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Communication

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Flexizyme-enabled benchtop biosynthesis of thiopeptides.

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Supporting Information Placeholder

ABSTRACT: Thiopeptides are natural antibiotics that are fashioned from short peptides by multiple layers of post-translational modification. Their biosynthesis, in particular the pyridine synthases that form the macrocyclic antibiotic core, has attracted intensive research but is complicated by the challenges of reconstituting multiple pathway enzymes. By combining select RiPP enzymes with cell free expression and Flexizyme-based codon reprogramming, we have developed a benchtop biosynthesis of thiopeptide scaffolds. This strategy side-steps several challenges related to the investigation of thiopeptide enzymes and allows access to analytical quantities of new thiopeptide analogs. We further demonstrate that this strategy can be used to validate the activity of new pyridine synthases without the need to reconstitute the cognate prior pathway enzymes.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a growing family of peptide-derived natural products that exhibit natural combinatorial biosynthetic logic.[1] RiPP biosyntheses initiate from a gene-encoded precursor peptide, which contains a core region that undergoes enzymatic post-translational modification, and a leader region, which is typically responsible for recruiting and coordinating these enzymes through specific recognition sequences (RSs). These RSs have affinity for select domains of RiPP biosynthetic enzymes, increasing substrate local concentration to the otherwise promiscuous enzyme active sites, and allowing the modification of diverse cores.[2,3] Natural pathways exhibit leader peptides with multiple RSs and recruit whole suites of post-translational modifying enzymes to convert precursor peptides into mature natural products. The combination of RS-programmable recruitment and promiscuous enzymes inspires recent efforts at repurposing this strategy to scaffold new-to-nature hybrid biosynthetic pathways. [4-8]

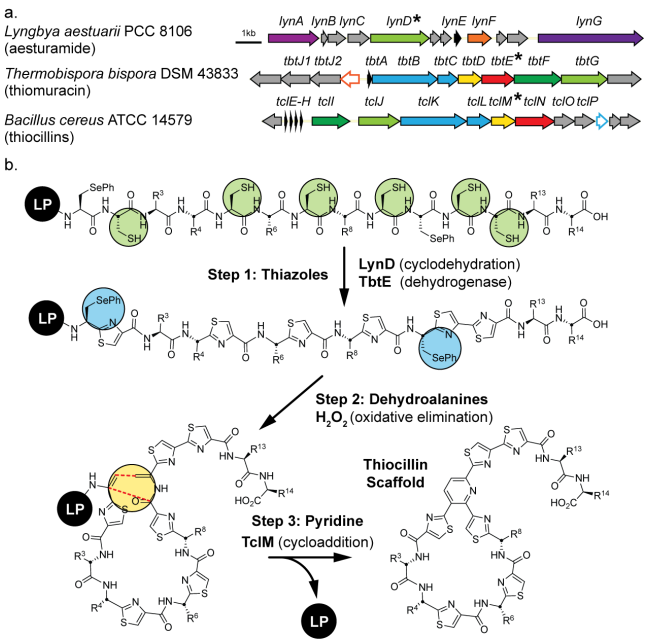

Figure 1. Flexizyme-enabled benchtop biosynthesis of thiopeptide scaffolds. a) Biosynthetic gene clusters of thiocillins, thiomuracin GZ, and aesturamide. Genes for key enzymes used in this work are highlighted with asterisks. b) Proposed hybrid route to the thiocillin core.

Thiopeptides are one of the most extensive natural examples of this combinatorial, RS-directed biosynthesis,[9,10] and the three class-defining transformations include the formation of azoles, dehydroamino acids, and pyridines from serine and cysteine residues (Figure 1a). Many of these enzymes are remarkably promiscuous and thiopeptide pathways have proven capable of generating many variants.[11,12] For example, hundreds of mutants of thiocillin, GE37468, and thiostrepton have been generated by gene replacement strategies.[13-15] However, competition between the pathway enzymes for functional groups on non-native substrates can give rise to complex mixtures of products and slower

processing of mutant substrates or host toxicity can restrict production of potential compounds. The *in vitro* reconstitution of whole thiopeptide biosynthetic pathways, which has recently been achieved for thiomuracin, can circumvent some of these problems, but relies on access to soluble, well-behaved proteins.[16,17] This can be especially challenging for the tRNA dependent Lantibiotic-type dehydratases. Additionally, this strategy still does not overcome enzyme competition. Alternative strategies, such as a chemoenzymatic approach, could allow rapid access to novel structural variants and ease characterization of new thiopeptides and thiopeptide-associated enzymes.[18]

We envisioned that carefully chosen RiPP enzymes might be combined with orthogonal chemical handles to create a flexible *in vitro* platform for the benchtop preparation of thiopeptides (Figure 1b).[19,20] More specifically, *in vitro* transcription-translation could be used to express designer hybrid leader peptide substrates displaying RSs for the cyclodehydratase LynD from aesturamide biosynthesis and pyridine synthase, TclM from thiocillin biosynthesis. Both enzymes have well-defined RS motifs and broad substrate promiscuity. Additionally, LynD exhibits excellent selectivity for Cys conversion to thiazolines while ignoring Ser/Thr residues.[21,22] Alternatively, if oxazoles are of interest, then PatD, which acts on Ser/Thr/Cys, could be used in place of LynD. [20] Oxidation of thiazolines to thiazoles might be effected by the azoline-oxidase, TbtE from thiomuracin biosynthesis, which acts in a leader peptide independent manner.[16] In place of Lantibiotic dehydratases, we would use robust Flexizyme technology to introduce the unnatural amino acid *Se*-phenylselenocysteine (SecPh), which undergoes oxidative elimination with H_2O_2 to generate dehydroalanines (Dhas) for the pyridine-forming cycloaddition.[23-25] Flexizymes are aptamers developed to condense a wide array of amino acid esters with tRNAs of choice (see Supplementary Fig. 6) , allowing codon reprograming in *in vitro* transcription/translation systems.[26,27] In total, this would cut the number of enzymes or proteins necessary to prepare a thiopeptide *in vitro* from, six, in case of thiocillin (TbtIJKLMN), to three (LynD, TbtE, and TclM, Figure 1). Although quantities of peptide made by *in vitro* transcription-translation and Flexizyme reprogramming are small relative to other technologies, such as amber codon suppression/in cell expression or solid phase peptide synthesis, the approach is rapid (~2 hr.), robust, and flexible for peptides of this size and complexity. Thus, we anticipate that this strategy might ultimately enable rapid characterization of new pyridine synthases and associated enzymes and aid elucidation of new thiopeptides.

In order to validate this strategy, we first had to confirm that existing Flexizymes could incorporate SecPh into *in vitro* translated peptides and that LynD and TbtE could accommodate SecPh at cysteine-adjacent positions. We confirmed that the dinitrobenzyl (Dnb) ester-specific Flexizyme dFx could ligate the Dnb ester of SecPh by means of an *in vitro* microhelix assay (see Supplementary Figure S2).[25] Hybrid substrates were prepared by codon reprogramming the tryptophan codon – although Flexizyme allows a number of codons to be reprogrammed – due to the scarcity of Trp residues in thiopeptides, using the AsnE2 tRNA body.[28] The loaded $AsnE2_{trp}$-tRNA was used to incorporate SecPh at positions Ser1 and Ser10 of the thiocillin core. For ease, initial DNA templates were prepared by cloning into plasmid pMCSG7 which necessarily incorporated an N-terminal sequence tag leading to the design of our test substrate (Figure 2a), and transcription-translation reactions were prepared on 2.5 µL analytical scale using NEB PURExpress. [26,27] In the

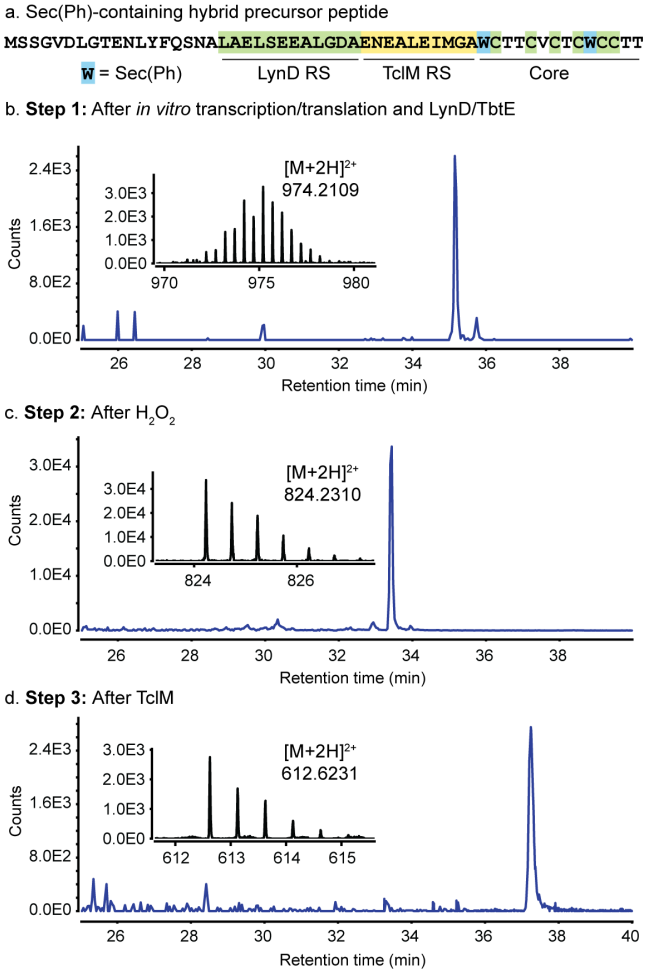

Figure 2. Flexizyme-enabled benchtop biosynthesis of a thiocillin scaffold. a) Sequence of the designer precursor peptide, including a pMCSG7-derived sequence, the LynD RS and TclM RS. The Trp-codon was reprogrammed to incorporate SecPh. EICs for SecPh and hexathiazole precursor peptide after LynD/TbtE treatment (b), hexathiazole and Dha-containing product after oxidative elimination (c), and fully cyclized thiocillin core (d).

event, LynD was able to convert all six cysteines in several test substrates into thiazolines, which were further processed to thiazoles by TbtE as confirmed by high resolution liquid chromatography and mass spectrometry (HR LC/MS) (Figure 2b and Supporting Information). Subsequent treatment with 1 M H_2O_2 efficiently converted both SecPh residues to Dhas (Figure 2c). In the last proof-of-concept step, the excess H_2O_2 could be quenched by addition of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and TclM added *in situ* provided complete conversion of the linear substrate into a cyclic thiopeptide (Figure 2d), confirming that TclM is compatible with this designed leader strategy.

We next began to explore the requirements of the designer leader peptide. The N-termini of natural

thiopeptide leader peptides have been implicated in an affinity-enhancing interaction with pyridine synthase enzymes.[29] Although our studies have shown this interaction dispensable for pyridine synthase processing, [22] we chose to test the potential impact of changes to the N-terminus on processing of designer leader peptide substrates. Thus, we prepared two new hybrid substrates in which we replaced the original pMCSG7-derived N-terminus with two different excerpts from the N-terminus of TclE, the native precursor peptide for TclM (Table 1A, entries 3 and 4). In a third substrate we truncated the leader peptide leaving only a short MSSQ tag before the LynD RS (Table 1A, entry 1). The relative impact of these changes was assessed by integration of extracted ion chromatograms (EICs) for the product (Table 1A, entry 2; Figure 2c,d). Interestingly, the TclE fragment sequences

Table 1. Results of flexizyme-enabled benchtop biosynthesis with mutant leader peptides (1A)[a] and Cores (1B).[b]

SecPh-containing hybrid precursor peptide

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                                                1                  14
MSSGVDLGTENLYFQSNALAELSEEALGDAENEALEIMGAWCTTCVCTCWCCTT
```

W = SecPh; LynD RS; TclM RS; Core

1A	Hybrid Leader Peptide Sequences	Thz	Pyr
1	MSSQLAELSEEALGDAENEALEIMGA	0.16	0.01
2	MSSGVDLGTENLYFQSNALAELSEEALGDAENEALEIMGA	1.00	1.00
3	MSEIKKALNTLEIEDFLAELSEEALGDAENEALEIMGA	0.76	0.69
4	MSEIKKALNTLEIEDFDAIEMVDVDAMPLAELSEEALGDAENEALEIMGA	0.73	0.22
5	MSSGVDLGTENLYFQSNALAELSEEALGDAAAAAAENEALEIMGA	0.80	0.31
6	MSSGVDLGTENLYFQSNALAELSEEALGDAGGGGGENEALEIMGA	0.13	0.11
7	MSSGVDLGTENLYFQSNALAELSEEALGDAKKKKKENEALEIMGA	0.18	0.09
8	MSSGVDLGTENLYFQSNALAELSEEALGDADDDDDENEALEIMGA	0.20	0.00
9	MSSGVDLGTENLYFQSNALAELSEEALGDAPPPPPENEALEIMGA	0.04	0.05
10	MLAELSEEALGDASEIKKALNTLEIEDFDAIEMVDVDAMPENEALEIMGA	0.04	0.03

1B	Core Amino Acid Sequences	Thz	Pyr
1	SCTTCVCTCSCCTT	✓	n/a
2	FCTTCVCTCFCCTT	✓	n/a
3	WCTTCVCTCWCCTT	✓	n/a
4	WCTTCKCTCWCCAA	✓	✓
5	WCTTCDCTCWCCAA	✓	✓
6	WCAACVSACWCCAA	✓	✓
7	WCTACACACWCA	✓	✗
8	WCTACACACWCC	✗	✗
9	WCTACACACWCCA	✓	✓
10	WCTACACACWCCT	✓	✗
11	WCTACACACWCCAA	✓	✓
12	WCTACACACWCCAG	✓	✓
13	WCTTCVCTCWCCAANSGGVS	✓	✓

[a]EIC area relative to entry 2 as a standard. [b]Checks indicate a detected EIC. An "X" indicates no EIC detected above the noise threshold of 1.0E2.

decrease thiopeptide formation and seem to negatively impact LynD processing. Moreover, removal of the pMCSG7-derived sequence greatly reduces processing by LynD/TbtE pair. Taken together, these results further confirm that the N-terminus of TclE is dispensable for TclM processing but LynD may be sensitive to the location of its cognate RS within the larger peptide context. To further probe the latter aspect, we designed a series of leader sequences, in which spacers were introduced between the LynD RS and TclM RS (Table 1A, entries 5-9).[20] In almost all cases, the substrates were converted to thiopeptides, suggesting that LynD is broadly tolerant of diverse sequence space between the RS and core, although at reduced efficiency. As an extreme example of this spacing promiscuity, a substrate with the LynD RS sequence N-terminal to the complete native TclE leader peptide was made and subsequently processed to the mature thiopeptide (Table 1A, entry 10). This last result suggests a potentially broadly applicable strategy for circumventing reconstitution of all pathway enzymes in thiopeptide formation: express the full leader as a C-terminal fusion to LynD RS.

We next examined allowable changes to the core sequence. The TclM-containing thiocillin pathway has been shown to tolerate a wide array of changes to the core peptide *in vivo*; we therefore focused on changes to the core that were unproductive in those studies (Table 1B, entries 4 and 5).[13,30] For example, Val6 of native thiocillin had been recalcitrant to charged or hydrophilic residues, such as lysine or aspartic acid. This limitation is a barrier to antibiotic development, as Val6 appears to be an ideal position for modulating the solubility.[31] In contrast, hybrid substrates bearing a V6D or V6K mutation were readily transformed to the cyclic thiopeptide *in vitro*. Additionally, the LynD/TbtE pair *in vitro* provided greater product control relative to the native thiocillin enzymes, TclI, TclJ, and TclN, as exemplified by a C7S mutant (Table 1B, entry 6). In the *in vivo* system, a similar mutant gave mixtures of different modifications and apparent misprocessing. LynD, however, modifies all cysteines indiscriminately and left the newly introduced serine untouched. We focused considerably more mutagenesis on the C-terminus of the core, because studies have suggested TclM might be sensitive to C-terminal modifications, [22,32] and such modifications would be necessary for linking the current hybrid substrates with mRNA display in the future (Table 1B, entries 7-13). Deletion of even one amino acid from the C-terminus was unfavorable for TclM and/or LynD processing (Table 1B, entries 7-10). In contrast, extending the C-terminus (Table 1B, entry 13) did not significantly impact enzymatic processing. These data suggest that the hybrid strategy is amenable to C-terminal extensions and new sequences, not previously accessible by *in vivo* engineering approaches, although, more extensive investigations will be needed to understand the limitations.

We last sought to test whether this strategy could be used to reconstitute new pyridine synthases. Recent work has suggested that only a fraction of genetically-encoded thiopeptides have been isolated.[33] Of the >500 predicted thiopeptide gene clusters, the largest family is comprised of members that contain a close homolog of LazC, the predicted pyridine synthase from lactazole biosynthesis.[34] Additionally, while LazC homology is high in this family, the core peptide diversity is broad, suggesting LazC and its homologs may exhibit unique substrate promiscuity (see Supplementary Fig. 5). Despite the preponderance of predicted LazC homologs, LazC has not yet been reconstituted *in vitro*. Therefore, we expressed and purified LazC as its MBP-fusion and designed three new hybrid sequences as potential substrates (Figure 3b). In one LazC substrate, we integrated LynD RS directly into the native lactazole leader at a site with apparent natural homology (termed LacHyb1, Figure 3a), while in the other two, LynD RS was encoded 15 or 25 residues N-terminal to the core (termed LacHyb2 and 3, respectively). Additionally, Trp2 and oxazole-forming Ser11 were replaced with a serine and thiazole-forming cysteine, respectively (see Supplementary Figure 3). Both mutations were previously produced by a gene-replacement

strategy suggesting that the double mutant may also be a LazC substrate and render the core compatible with Trp-reprogramed SecPh incorporation. Upon treatment with LynD/TbtE, the four cysteines in each substrate readily underwent conversion to thiazoles and subsequent H_2O_2 oxidation introduced the four Dhas (Figure 3c). Lastly, treatment with LazC efficiently yielded the new, pyridine-containing masses, thus confirming activity of LazC as a pyridine synthase (Figure 3d). This result was consistent for all three hybrid lactazole leader peptides (Supplementary Fig. 4, traces S22-24 and S42-44) and suggests that the hybrid *in vitro* strategy should work on many other, yet uncharacterized pyridine synthases and could ultimately allow elucidation of new thiopeptides.

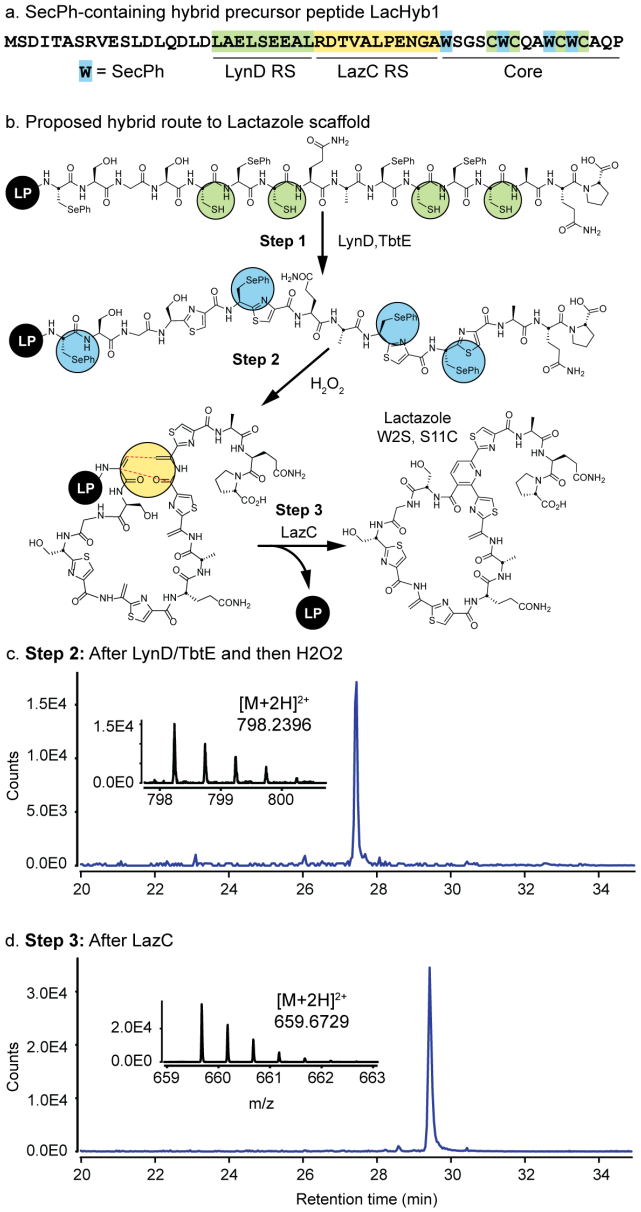

Figure 3. Flexizyme-enabled benchtop biosynthesis of lactazole W2S, S11C. a) Sequence of designer precursor peptide. b) Proposed route to lactazole scaffolds. EICs for the hexathiazole and Dha-containing product of treatment with LynD/TbtE and H_2O_2 (c), and fully cyclized lactazole core (d).

In summary, we have developed a new, facile strategy to access thiopeptide backbones. This approach combines robust, Flexizyme-assisted incorporation of chemical handles into *in vitro* transcribed/translated peptides with three unrelated RiPP enzymes LynD, TbtE, and TclM by using designer leader peptides. We demonstrate the ability to make thiocillin variants previously unattainable through natural biosynthetic processes and use this strategy to reconstitute the pyridine synthase LazC to make lactazoles for the first time *in vitro*. We anticipate this approach will be useful in making new thiopeptide variants with therapeutic potential, studying more pyridine synthases and associated enzymes, and may aid elucidation of new thiopeptide structures. Finally, we anticipate that this strategy will be compatible with high throughput screening techniques, such as mRNA display, which is a current focus in our lab.

ASSOCIATED CONTENT

Experimental details, synthetic schemes, figures available at http:// pubs.acs.org.

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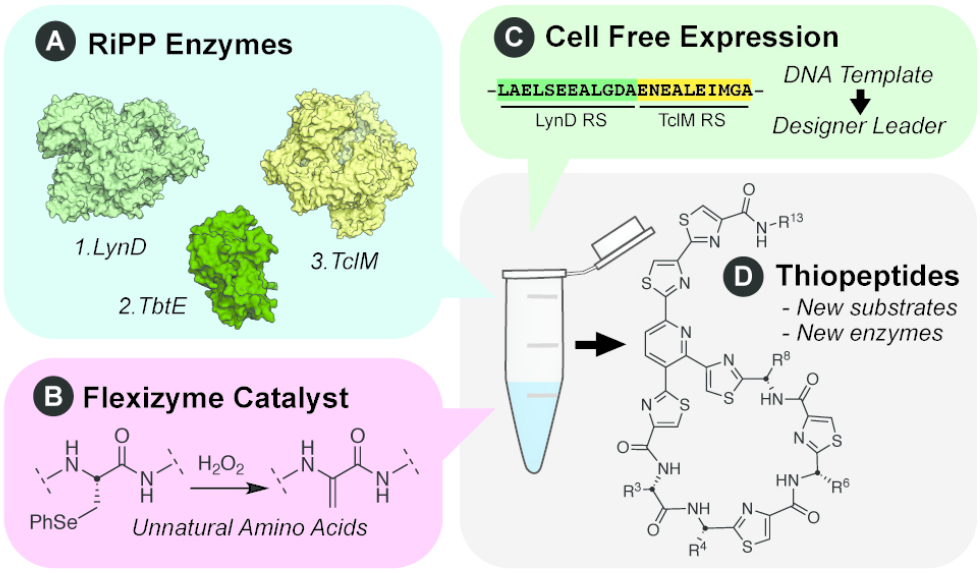
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