of the precursor into the final product (Figure S8). These results show that all three proteins, TfuB1, TfuB2, and TfuC are required for a functional fuscanodin synthetase. As the optimal growth temperature for *T. fusca* is 45–50 °C, and enzymes from the organism are known to function at elevated temperatures,³⁷ we tested the effect of temperature on lasso peptide conversion. The reaction appeared to be more efficient at 50 °C than at 37 °C, but no lasso peptide was formed when the reaction was run at 65 °C (Figure S9).

Measuring initial rates of lasso peptide maturation

Having established that fuscanodin biosynthesis proceeds at both 37 °C and 50 °C, we next turned our attention to determining the rate of fuscanodin formation *in vitro*. Lasso peptide biosynthesis requires three distinct steps: cleavage of the leader peptide, folding of the substrate from a linear to pre-lasso shape, and formation of the isopeptide bond between the N-terminus of the core and the acidic side chain. While there have been previous studies of microcin J25 biosynthesis *in vitro* as mentioned above, this work did not provide any information about the kinetics of lasso peptide biosynthesis. We reasoned that the well-behaved fuscanodin biosynthesis enzymes could allow us to gain the first insights into the rate and turnover in lasso peptide biosynthesis. To this end, we set up reactions as described in the previous section and quantified the formation of the isopeptide-bonded product using LC-MS. Reactions had a 5-fold excess of the TfuA substrate (2.5 μM) relative to the enzymes (500 nM) and a large excess of ATP/Mg²⁺ (10 mM). The reaction was initiated by the addition of ATP, and aliquots were analyzed after 5 min, 10 min, 20 min, 30 min, and 40 min. At least 5 replicates at each time point and temperature were carried out to ensure repeatability in the LC-MS quantification, and this data was also compared to a standard curve generated from purified unthreaded fuscanodin. The data in this time range fit a linear curve well (Figure 5) and indicate that at 50 °C, ~1.25 equivalents of the lasso peptide (relative to the enzymes) are produced over the course of the 40 min reaction. At 37 °C, ~0.7 equivalents of lasso peptide are produced over 40 min. This data allowed us to calculate an initial rate of lasso peptide formation of 15.3 nM/min at 50 °C and 8.3 nM/min at 37 °C. We next carried out a study at 50 °C in which the TfuA substrate concentration was varied between 1 μM (2 equivalents relative to the enzymes) and 10 μM (20 equivalents). These reactions were analyzed at the 30 minute timepoint, and surprisingly we saw similar turnover (~1 equivalent of lasso) across all substrate concentrations (Figure S10).

Having demonstrated that fuscanodin biosynthesis was insensitive to changes in the substrate TfuA concentration, we next varied other components of the reaction. In other RiPPs systems, such as lanthipeptides^{38, 39} and cyanobactins⁴⁰, it has been demonstrated that the leader peptide of a RiPP precursor can be added in *trans* and still effect efficient enzyme turnover. We first tested adding additional leader peptide to the full reaction comprised of full-length TfuA, TfuB1, and the TfuB2/TfuC complex. Addition of 1 or 10 equivalents of leader peptide relative to TfuA did not cause any appreciable change in the turnover of TfuA at the 30 minute timepoint at 50 °C (Figure S11). Addition of excess TfuB1, the leader peptide binding protein, led to slightly improved turnover (Figure S12).

We next set up a reaction in which the TfuA leader peptide and core peptide (5 eq each, relative to the enzymes) were added as the substrate, rather than intact TfuA. Under these conditions, fuscanodin was still produced, albeit at ~10% of the level of the reaction with intact TfuA (Figure S13). Interestingly, this same amount of turnover was observed even when omitting the leader peptide (Figure S13). Given that TfuA core peptide alone could be converted into fuscanodin, and since TfuB1 is a leader peptide binding protein,⁹ we next set up a reaction that included TfuA core peptide and the TfuB2/TfuC complex, omitting both leader peptide and TfuB1. At both 37 °C and 50 °C, no fuscanodin production was observed (Figure S14). This result further supports the idea that TfuB1 is required for a catalytically competent lasso peptide synthetase.

Promiscuity of fuscanodin biosynthesis

Like *T. fusca*, the *Thermobifida cellulosilytica* genome also encodes a lasso peptide with a Trp residue at the N-terminus of the core peptide. The core peptide sequences for these two peptides are highly similar (8/18 identities) as are the maturation enzymes (Figure S15). To test the promiscuity of the fuscanodin synthetase, we carried out an experiment in which the precursor protein from *T. cellulosilytica*, TceA, was added to the *T. fusca* enzymes TfuB1, TfuB2, and TfuC. TceA is turned over by these enzymes, but the enzymes are much less efficient than they are with their cognate substrate TfuA (Figure S16). We also attempted to swap in the *T. cellulosilytica* B1 protein (TceB1) to the reaction so that the TceA substrate could interact with its cognate leader peptide binding protein. The matching of substrate to B1 protein did not appreciably improve turnover (Figure S16).

Lasso peptides are highly tolerant of amino acid substitutions within the core peptide,⁴¹ so we decided to test whether the fuscanodin synthetase could tolerate TfuA substrates with amino acid substitutions. Specifically, since we were unable to maintain fuscanodin in a threaded state, we sought to investigate putative steric lock residues. The Phe residue at position 17 of fuscanodin is a candidate for a steric lock residue that would result in a lasso topology with a short C-terminal tail reminiscent of MccJ25 (Figure 6A, Figure S17). For these experiments, we generated a recombinant (protein G)-TfuA fusion protein, which affords two advantages. First, these fusion proteins were soluble in water, allowing us to eliminate methanol from the reaction. Second, amino acid substitutions could be introduced using recombinant DNA methods rather than having to carry out peptide synthesis for each new substrate. We first established that wild-type (protein G)-TfuA fusion was as good of a substrate for the fuscanodin synthetase as synthetic TfuA (Figure S18). Variants of the fusion protein in which the F17Y and F17W substitutions were present were tested next. Both of these variants were converted into product, but much less efficiently than the native substrate (Figure S18).

With evidence that the F17Y and F17W variants of fuscanodin were substrates for the fuscanodin synthetase, we returned to our heterologous expression system to determine whether these variants could be produced in cells. Both of these variants were successfully produced with yields of roughly one quarter to one half of the wild-type peptide as judged by HPLC (Figure S19). We incubated extracts of both of these peptides in 50:50 ACN/water to determine whether the peptides exhibited increased stability. For the F17Y variant, the

peptide still clearly underwent a conformational change, albeit at a slower rate than the wild-type peptide (Figure 6B, Figure S20). The F17W fuscanodin variant unthreads more slowly, with the confounding factor that much of this peptide runs as a broad peak on both HPLC and LC-MS (Figure 6C, Figure S21). Collectively this data suggests that increasing sidechain size at the F17 position of fuscanodin retards unthreading of the peptide, supporting the idea that F17 is a steric lock residue. However, neither substitution with Tyr or Trp at this position is able to completely prevent unthreading.

Discussion

In this work we report a unique lasso peptide, fuscanodin, in the genome of a thermophilic actinobacterium *Thermobifida fusca*. We searched for lasso peptide gene clusters in thermophilic organisms with the hope that these organisms would yield lasso peptide biosynthetic enzymes with improved solubility and stability. While there is a body of work on the biosynthetic enzymes for microcin J25, the enzymes often copurify with other proteins such as chaperones. We show here that the fuscanodin synthetase, comprised of the three proteins TfuB1, TfuB2, and TfuC, can be purified to homogeneity and can catalyze fuscanodin biosynthesis *in vitro*. The expression of TfuB2, the cysteine protease that cleaves the precursor TfuA, was only possible when coexpressed with TfuC. We also show that these two enzymes form a complex, an idea that has been proposed previously in the literature.³⁴ The ability to produce a soluble, well-behaved lasso peptide synthetase has allowed us, for the first time, to quantify lasso peptide production *in vitro* and thus measure initial rates of lasso peptide biosynthesis.

The fuscanodin synthetase is insensitive to the concentration of the lasso peptide precursor substrate TfuA (Figure S10). The fuscanodin gene cluster (and many other lasso peptide gene clusters) is organized in a single operon, suggesting that the expression level of the precursor protein TfuA in cells does not differ greatly from the expression levels of the proteins making up the synthetase. In this scenario, each fuscanodin synthetase would only need to turn over a small number of TfuA substrates in cells, which agrees with what we observe *in vitro* (Figure 5). We also cannot exclude the possibility that our choice of buffer or some other reaction conditions leads to the observed single turnover behavior.

Using heterologous expression, our group has previously demonstrated that MccJ25 could be produced even if up to 28 of the 37 aa of its leader peptide were removed from its precursor McjA.⁴² Truncation of the McjA leader peptide led to a precipitous drop in production of MccJ25. The drop in production could be explained by a variety of factors in heterologous expression experiments. Truncation of the leader peptide leads to a shorter transcript and a shorter precursor protein, both of which could be subject to degradation. Alternatively, precursors with truncated leader peptides could be poorer substrates for the lasso peptide synthetase. *In vitro* experiments allow for the deconvolution of these different effects. In *in vitro* experiments with MccJ25, Rebuffat and co-workers showed low but detectable conversion of core peptide alone to MccJ25.²⁰ In a similar experiment with fuscanodin, we show that the TfuA core peptide can be converted to fuscanodin, but only at ~10% of the turnover observed with full-length TfuA (Figure S13). Addition of leader peptide *in trans* does not lead to any further improvement of fuscanodin turnover (Figure

S13), in contrast to other RiPPs enzymes. These studies suggest a model in which the leader peptide improves the turnover of the core peptide, but only when covalently attached.

Our results also provide some new insights into the role of B1 proteins in lasso peptide biosynthesis. This class of protein, homologous to PqqD,^{9, 43} was originally found to be indispensable for the biosynthesis of the lasso peptide lariat in experiments in *Rhodococcus*.²³ Later work showed that these B1 proteins bind the leader peptide of their cognate lasso peptide precursors with submicromolar affinity.⁹ We have further shown, using photocrosslinking, that B1 proteins engage the core peptide of lasso peptide precursors as well.¹⁷ In agreement with the work on the lariat system described above, we observe that TfuB1 must be present for a catalytically competent fuscanodin synthetase (Figure S8). TfuB1 is required even if only core peptide is present as a substrate (Figure S13, S14). This suggests a role for TfuB1 that goes beyond just leader peptide binding; for example, this protein may be interacting with the TfuB2/TfuC complex and contributing to its stability.

Lasso peptides are often touted for their stability, both to proteolysis⁴⁴ and thermal challenge.⁴⁵ Fuscanodin, in contrast, is structurally labile and unthreads in the organic solvents required to purify it. The ring of fuscanodin is formed via isopeptide linkage of the Trp-1 amine and the sidechain of Glu-9, resulting in a ring size of 29 atoms. Other lasso peptides such as caulosegnin II²⁴ and caulonodins V and VII²⁵ have a similarly large ring size, but remain threaded even with heating. We propose that the unthreading of fuscanodin in organic solvents is due both to its large ring size and its high hydrophobicity. While most studies of lasso peptide unthreading have been carried out in water,^{5, 10, 46, 47} there is some precedent for lasso peptide unthreading in organic solvents. The anti-mycobacterial peptide lassomycin, mentioned briefly above, was isolated from acetone extracts of *Lentzea kentuckyensis*. NMR studies on lassomycin were carried out on a sample of the peptide in DMSO that had been heated to 40 °C,¹² and structure calculations indicated an unthreaded structure. This unthreaded lassomycin species has been synthetically prepared, however, and lacks bioactivity.^{48, 49} This suggests that the active form of lassomycin is threaded, while the unthreaded lasso form loses all activity. Early work on microcin J25, the most well-studied lasso peptide, showed that the threaded form of the peptide was bioactive while a synthetic unthreaded species lost all activity.³¹ In summary, our findings here on fuscanodin indicate that the stability of the lasso peptide fold cannot be taken for granted, and that careful analysis of the lasso topology is important for correlation with potential bioactivities of these peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Gene cluster and core peptide sequence of a novel lasso peptide from *Thermobifida fusca*.

A: Lasso peptide gene cluster from *T. fusca* (bottom) compared to the gene cluster for lasso peptide microcin J25 (top). B: Schematic of putative binding events and catalytic steps in lasso peptide biosynthesis. The B1 protein binds the lasso peptide precursor leader peptide (black circles). The B2 enzyme cleaves the leader peptide from the core peptide (colored circles). The C enzyme cyclizes the peptide into the lasso shape. Blue circles correspond to the lasso peptide ring and gray circles are the lasso peptide thread. The red circle shows the position of the isopeptide bond and orange circles are steric lock residues. C: Sequences of the microcin J25 precursor protein and the precursor from *T. fusca*. The N-terminal amino acid of the core peptide is underlined. Color scheme as in part B.

Figure 2:

Characterization of fuscadin. A: Fuscadin isolated from *E. coli* and dissolved in 50:50 acetonitrile/water elutes as two peaks initially. With prolonged incubation in this solvent, the peptide shifts to a single peak. B: Tandem mass spectrum of fuscadin showing fragmentation of the lasso peptide thread.

Figure 3: Purification of fuscanodin biosynthetic enzymes. A: Elutions from buffer exchange column of purified Tfub1-His. B: Elutions from buffer exchange column of purified coexpressed His-TfuB2 and His-TfuC. C: Size exclusion chromatogram of coexpressed Tfub2 and TfuC showing that these proteins elute as a single peak. Inset: anti-His Western blot of size exclusion column fractions starting at 35 min showing that Tfub2 and TfuC coelute and form a complex.

Figure 4:

Analysis of *in vitro* fusicanodin production. A: Extracted ion chromatogram of *in vitro* fusicanodin biosynthetic reaction (2.5 μ M TfuA, 500 nM TfuB1, 500 nM each of TfuB2 and TfuC, 10 mM ATP, 10 mM Mg^{+2} , 37 $^{\circ}C$, 30 min) after overnight incubation in 50:50 acetonitrile/water. B: The same reaction as part A, but injected into the LC-MS immediately after the 30 min reaction time without addition of acetonitrile. A putatively threaded species is present at a retention time of 18.9 min. C: Mass spectra of the +2 and +3 charge states of fusicanodin produced *in vitro*.

Figure 5:

Initial rates of fuscanodin biosynthesis. Red circles are data at 37 °C and blue triangles are data at 50 °C. Error bars reflect the standard error of the mean and are calculated from 5–9 individual measurements at each time point. Error bars are present for the 5, 10, and 20 min time points of the 37 °C data, but are smaller than the symbols. The extracted ion counts were calibrated to a standard curve generated from purified fuscanodin.

Figure 6: Altering the steric lock of fuscanodin. A: Cartoon of the predicted threaded topology of fuscanodin showing Arg16 and Phe17 as steric locks. B: HPLC traces of cell extract containing heterologously expressed F17Y fuscanodin injected either immediately after resuspension in 50:50 acetonitrile/water (initial) or after an overnight incubation in this solvent at room temperature (overnight). C: As in B, but for the F17W variant of fuscanodin. Increasing the steric bulk of the F17 position retards the putative conformational change from the threaded to the unthreaded state.