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Chimeric Leader Peptides for the Generation of Non-Natural Hybrid RiPP Products

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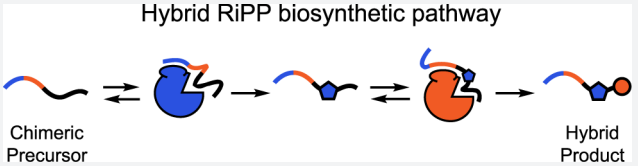
Supporting Information

ABSTRACT: Combining biosynthetic enzymes from multiple pathways is an attractive approach for producing molecules with desired structural features; however, progress has been hampered by the incompatibility of enzymes from unrelated pathways and intolerance toward alternative substrates. Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are a diverse natural product class that employs a biosynthetic logic that is highly amenable to engineering new compounds. RiPP biosynthetic proteins modify their substrates by binding to a motif typically located in the N-terminal leader region of the precursor peptide. Here, we exploit this feature by designing leader peptides that enable recognition and processing by multiple enzymes from unrelated RiPP pathways. Using this broadly applicable strategy, a thiazoline-forming cyclodehydratase was combined with enzymes from the sactipeptide and lanthipeptide families to create new-to-nature hybrid RiPPs. We also provide insight into design features that enable control over the hybrid biosynthesis to optimize enzyme compatibility and establish a general platform for engineering additional hybrid RiPPs.



INTRODUCTION

With the considerable success of natural products as drugs,[1−3] great efforts have been invested in using synthetic biology to engineer analogues of natural products with desired chemical and biological properties.[4−6] One popular approach has been to combine enzymes from different biosynthetic pathways to generate new products in a process known as combinatorial biosynthesis.[7,8] Most commonly, this procedure was applied to polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) pathways where different domains or modules were swapped or deleted. This approach enabled alteration of the loaded amino acid in nonribosomal peptides or the loading and/or extender unit and its subsequent oxidation state in polyketides.[9,10] Despite these feats, the approach has been largely limited to producing new variants of a given pathway rather than building entirely new products or pathways because of the incompatibility of interdomain linker regions, module interfaces, and other requirements.[9,10]

Ribosomally synthesized and posttranslationally modified peptides (RiPPs)[11] represent another group of natural products that provide an attractive starting point for engineering new molecules. The potential for engineering RiPPs derives from their leader peptide-guided biosynthetic logic and the reduced size of the biosynthetic gene clusters.[12] Despite their genetic simplicity, RiPPs are structurally and functionally diverse[11,13,14] and their biosynthesis entails a well-orchestrated modification of a ribosomally produced precursor peptide (Figure 1a). RiPP biosynthetic proteins bind their respective precursor peptide(s) through specific recognition sequences (RS) typically located in the N-terminal leader region.[12] The majority of prokaryotic RiPP pathways rely on a ∼90-residue PqqD-like domain, known as the RiPP recognition element (RRE),[15] to engage the RS, but some notable examples of leader peptide-dependent enzymes lack this domain or do not use it.[16−18] Once the leader peptide is bound, the C-terminal core region of the precursor peptide undergoes posttranslational modification, sometimes extensively, to form azol(in)e heterocycles, lanthionine or sactionine cross-links, D-amino acids, various types of macrocycles, and other modifications.[11] The modified core region ultimately becomes the natural product as the unmodified leader region is enzymatically removed during biosynthetic maturation. Beyond the primary, class-defining modifications installed by leader-dependent enzymes, RiPPs can also be endowed with multiple secondary modifications by "tailoring" enzymes. In most cases, these tailoring enzymes act on the core region independently of the leader peptide to increase the chemical diversity of the core peptide.[11]

The physical separation of leader peptide binding and sites of modification allow RiPP enzymes to solve the paradoxical problem of being specific for a substrate yet promiscuously acting upon it.[19−25] Indeed, many natural RiPP pathways have

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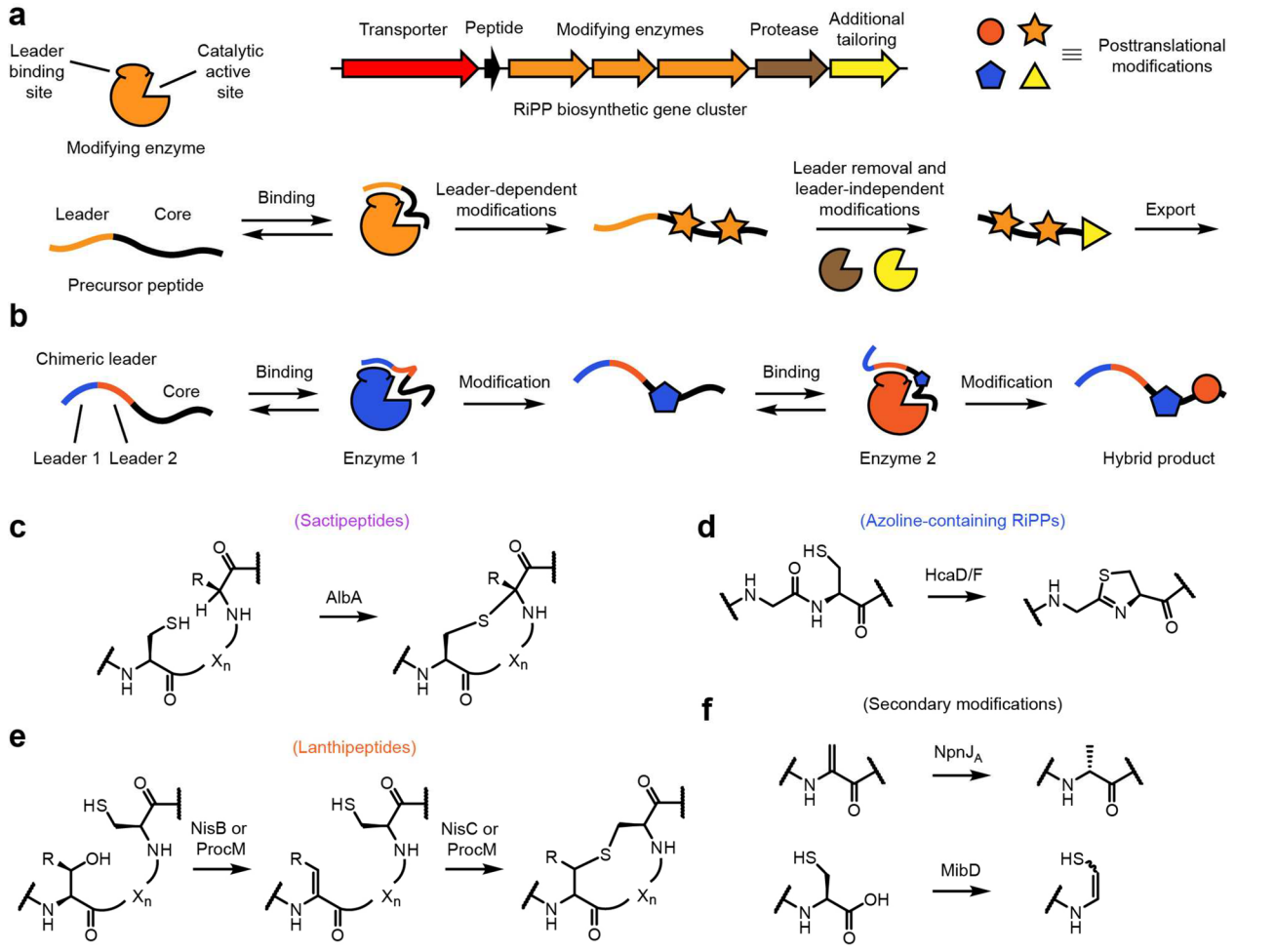

Figure 1. Overview of RiPP biosynthesis. (a) A generic RiPP gene cluster and the key maturation steps. (b) Chimeric leader peptide strategy for combining modifying enzymes from unrelated pathways. Examples of primary RiPP modification enzymes combined in this work include (c) Cα-cross-linked sactipeptides, (d) azoline-containing RiPPs, and (e) Cβ-cross-linked lanthipeptides. (f) Examples of secondary RiPP modification enzymes used in this work to install D-Ala residues and decarboxylate C-terminal Cys residues.

highly variable core regions but retain nearly identical biosynthetic enzymes and leader peptides.[26−29] RiPPs also increase structural diversity by acquiring new enzymes from different pathways to create natural "hybrid" RiPPs,[30] sometimes leading to entirely new classes as exemplified by the elaboration of linear azol(in)e-containing peptides into the cyanobactin and thiopeptide classes (Figure S1).[11,31] A few reports have demonstrated artificial combinations of RiPP enzymes,[32−36] but these examples have not led to combinations of modifications from different RiPP classes because they primarily swap enzymes between pathways that produce structurally related molecules.[32−36] Ideally, more diverse combinations of structural modifications can be harnessed using RiPP enzymes, but no general approach has been reported for the production of peptides with posttranslational modifications from different RiPP classes.

In order to address this unmet challenge, we envisioned a "chimeric leader peptide" strategy that would enable the rational combination of RiPP modifications. In this method, RSs from two leader peptides are concatenated to form a new bifunctional leader peptide within a single precursor peptide (Figure 1b). With their native RSs present, we hypothesized that two primary modifying enzymes would bind to their respective regions on the chimeric leader peptide and sequentially install posttranslational modifications, resulting in a hybrid RiPP product. We demonstrate that, after optimizing the core sequence and properly designing the coexpression plasmids, this approach successfully combines a thiazoline-forming cyclodehydratase (HcaD/F) with primary modifying enzymes from several different RiPP classes including sactipeptides (AlbA) and class I and II lanthipeptides (NisB/C and ProcM, respectively; Figure 1c−e). We also demonstrate how leader-independent tailoring enzymes such as MibD and NpnJ$_A$ (Figure 1f) can be included. Thus, this work lays a broad foundation for the generation of designer RiPP hybrids.

RESULTS AND DISCUSSION

Thiazoline−Lanthipeptide (Class I) Hybrid. To probe the feasibility of a chimeric leader peptide strategy to mix-and-match disparate RiPP biosynthetic pathways (Figure 1b), we first sought to combine an azoline-forming cyclodehydratase (HcaD/F) with a lanthipeptide synthetase (NisB/C). HcaD/F is a thiazoline-forming cyclodehydratase (Figure 1d)[31,37] and was chosen in part because the RS of the precursor peptide (HcaA) was previously defined.[37] The other enzyme, NisB/C, originates from the nisin biosynthetic gene cluster and was chosen because of the large body of knowledge pertaining to how the peptide substrate (NisA) is recognized and processed

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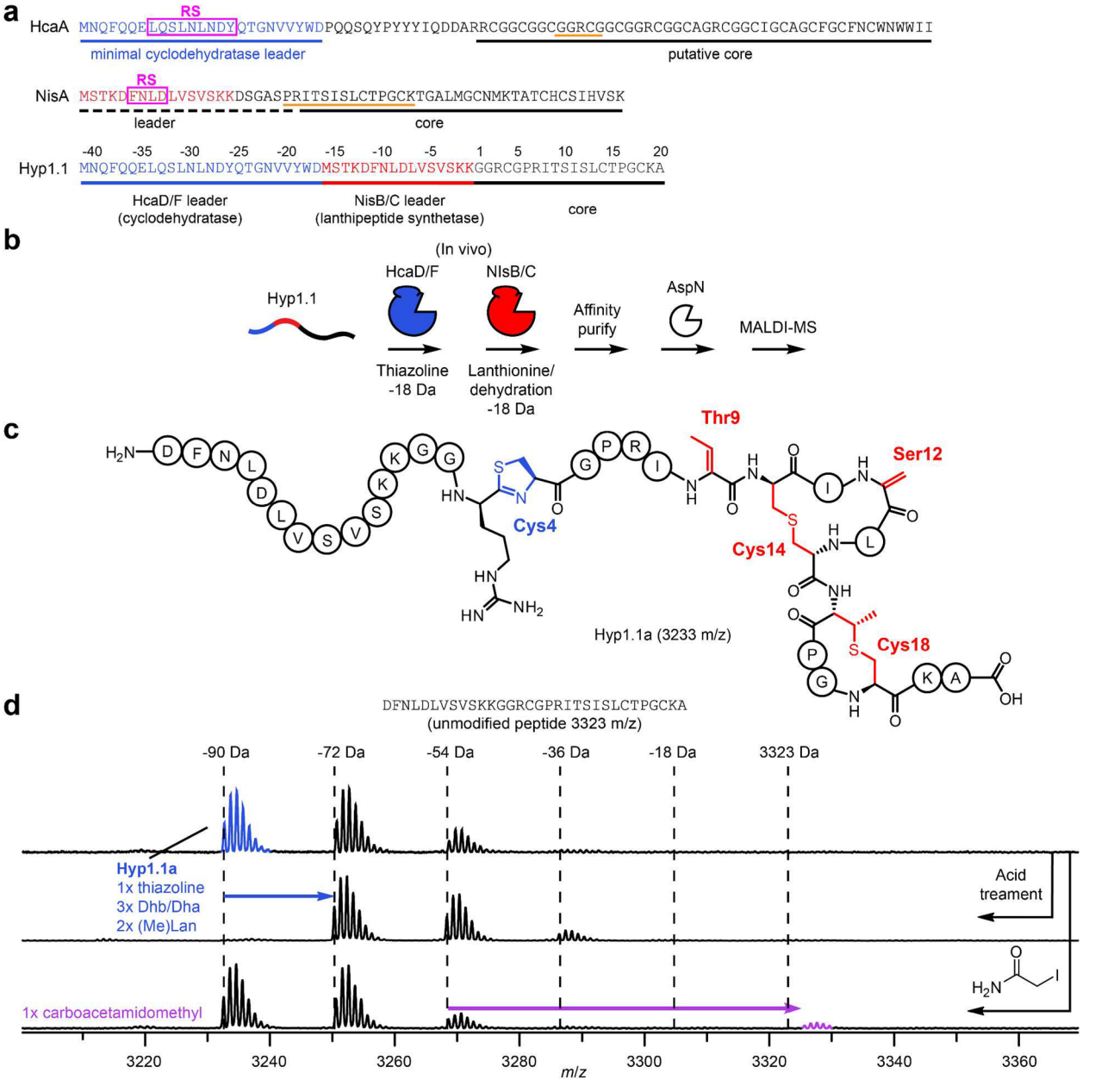

Figure 2. Production of a thiazoline–lanthipeptide (class I) hybrid. (a) Design of the Hyp1.1 sequence from HcaA and NisA precursor peptides. Portions of the leader peptides that were combined are colored. The recognition sequences (RSs) bound by the RREs of the cognate enzymes are indicated with a pink box. The orange underlined regions were combined to generate the chimeric core region. (b) Overview of experimental procedure. (c) Structure of thiazoline–lanthipeptide (class I) hybrid Hyp1.1a upon AspN digestion. Thiazolines are blue while dehydrations and (Me)Lan are red. Numbering is based on the core position, not peptide length after digestion. (d) The mass of Hyp1.1a (MALDI-TOF-MS) is consistent with one thiazoline, two dehydrations, and two (Me)Lan. These modifications are supported by acid hydrolysis (blue arrow, +18 Da) and by lack of labeling by iodoacetamide (green arrow, +57 Da).

(Figure S2).[38] As a class-defining enzyme, NisB/C forms the characteristic lanthionine (Lan) or methyllanthionine (MeLan) residues found in lanthipeptides through a two-step process.[38] First, NisB catalyzes dehydration of Ser/Thr residues to form the corresponding dehydroalanine (Dha) or dehydrobutyrine (Dhb). Next, NisC catalyzes a 1,4-nucleophilic addition of a Cys thiol to the *Cβ* of the dehydro amino acid to form a thioether ring (Figure 1e). Being a class I lanthipeptide dehydratase, NisB forms Dha/Dhb moieties in a tRNA-dependent manner while other classes of lanthipeptide dehydratases employ an ATP-dependent mechanism.[38−40]

A chimeric precursor peptide was constructed from the HcaA and NisA precursor peptides to serve as a potential substrate for both HcaD/F and NisB/C (Figure 2a). The sequence of this hybrid peptide (Hyp1.1) was designed to resemble key segments of each parent peptide. HcaD/F and NisB/C both have an RRE domain for leader peptide recognition, and the RS residues of their substrates are known (Figure 2). To incorporate both the RS from HcaA and NisA, we replaced the nonessential C-terminal region of the HcaA leader peptide[37] with the essential region of the NisA leader peptide.[41,42] We next engineered the core region of the chimeric precursor peptide. Leader-dependent enzymes are

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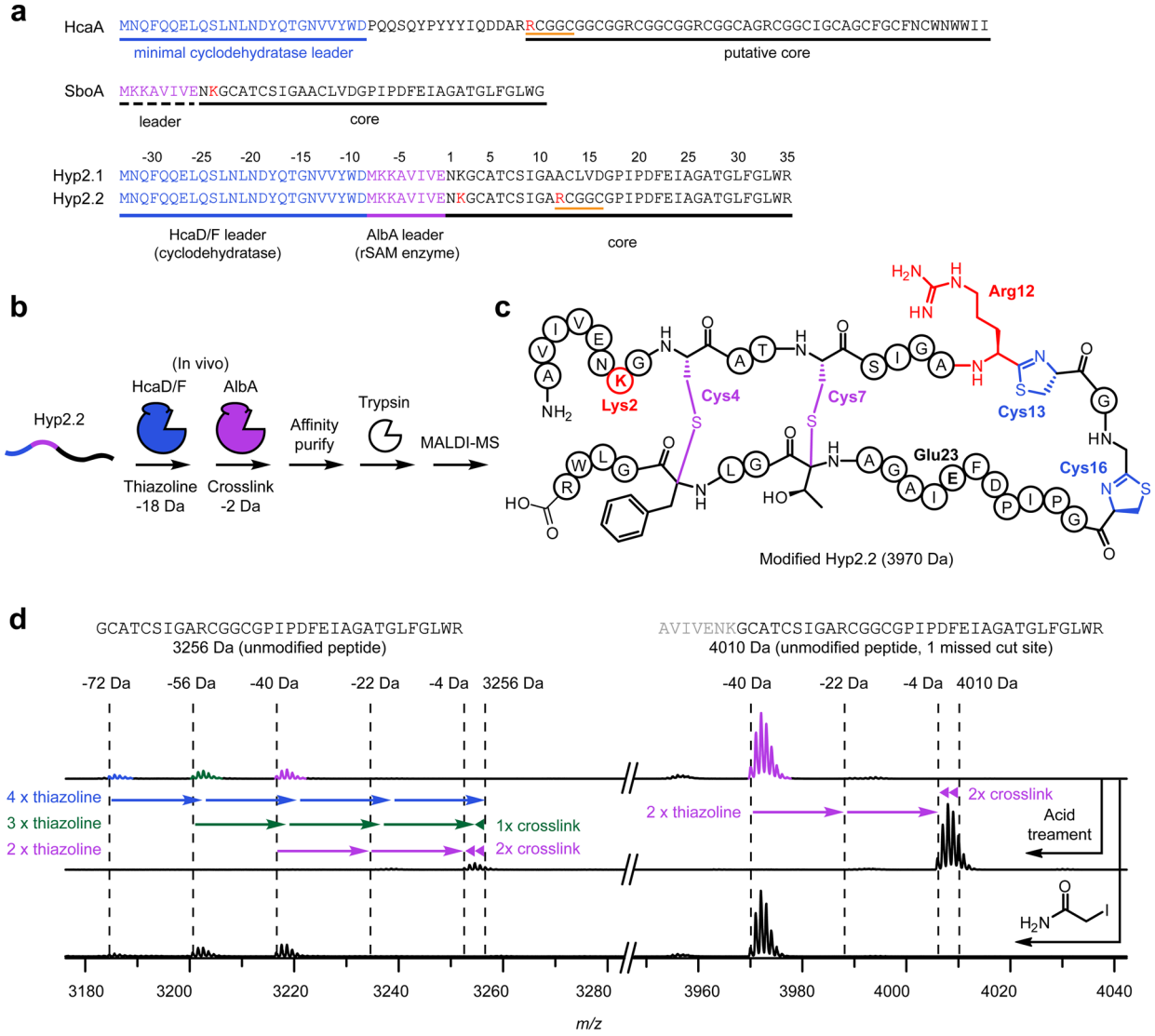

Figure 3. Production of a thiazoline−sactipeptide hybrid. (a) Design of hybrid peptides. Orange underlining indicates edited sequences. (b) Overview of the experimental procedure for combination of HcaD/F and AlbA. (c) Structure of thiazoline−sactipeptide hybrid (modified Hyp2.2). Numbering is based on the core position, not peptide length after digestion. (d) The mass of the modified peptide is consistent with two thiazolines and two sactionine linkages. The two sets of peaks correspond to protease digestion at different sites (gray font). The structure is supported by acid hydrolysis, lack of labeling by iodoacetamide, and resistance to proteolytic digestion (see also Figure S6). Arrows and triangles represent +18 and −2 Da, respectively. Red coloring indicates positions resistant to trypsin digestion.

known to require a certain minimum spacer region between the RS and the core peptide,[36,39,40] whereas extended core sequences are generally accepted.[43−46] Therefore, we first introduced a "GGRCG" motif, which derives from HcaA, at a position that would be appropriately distanced from the RS of HcaA to facilitate HcaD/F processing but too close to the NisA RS to allow for NisB/C processing. This motif was then followed by a segment of native NisA core sequence so that it would approximate the natural spacing from the NisA RS on the chimeric leader peptide (Figure 2a). Only 12 residues of the NisA core peptide were used to simplify analysis by mass spectrometry.

As diagrammed in Figure 2, the hybrid was tested by expressing HcaD/F, NisB/C, and His_6-Hyp1.1 in *Escherichia coli* (see Table S1 for plasmids). The modified peptide was affinity purified, digested with endoproteinase AspN, and analyzed via matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). A mixture of three products was evident. The most extensively processed peptide was dubbed Hyp1.1a and contained one thiazoline, two Dhb, one MeLan, and one Lan (Figure 2). To confirm the structure, we leveraged the fact that thiazolines are readily hydrolyzed to Cys under mild acid treatment with a concomitant gain of 18 Da. Treatment with iodoacetamide under reducing conditions, which would alkylate free Cys, yielded very little product, suggesting that the thioether rings were formed in high yield.

While previous nisin biosynthetic manipulation introduced new tailoring enzymes from related pathways[32,33] and hybrids have been synthetically prepared,[47,48] the enzymatic incorporation of a non-native modification (thiazoline) has not been reported. Our data show the feasibility of combining disparate RiPP pathways to generate new-to-nature hybrids by rationally designing a chimeric leader peptide. These results also demonstrate that two RiPP enzymes that compete for modification of the same residue (Cys) can still be rendered

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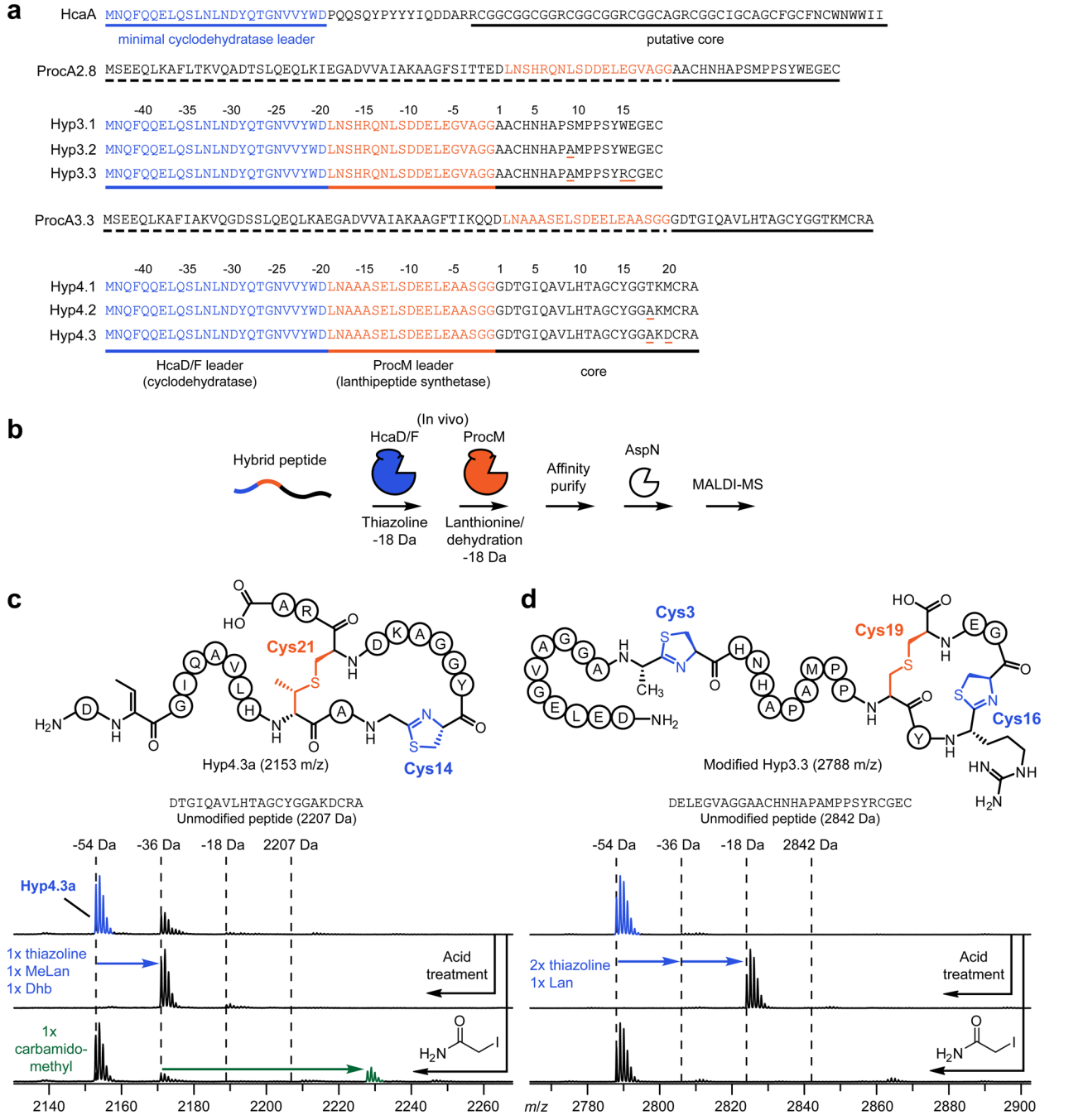

Figure 4. Production of hybrid thiazoline–lanthionine (class II) hybrids. (a) Design of hybrid peptides. Orange underlining indicates edited positions of the core peptide. The dashed line indicates the leader peptide of the ProcA2.8 and Proc3.3. (b) Experimental overview for combination of HcaD/F and ProcM. (c) Deduced structure and MALDI-TOF-MS support of posttranslational modifications for Hyp4.3. Numbering is based on the core position, not peptide length after digestion. (d) Same as in panel c but for Hyp3.3.

compatible. In this first example, discrete regions in Hyp1.1 were used to mimic the native peptides, and a similar approach could be used to append different modified structures to the N-terminus of other lanthipeptides.

Thiazoline–Sactipeptide Hybrid. Building on these initial results, we next sought to combine HcaD/F with an enzyme from another RiPP class that also modifies Cys. For this, the radical *S*-adenosylmethionine (rSAM) enzyme, AlbA, from subtilosin biosynthesis was chosen (Figure S3). AlbA is oxygen-sensitive and forms thioether cross-links between Cys thiols and the *Cα* of an acceptor residue. These linkages are called sactionines and gave rise to the RiPP class of sactipeptides (Figure 1c, note the difference from *Cβ*-linked Lan).[49,50] AlbA and HcaD/F both harbor RREs,[15] and hence this would be a second test of whether structurally similar binding domains on two enzymes that both act on Cys could be used to site-selectively modify a peptide substrate. Given the short natural leader peptide of SboA, the native substrate for AlbA, a simpler hybrid peptide (Hyp2.1) was designed that merely appended the minimal HcaA leader sequence to the N-terminus of SboA (Figure 3a). A C-terminal Arg was included in the core peptide to enhance MS detection. Because the core peptide of SboA has three Cys residues, we expected to see a mixture of modified products, which would allow us to assess

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competition between the two enzymes in the absence of any core peptide engineering.

The potential thiazoline−sactipeptide hybrid system was first tested by expressing AlbA, HcaD/F, and Hyp2.1 in *E. coli* (Table S1). Under high culture aeration (shaking at 250 rpm in baffled flasks), only cyclodehydration was observed. Upon reducing the culture aeration (shaking at 100 rpm in nonbaffled flasks),[50] we observed either sactionine-containing (strong signal) or thiazoline-containing (weak signal) products. No observable products contained both modifications (Figure S4). Although both enzymes were active under lower aeration conditions, AlbA appeared to outcompete HcaD/F given the low intensity signals for cyclodehydrated products (Figure S4). Prior work indicates that both HcaD/F and AlbA are highly processive with their native precursor peptides (i.e., substrate processing is faster than intermediate release).[37,51] We reasoned that intermediates with Hyp2.1 may also not be released during processing, thus preventing formation of a hybrid product. Additionally, if AlbA was acting faster because its native core sequence was used in Hyp2.1, thioether formation could preclude subsequent azoline formation.

In order to overcome this challenge, we enhanced the rate of azoline formation by editing the core peptide to include a more native-like "RCGGC" motif resulting in a second hybrid peptide with four Cys (Hyp2.2; Figure 3a). Presumably, the "RCGGC" motif would be more efficiently processed by HcaD/F while the remaining two Cys native to SboA would remain available for modification by AlbA. Upon coexpression of Hyp2.2 with the enzymes (Table S1), a mass was detected that was consistent with two sactionines and two dehydrations (Figure 3c). Mild acid treatment supported the presence of two thiazolines, as indicated by a gain of 36 Da. The presence of two sactionines was supported by lack of reactivity toward iodoacetamide. Analysis of high-resolution tandem MS spectra localized these modifications and corroborated the proposed structure (Figure S5). Additional evidence for the structure is provided by the following observations: (i) trypsin cuts very slowly at Lys2 near the first cross-link, presumably due to steric congestion (for structure, see Figure 3c); (ii) Arg12-related tryptic fragments were not detected (because the scissile bond was transformed to a thiazoline); and (iii) treatment with endoproteinase GluC did not result in digestion at Glu23 (Figure S6). Other low intensity signals consistent with a trithiazoline/monosactionine and tetrathiazoline species were also detected, suggesting that the hybrid pathway does not necessarily follow a strict processing order (Figure 3c).

Based on the results of Hyp2.1 and 2.2 (Figures 3 and S4), a change in the sequence context of a modifiable residue can change which RiPP enzyme will act at that position. Alteration of surrounding residues has been previously shown to enhance or diminish the processing rate of cyclodehydratases.[25,52,53] The use of the SboA core sequence initially provided an advantage to AlbA. However, the processing efficiency of HcaD/F could be enhanced through insertion of a more native-like RCGGC motif, ultimately producing the desired hybrid product.

Because AlbA and HcaD/F were transplanted from unrelated pathways, we could not use evolved biosynthetic controls to ensure that both enzymes act in the desired order to produce a hybrid product. Such controls include using protein−protein interactions that channel the substrate from one enzyme to the next or tuning biosynthetic enzymes to favor core processing only in a specific order (e.g., LanP and PatG proteases preferentially remove recognition sequences after earlier modifications are completed).[18,38] In the absence of such controls that are difficult to engineer, a key design feature for controlling hybrid RiPP biosynthetic schemes is using the local sequence context to alter the rate of enzymes that compete for the same residues or install modifications that block the activity of subsequent enzymes.

Thiazoline−Lanthipeptide (Class II) Hybrid. Thus far, three different primary RiPP enzymes have been shown to be tolerant toward substrates with non-native elements, but it has yet to be determined if a single enzyme pair will be generally able to process several different peptide sequences. For convenience, HcaD/F was again chosen for incorporation into another hybrid biosynthetic pathway to investigate this question. The class II lanthipeptide synthetase ProcM was selected as the second enzyme because it is mechanistically unique compared to the class I NisB/C enzyme and provides another opportunity to test different enzyme types using the hybrid approach. ProcM is known to be highly substrate-tolerant, having 30 different native precursor peptides (ProcAs) and forming a structurally diverse set of lanthipeptides (Figure S7).[28,29,54−56] Thus, we envisioned that it would be well suited for producing structurally diverse hybrid RiPPs. Furthermore, use of ProcM would extend the scope of the method because it does not contain an RRE;[16] in fact, the site of leader peptide binding on ProcM is currently not known.

A chimeric leader peptide for ProcM and HcaD/F was designed analogously to the previous hybrids. The minimal HcaA leader peptide was joined with the C-terminal portion of ProcA2.8 because the majority of the ProcA N-terminus is dispensable for processing.[28] Accordingly, the final 19 residues of the ProcA2.8 leader peptide were placed after the first 25 residues of the HcaA leader region (Figure 4a). As first trial, the native core sequence of ProcA2.8 was used to create Hyp3.1, which contained two Cys. Coexpression of Hyp3.1, HcaD/F, and ProcM (Table S1) produced a peptide with two Lan; no thiazolines were detected (Figure S8). Thus, as with the sactipeptide hybrid Hyp2.1, use of a native ProcA core peptide in the chimeric peptide resulted in ProcM outcompeting HcaD/F. Rather than improving HcaD/F catalysis by altering the local sequence of the core peptide, we decided to investigate a different control mechanism. We placed the gene for Hyp3.1 on the same plasmid as that for HcaD/F and lowered the copy number of the plasmid encoding ProcM to tune the activity in the desired direction (Table S1). This arrangement afforded a hybrid product with one thiazoline, as indicated by mild acid treatment, and one Lan (Figure S8). Analysis of MS/MS fragmentation indicated that the thiazoline was located at the N-terminal Cys while the Lan was at the C-terminus (Figure S9). It appeared, however, that the thiazoline at core position 3 (residue 13 of the AspN-digested peptide) inhibited dehydration at Ser9, indicating that thiazolines may occasionally interfere with downstream hybrid processing. A low intensity ion consistent with a peptide containing two Lan was also observed, indicating that the hybrid was not formed in quantitative yield. Substitution of the acceptor Ser9 to Ala in Hyp3.2 eliminated this side product (Figures S10 and S11). These data demonstrate how modest sequence alterations readily lead to the production of a single hybrid product without resorting to insertion of longer motifs.

The general tolerability of HcaD/F and ProcM encouraged us to design a new hybrid based on a peptide with a completely different core sequence, ProcA3.3 (Figure S7). Like Hyp3.1, this hybrid (Hyp4.1) appended the HcaA minimal leader

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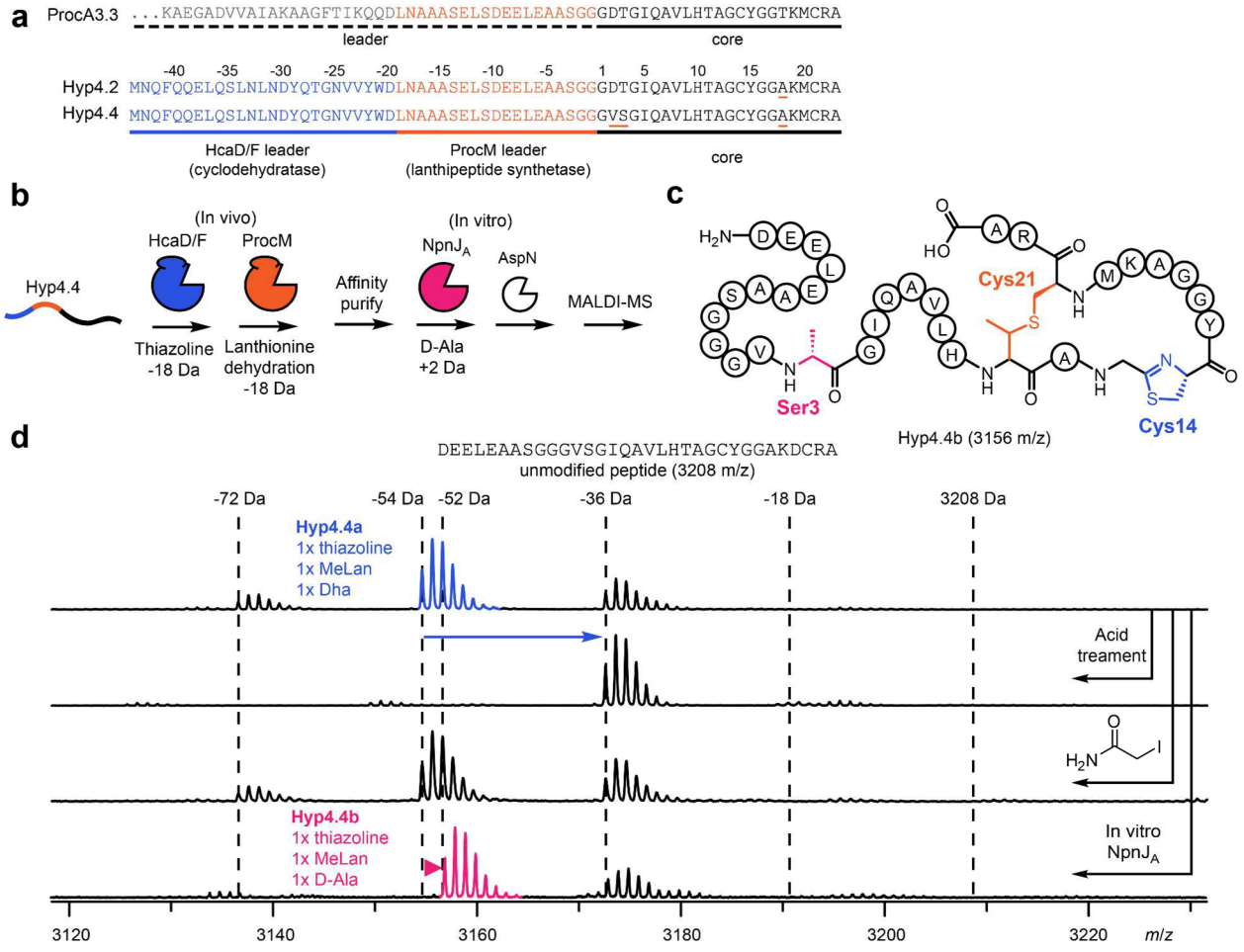

Figure 5. Combination of two primary and one secondary RiPP modifications. (a) Design of hybrid peptide. Orange underlining highlights changed residues. (b) Experimental overview for combining HcaF/D, ProcM, and $NpnJ_A$. (c) Deduced structure of Hyp4.4b. (d) MALDI-TOF-MS analysis of Hyp4.4 products. The initially formed hybrid has a Dha, thiazoline (arrow indicates hydrolysis), and MeLan. The formation of D-Ala from Dha is indicated by the +2 Da shift (triangle) of the Hyp4.4a species upon addition of $NpnJ_A$. The weak ion labeled as −72 Da is likely a peptide that underwent two cyclodehydrations and two dehydrations.

sequence to the C-terminal portion of the ProcA3.3 leader peptide (Figure 4a). The native ProcA3.3 core sequence contains two Cys that normally form a dual MeLan "ring-within-a-ring" topology in prochlorosin 3.3 (Figure S7). Coexpression of Hyp4.1, ProcM, and HcaD/F resulted in two major products: Hyp4.1a and Hyp4.1b (Figure S12). Hyp4.1a contained one Dhb, one thiazoline, and a non-natural MeLan between Thr18 and Cys21 (Figure S13). Hyp4.1b displayed modifications similar to native prochlorosin 3.3, suggesting that ProcM had processed the substrate before HcaD/F.

With HcaD/F and ProcM both able to act upon two different core peptides derived from native ProcA sequences, we next designed additional hybrids to direct enzymatic processing with greater precision. In the product of Hyp4.1, Cys21 forms a MeLan with Thr18, which places the thiazoline at Cys14 outside the MeLan macrocycle (Figure S13). We then wondered if we could create a hybrid in which a thiazoline would be within a (Me)Lan macrocycle. A Thr18Ala mutation was introduced (Hyp4.2; Figure 4a) to prevent Cys21 from generating a MeLan with Thr18 with the intention that it would then form a larger thioether ring with Thr11. After coexpression, Hyp4.2 was converted into a mixture of modified products (two thiazolines, thiazoline and Lan, or two Lan), indicating that each Cys could be modified by either enzyme (Figure S12). Although diversity generating systems can be advantageous, a mixture of products was not our desired outcome, and thus, we aimed to again tune the hybrid pathway to produce a single RiPP product. Accordingly, we designed Hyp4.3 in which we placed Asp before Cys21 to decrease the efficiency of HcaD/F processing at this position (i.e., Met20Asp substitution to Hyp4.2; Figure 4a). Expression of Hyp4.3 with HcaD/F and ProcM yielded primarily one product (Hyp4.3a) that contained one Dhb, one thiazoline, and one MeLan based on mild acid hydrolysis and iodoacetamide labeling (Figure 4c). MS/MS indicated that Cys21 was converted to MeLan, supporting our prediction that HcaF/D is less efficient in processing Cys with a preceding Asp residue (Figure S14; Figure 4c). The thiazoline was verified to be within the macrocycle through linearization by thermolysin digestion and subsequent MS/MS analysis (Figure S15). The stereochemistry of the MeLan was confirmed to be the native DL configuration by chiral GC/MS (Figure S16), providing support that this ring was likely still formed enzymatically.[38] The other low intensity ions observed from coexpression with Hyp4.3 appeared to be a mixture of isobaric species (Figure S17).

The previous example demonstrates that a thiazoline can be installed within a large, 11-residue MeLan macrocycle. We next

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investigated whether a thiazoline could be placed within a smaller (Me)Lan macrocycle. For this purpose, we generated another variant of the ProcA2.8 core (Hyp3.3; Figure 4a). We introduced an additional Arg-Cys motif that we envisioned would become a thiazoline within a 7-residue Lan macrocycle. Upon coexpression, the modified peptide had three dehydrations (−54 Da), two of which were susceptible to mild acid treatment, indicating the presence of two thiazolines and one Lan (Figure 4d). Lack of iodoacetamide labeling further indicated that all Cys were modified, and MS/MS fragmentation data support the proposed modified Hyp3.3 structure (Figure S18).

Overall, these experiments with HcaD/F and ProcM suggest that the chimeric leader peptide strategy will be amenable to creating libraries of modified peptides. After plasmid design optimization, ProcM and HcaD/F processed several peptides with altered core sequences and produced different modified structures with different arrangements of thiazoline heterocycles and (Me)Lan rings. The ability to further tune hybrid biosynthesis toward a single desired product through simple core sequence changes was also demonstrated. HcaD/F-dependent azoline formation can apparently be inhibited by employing a C-terminal Cys or by using an "Asp-Cys" motif, which reduces residue competition. Conversely, a simple "Arg-Cys" motif was robustly cyclodehydrated in Hyp3.3. This is notable as the earlier examples relied on larger motifs to introduce thiazolines. With the use of minimalistic motifs to tune enzyme activity, and the usage of enzymes with diverse sequence preferences, we anticipate that a wide diversity of new-to-nature RiPPs will be accessible through the chimeric leader peptide engineering approach.

Hybrids with Secondary Tailoring Enzymes. Although our chimeric leader peptide strategy was designed for combining leader-dependent "primary" RiPP biosynthesis enzymes, the method should also allow interfacing with secondary tailoring enzymes. Many RiPP pathways have tailoring enzymes that install functional groups critical for bioactivity.[11] We chose to combine the D-Ala-forming Dha reductase $NpnJ_A$ from *Nostoc punctiforme* PCC 73102 (Figure 1f) with the HcaD/F and ProcM hybrid system.[57] $NpnJ_A$ appears to prefer hydrophobic flanking residues, so a D2V/T3S double substitution was introduced into the Hyp4.2 peptide to create Hyp4.4 (Figure 5a).[57] Coexpression of Hyp4.4, HcaD/F, and ProcM initially produced three detectable species (Figure 5). The most intense ion (labeled as Hyp4.4a) was a hybrid containing one thiazoline, one MeLan, and one Dha (from dehydration of the newly introduced Ser3). Upon treating Hyp4.4a with $NpnJ_A$ and NADPH in vitro, a new +2 Da product resulted (Hyp4.4b; Figure 5d), indicative of D-Ala formation. We also successfully combined HcaD/F with MibD, a flavin-dependent enyzme from *Microbispora* sp. 107891,[58] to produce a C-terminally decarboxylated, linear azoline-containing peptide (Figure S19).

These results indicate that our approach for creating hybrid RiPPs can be extended to leader-independent modifications, provided the proper modifiable sequence is present. Even though tailoring enzymes tend to bind directly to the core peptide, inserting short motifs into the core in conjunction with a chimeric leader peptide led here to three distinct posttranslational modifications from unrelated pathways.

CONCLUSION

Rational design of biosynthetic pathways to produce custom molecules has long been a goal of combinatorial biosynthesis and natural product synthetic biology. Despite progress in understanding natural pathways, the chemical space that can be explored by current engineering techniques is limited compared to what is theoretically possible.[9,10] In this work, we demonstrate how a chimeric leader peptide has the potential to unlock the vast chemical space afforded by RiPPs. This concept was inspired by how natural pathways can combine different enzymes (Figure S1) and represents a major step forward in RiPP engineering. Although prior work has demonstrated enzyme swapping within related pathways, such as for nisin and the cyanobactins,[27,30,32−36] this study is to our knowledge the first demonstration of leveraging the recognition sequences and programmability of RiPP enzymes to mix-and-match the primary, class-defining structural features from unrelated classes. In total, four mechanistically unique enzymes were shown to be tolerant to manipulation of their leader peptides and insertion of non-native sequences.

The primary difficulty we encountered was ensuring that the enzymes acted at the desired locations and in the correct order. Being from unrelated pathways, there were no natural biosynthetic checkpoints to guide processing toward a single hybrid product, meaning the enzymes could compete for the same residue or act in a fashion that blocked the other enzyme. However, modest editing of the core sequence was sufficient to exploit the innate selectivity of an enzyme to yield the desired product. Future work with other enzymes that exhibit different substrate preferences may lead to a toolbox of enzymes for use in specific applications. Optimizing expression levels of each enzyme through plasmid design also adds another layer of control. Despite the successful use of these design principles in this work, there are likely some RiPP modifications that are inherently incompatible or too difficult to generally combine given their different structural requirements or limited promiscuity of their enzymes. Nonetheless, based on the enzymes combined here, we predict that many other hybrid RiPP combinations will be feasible.

In summary, chimeric leader peptides appear to be a broadly applicable and effective platform for creating hybrid RiPPs. In the cases shown here, the method afforded significant product yields (~1 mg/L of culture for the core peptide without any optimization) and employed a standard coexpression plasmid system. Moreover, RiPP enzymes can be chosen even if the enzymes act on the same residue. Our approach was successful regardless of native leader peptide length (8 vs 60+), its binding affinity (~5 μM for ProcA/ProcM vs ~50 nM for HcaA/HcaF),[37,55] how it was bound (RRE or non-RRE binding site), or whether the binding site was previously known. Further, our approach is amenable to the inclusion of leader-independent enzymes. Accordingly, the chimeric leader peptide strategy holds much potential for combinatorial RiPP biosynthesis and opens the door for the generation of additional hybrid RiPP compounds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00141.

Experimental methods, Tables S1 (new plasmids) and S2 (nucleotide sequences), and Figures S1−S19 (PDF)

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