De Novo Discovery of Thiopeptide Pseudo-natural Products Acting as Potent and Selective TNIK Kinase Inhibitors

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ABSTRACT: Bioengineering of ribosomally synthesized and post-translationally modified peptides (RiPPs) is an emerging approach to explore the diversity of pseudo-natural product structures for drug discovery purposes. However, despite the initial advances in this area, bioactivity reprogramming of multienzyme RiPP biosynthetic pathways remains a major challenge. Here, we report a platform for de novo discovery of functional thiopeptides based on reengineered biosynthesis of lactazole A, a RiPP natural product assembled by five biosynthetic enzymes. The platform combines in vitro biosynthesis of lactazole-like thiopeptides and mRNA display to prepare and screen large (≥10¹²) combinatorial libraries of pseudo-natural products. We demonstrate the utility of the developed protocols in an affinity selection against Traf2- and NCK-interacting kinase (TNIK), a protein involved in several cancers, which yielded a plethora of candidate thiopeptides. Of the 11 synthesized compounds, 9 had high affinities for the target kinase (best \( K_D = 1.2 \) nM) and 10 inhibited its enzymatic activity (best \( K_i = 3 \) nM). X-ray structural analysis of the TNIK/thiopeptide interaction revealed the unique mode of substrate-competitive inhibition exhibited by two of the discovered compounds. The thiopeptides internalized to the cytosol of HEK293H cells as efficiently as the known cell-penetrating peptide Tat (4–6 \( \mu \)M). Accordingly, the most potent compound, TP15, inhibited TNIK in HCT116 cells. Altogether, our platform enables the exploration of pseudo-natural thiopeptides with favorable pharmacological properties in drug discovery applications.

INTRODUCTION

Biosynthesis of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products is often amenable to bioengineering. Improving the bioactivities and/or pharmacological profiles of RiPP natural products is a common goal pursued by many recently developed bioengineering approaches, as exemplified by mutagenesis techniques and strategies to create hybrid RiPPs. Even complete bioactivity reprogramming, achieved by the screening of combinatorial libraries of natural product analogs, has been accomplished with the use of catalytically promiscuous lanthipeptide or ranthipeptide synthetases. Despite these initial advances, the logical extension of this idea, that is, leveraging multienzyme biosynthetic pathways for the de novo discovery of compounds with natural product-like structural complexities, remains an unsolved challenge. Such applications demand not only a broad substrate scope for all participating enzymes, but also the ability of the biosynthetic pathway to maintain its integrity in a combinatorial context. That is, the enzymes must cooperate to avoid the formation of complex product mixtures from a single substrate for most sequence-randomized precursor peptides in a combinatorial library.

The significance of these challenges is highlighted by the outcomes of thiopeptide reengineering efforts to date. Known as one of the most structurally complex RiPPs, thiopeptides are assembled by multiple biosynthetic enzymes, which carefully orchestrate the installation of post-translational modifications (PTMs) into thiopeptide precursor peptides. The enzymes usually manage to avoid multiple points of potential competition (e.g., Ser/Thr residues of precursor peptides can be either cyclized to oxazolines by promiscuous YcaO cyclodehydratases or dehydrated by Ser/Thr dehydratases) and furnish homogeneous natural products. Over a hundred known thiopeptides are renowned for their strong anti-
bacterial activities that overcome existing antibiotic resistance, and as such, much effort has been dedicated to thiopeptide engineering.

However, such endeavors usually found it difficult to significantly alter the natural product structures, as most single-point mutations either abrogated biosynthesis or led to the formation of product mixtures, an observation which underscores the challenges of modifying multienzyme assembly lines.

A notable exception to these results is bioengineering of lactazole A, a cryptic thiopeptide from *Streptomyces lactacystinaeus* (Figure 1a). 

Previously, we reconstituted the biosynthesis of lactazole A in vitro by combining the flexible in vitro translation (FIT) system—utilized for LazA precursor peptide expression—with five recombinantly produced lactazole biosynthetic enzymes (LazBCDEF). This platform, termed FIT-Laz, enabled simplification of lactazole biosynthesis that resulted in the identification of LazA<sub>min</sub>, a precursor peptide, in which only seven amino acids (Ser1, Trp2, Ser10, Ser11, Ser12, Cys13, and Ala14) are required for the maturation to macrocyclic lactazole-like structures. Enabled by the unique promiscuity of Laz enzymes, these results suggest that the construction and screening of combinatorial thiopeptide libraries are feasible. Nevertheless, the development of such a platform would require addressing an array of associated challenges: primarily, the aforementioned issue of biosynthesis integrity, structure deconvolution of hit compounds, and at-scale production of the discovered thiopeptides.

Here, we report the development of an mRNA display-based screening platform that enables the construction and screening of large (≥10<sup>12</sup> unique compounds) combinatorial libraries of lactazole-like thiopeptides. We reengineered lactazole biosynthesis to improve the maturation efficiency and homogeneity of the resulting products and developed library construction protocols. We then designed, assembled, and screened a large combinatorial library of thiopeptides against Traf2- and NCK-interacting kinase (TNIK), a protein implicated in several forms of cancer, to identify a series of compounds acting as potent and selective kinase inhibitors (the best compound, TP15, had K<sub>i</sub> of 3 nM). Two X-ray crystal structures of TNIK/thiopeptide complexes revealed how multiple PTMs of the discovered thiopeptides promote their folding and contribute to the interaction with the target protein. TP15 inhibited TNIK in HCT116 cell line assays, indicating that like many natural thiopeptides, de novo discovered compounds can access intracellular targets. Overall, the established platform opens new possibilities to utilize pseudo-natural thiopeptide scaffolds in early drug discovery.

**RESULTS AND DISCUSSION**

**Construction of the Selection Platform.** The biosynthesis of lactazole A commences with the formation of azolines (catalyzed by LazDE), their dehydrogenation to azoles (thiazoles, Thz, and oxazoles, Oxz; conducted by LazF, dehydrogenase domain), and dehydroalanine installation (Dha; a two-step process executed by LazBF) into the C-terminal portion of LazA (core peptide).

The remaining enzyme, LazC, then utilizes Dha1 and Dha12 in the core peptide to perform macrocyclization and eliminate the N-terminal recognition sequence (leader peptide) as the C-terminal linker.
terminal amide (leader-NH$_2$). LazA$^{\text{min}}$ is constructed from LazA by mutating out four modifiable amino acids dispensable in the macrocyclization process (Ser4, Cys5, Ser6, and Cys7), leading to a biosynthetic pathway composed of 11 chemical reactions. The broad substrate scope of all Laz enzymes enables randomization of the seven internal residues in the LazA$^{\text{min}}$ core (positions 3–9), but the low diversity of the resulting combinatorial library (∼10$^9$ peptides) would not take advantage of the throughput offered by mRNA display (routine screening >10$^5$-member libraries). Therefore, we designed an mRNA library v.t.2 by inserting additional 3 or 4 amino acids into the LazA$^{\text{min}}$ core for a total of 10 or 11 randomized residues and the combined theoretical diversity of 9 × 10$^{13}$ peptides (Figure 1b). Because Laz enzymes show some preference for amino acids adjacent to the modification sites, positions 3 and 12/13 were only partially randomized (encoded by NBK rather than fully randomized NNK codons) to increase the chance for complete maturation. Notably, library v.t.2 contained Ser/Thr/Cys, that is, the residues that can be modified by the enzymes, in the random region, thereby opening a chance for the installation of additional PTMs inside the macrocycle. With these designs, we envisaged that the standard combination of the FIT system and mRNA display protocols could be utilized to prepare mRNA-barcoded v.t.2 precursor peptides which could be converted to a genetically encoded library of lactazole-like thiopeptides upon the treatment with Laz enzymes.

However, when we treated six randomly chosen v.t.2 library peptides with LazBCDEF, complex product mixtures formed in 5 out of 6 cases (Figures S1–S6). LC/MS analysis of the reaction products revealed incomplete modification, formation of various linear shunt products and mixtures of thiopeptides as the main sources of heterogeneity (see Figure S7 for the breakdown of major products). The many identified points of potential failure had both enzymatic (for instance, linear shunt product formation due to LazBF-mediated conversion of Ser14/15 to Dha rather than the LazDEF-catalyzed cyclodehydration as designed) and nonenzymatic (lantipeptidyl-like cyclization of Cys inside the random region to Dha1) origins. These results reiterated the notion that for multi-enzyme biosynthesis catalytic promiscuity is not the only requirement for efficient library construction, and the innate cooperativity of Laz enzymes is insufficient to ensure clean thiopeptide biosynthesis in a library format. Because the deconvolution of product mixtures and unambiguous structural annotations for individual components pose considerable challenges, we set out to redesign the enzymatic treatments to improve the homogeneity and efficiency of the maturation process. Based on the aforementioned analysis of side-product distributions, we separated the azoline and Dha formation stages to mitigate the extent of enzyme competition and added an iodoacetamide alkylation step to minimize nonenzymatic reactions between thiols and Dha. Thus, the reengineered maturation protocol employed a 3 h treatment of v.t.2 precursor peptides with LazDEF, followed by iodoacetamide alkylation and 8 h LazBCF incubation steps (Supporting Information S2.2). This procedure substantially improved the efficiency and purity of the maturation process in every case as evident from LC/MS analysis (Figures S1–S6). To further increase the fraction of thiopeptides in the library, we decided to include streptavidin pulldown during the library construction process (Figure 1c). Aimed at removing shunt products and partially modified precursors ("linear forms"), this step utilizes in vitro genetic code reprogramming to install N$^\text{6}$-biotin-Phe as the translation initiator. LazC cleaves the leader peptide during the last step of maturation, thus removing the biotin-Phe tag from the constructs, whereas all linear forms remain biotinylated and can be selectively eliminated from the library prior to selection.

**Selection of Thiopeptides Targeting TNIK.** Next, we performed an affinity selection of pseudo-natural thiopeptides against TNIK as a proof-of-concept of the de novo discovery platform. TNIK belongs to the Ste20 family of Ser/Thr protein kinases, and its kinase activity is linked to tumorigenesis in Wnt-driven colorectal cancers and in lung squamous cell carcinomas. Several reports confirmed the therapeutic efficacy of TNIK inhibition, and therefore, selective TNIK inhibitors could serve as promising anticancer drug candidates. A large thiopeptide-mRNA/cDNA library (initial diversity: 10$^{14}$ mRNA molecules; v.t.2 design) was prepared according to the aforementioned techniques, and the binding species were isolated by pulldown against the biotinylated kinase domain of human TNIK immobilized onto streptavidin M280 Dynabeads (Supporting Information S2.1 and S2.2). Genetic code reprogramming to install biotin-Phe and the associated streptavidin pulldown were not performed in round 1. Counterselection, which consisted of three successive incubations of the library with M280 Dynabeads without the target protein, also commenced at round 2.

Both the absolute amount of isolated cDNA and the ratio between cDNA recoveries for the TNIK pulldown and counterselection fractions increased from round to round as measured by qPCR (Figure 2a), which indicated a steady enrichment in binding species over the course of the selection. In total, we performed five rounds of affinity selection. Next generation sequencing of cDNA and the following analysis of the random insert regions revealed that the library converged to ∼15 distinct families, each characterized by 4- to 8-residue motifs (Figure 2b, Table S3). The three main families [x(F/Y)xYKxLxxSG, WxxxxxYGC(T/S), and WSxxxNxGxYM] comprised ∼60% of the total sequencing reads, and the most abundant sequence, TPP1, accounted for 5% of the library. Altogether, the selection yielded an abundance of candidates for testing. We chose 15 precursor peptides (TPP1-1S; Figure 2b) for further study.

First, we investigated whether the selected sequences undergo conversion to thiopeptides under the selection conditions (Figure 2c). To this end, in vitro-translated TPP1-15 were treated with Laz enzymes to recapitulate the display conditions, and the reaction outcomes were analyzed by LC/MS. Chemical structure assignments of the resulting products were guided by mass shifts, HPLC retention times, and the available substrate specificity data for Laz enzymes (Supporting Information S2.5). All tested precursors underwent efficient maturation to thiopeptides as judged by the formation of leader-NH$_2$, which was the major reaction product in every case (Figure 2c). Modification of 11 constructs yielded a single major thiopeptide product, despite the presence of potentially modifiable Ser/Thr/Cys residues in the random regions of 13 peptides. Notably, LazBF cleanly dehydrated Ser11-Ser12 in TPP1 and TPP2 to Dha11-Dha12, and LazDEF converted Cys13 to Thz13 in TPP15.

In a separate experiment, we utilized a qPCR-coupled mRNA display assay to qualitatively assess the binding affinity of TPP1-15 toward TNIK (Figure S12). We found that all selected sequences had an affinity for the target protein but...
only after the treatment of precursors with Laz enzymes. This result suggests that the macrocyclic thiopeptide structures, and not their linear epitopes, are responsible for binding to TNIK. Together, these results prompted us to focus on 11 candidate sequences, primarily those modified to a single thiopeptide, for further characterization.

**Synthesis and Biochemical Characterization of the Identified Thiopeptides.** To access the target compounds, we sought a general and scalable synthetic strategy. Despite the high efficiency of FIT-Laz, which can yield up to 7.5 pmol/μL lactazole A and analogs, the platform primarily excels as a tool for rapid analysis of thiopeptide biosynthesis on an analytical scale (∼10−102 pmol of material), whereas preparative production is cost prohibitive. Therefore, we opted for a fully synthetic approach which included a convergent synthesis of the key pyridine-bisazole-containing amino acid and solid-phase peptide synthesis for chain assembly as detailed in a separate report.

Linear peptides were then macrocyclized in solution, and in the last step, the oxidation of Se-alkylated selenocysteine derivative-furnished Dha-containing thiopeptides. To simplify the synthesis, the original selection hit structures were modified by truncating the tail and by introducing an Oxz14/15 to Thz14/15 mutation (Figure 3a). Overall, this approach enabled modular and scalable access to lactazole-like thiopeptides, enabling us to prepare all target compounds (TP1, 3, 4, 6−8, and 11−15 derived from the eponymous TPP precursor peptides) in multi-mg quantities.

To characterize the binding affinities of the discovered thiopeptides to TNIK, we employed surface plasmon resonance (Supporting Information S2.7). We found that 9 out of the 11 compounds strongly bound the target protein as evidenced by single- or double-digit nanomolar dissociation constants (K_D; Figures 3b and S13). The most potent compound, TP15, had a K_D of 1.2 nM. TP15 was also the strongest inhibitor in an ADP-Glo-based kinase inhibition assay performed with MSTtide as the consensus TNIK substrate (Supporting Information S2.8), with an IC_{50} value of 0.014 μM (Figures 3b, S14). In total, 10 thiopeptides inhibited TNIK, most with IC_{50} in the single-digit μM range, while TP1 (IC_{50} = 0.13 μM) and TP8 (IC_{50} = 0.6 μM) stood out as sub-μM inhibitors. According to the surface plasmon resonance results, the compounds (TP1, 8, 14, and 15; the rest not tested) also were bound TNIK in the presence of a saturating concentration of ATP (1 mM; 15-fold higher than K_M; Figures S13 and S15), suggesting that the thiopeptides
target an interface other than the ATP binding site. Consistent with this data, kinetic analysis of TNIK inhibition by TP15 established that the compound acts as a substrate-competitive inhibitor of the enzyme, with an inhibitor constant (\(K_i\)) of 3 nM (Figures 3c, S16).

The size (>500 kinases) and redundancy of the human kinome make the discovery of selective inhibitors a challenging task. In a kinase selectivity profiling experiment performed using a panel of diverse human kinases (67 proteins; Supporting Information S2.9 and Table S4), TP1 and TP15 demonstrated good selectivity for the target enzyme. At 1 μM concentration, TP15 had a standard selectivity score [S(0.1)] of 0.03, pointing to the selective action (Figure 3d). In addition to TNIK, TP15 also inhibited two other Ste20 family kinases, mammalian sterile twenty-like 1 (MST1) and, to a lesser extent, lymphocyte-oriented kinase (LOK). TP1 was even more selective [S(0.1) = 0.01], although a higher concentration (10 μM) was required to completely inhibit TNIK (Figure S17). Taken together, these data indicate that the selection afforded potent and selective inhibitors of the target kinase.

Next, we explored the metabolic stability of the five most active thiopeptides (TP1, 4, 8, 14, and 15). The compounds were incubated with human serum at 37 °C, and the amounts of the remaining analytes at various timepoints were quantified by LC/MS against an internal standard (Supporting Information S2.10). TP4 and TP8 showed good stabilities, with half-lives (\(\tau_{1/2}\)) of 88 and 14 h, respectively, whereas TP1, TP15, and in particular, TP14 were less stable (Figures 3b, S18). We then investigated whether the half-life of the compounds is affected by the reactivity of Dha residues, which can potentially conjugate to various serum thiols. To this end, we incubated TP1, 14, and 15 with increasing concentrations of glutathione and analyzed reaction outcomes by LC/MS. We found that Dha11 and Dha12 in TP1 reacted with glutathione but the 2-(1-aminovinyl)-thiazole moiety present in every peptide (Dha13/14) did not. Accordingly, TP14 and TP15 were stable (>95% remaining) in the presence of 10 mM glutathione after 24 h (Figure S19), and as such, their short half-lives in serum stem primarily from proteolytic degradation. Indeed, several products of TP14 and TP15 proteolysis were observed by LC/MS (Figures S20 and S21).

**Figure 3.** Biochemical characterization of the synthesized compounds. (a) TP15 as the prototypical lactazole-like thiopeptide; the chemical structures of other compounds can be inferred by analogy based on their insert sequences. The common structural element is highlighted in grey. (b) Binding affinity to TNIK (\(K_D\) values) and its inhibition (IC\(_{50}\)) by the discovered thiopeptides. Half-life times in human serum (\(\tau_{1/2}\)) are also shown. The values were obtained via non-linear regression of experimental data and are reported with standard errors of the fits. [a]: an S highlighted in green indicates a Dha residue; C in red—S,SGln (the product of iodoacetamide alkylation of Cys); C in blue—Thz; [b]: no measurable binding/inhibition; [c]: n. d.: not determined. [d]: for TP8, binding was measured in the presence of 1 mM ATP. [e]: for TP15, \(K_i\) was determined as 3 nM (Figure S16). (c) Lineweaver–Burk plot of the inhibition of TNIK-mediated MSTtide phosphorylation by TP15. The thiopeptide acts as a substrate-competitive inhibitor of the enzyme. (d) Color-coded kinase selectivity profiling outcomes for TP15 against a panel of 67 human kinases. Numerical values are listed in Table S4. The compound shows good selectivity for the target enzyme. Analogous data for TP1 is summarized in Figure S17.

**Structural Analysis of TNIK Inhibition by Thiopeptides.** To understand the structural basis for the interaction, we determined X-ray cocrystal structures of TNIK·TP1 and TNIK·AMPPNP·TP15 complexes to 2.1 and 2.3 Å resolution, respectively (Figures 4, S22 and S23). Consistent with the biochemical characterization, the structural analysis indicated that both thiopeptides engage with the substrate binding site of the enzyme (Figure S24). Substrate mimicry is especially apparent for TP15. The thiopeptide makes extensive contacts with the protein, creating a nearly 1570 Å\(^2\) binding interface that incorporates both N- and C-lobes of TNIK (Figure 4a). The triheterocyclic core of TP15 (Thz15-Py1/16-Thz17) is...
Figure 4. Structural analysis of TNIK inhibition by TP15. See Figure S22 for analogous data for TP1. (a) Overview of the X-ray crystal structure of the TNIK-AMPPNP-TP15 complex (pdb 7xpr). TNIK protein surface is shown in grey; TP15 and AMPPNP are displayed as ball and stick models. The thiopeptide engages with the substrate-binding region of TNIK. (b) The interaction between αG-helix of TNIK and the heterocyclic fragment of TP15. Atoms within 4 Å distance are connected with blue dotted lines. (c) The interaction between αC-helix of TNIK and Arg5, 7, 9 of TP15. The thiopeptide makes multiple ionic contacts with the protein. (d,e) Folding of TNIK-bound TP15 (d) and TP1 (e). The protein and amino acid side-chains of the ligands are omitted for clarity. Comparison of the structures reveals that lactazole-like thiopeptides can fold into β-hairpins in two different ways, as determined by the orientation of Py1 carbonyl moiety.

stacked against the N-terminal side of the αG-helix, with Thz15 facing and partially occupying the P-2 site (Figure 4b). The steric complementarity of the polyaromatic structure to the protein surface is reminiscent of how naturally occurring thiopeptides interact with their targets.\(^{32,54}\) TP15’s Thr3 and Ile4 reside in the P and P + 1 pockets, respectively, and Arg5, 7, and 9 interact with the E\(^{61}\)DE\(^{65}\) motif located on the N-terminal side of the αC-helix via multiple ionic contacts (Figure 4c). As such, the Thr3-Ile4-Arg5 motif of TP15 resembles a prototypical TNIK substrate.\(^{48}\) TP15-bound enzyme adopts a catalytically active conformation: the R-spine and the Lys54/Glu69 salt bridge are properly assembled, while the αC-helix moves inward (Figure S25a–d). It appears then that the phosphate transfer is primarily obstructed by the half-closed Gly-rich loop, particularly by Thr35, which dislocates the γ-phosphate of ATP and sterically occludes it from the substrate (Figure S25e–g). The unique inhibition mode revealed by our analysis suggests a rational structure-guided development of a new class of kinase inhibitors.

The structure also reconciles TP15’s selectivity profile. The architectures of MST1 and LOK bear a striking resemblance to that of TNIK,\(^{34}\) especially around the N-terminal side of the αG-helix, P-2, P, and P + 1 sites (Figure S26). MST1 further features a near identical substrate discrimination motif in its αC-helix (\(^{60}\)Dx\(^{62}\)E), which helps in explaining the potency of MST1 inhibition by TP15 (IC\(^{50}\) = 120 nM; Figure S27).

TP15 folds into a twisted β-hairpin which stems from the polyaromatic scaffold (Figure 4d). The resulting structure contains 4 intramolecular backbone and 4 side-chain hydrogen bonds, leading to a compact fold. TP1 also contains a β-turn facing the aromatic scaffold, but the two folds differ in many ways (Figure 4e). Whereas TP15 adopts a “Py1 carbonyl” out” configuration that, in combination with Thz13, helps with the antiparallel positioning of the strands; the “Py1 carbonyl in” in TP1 leads to a twisting of the Dha11-Dha12-Gly13 motif. As a result, despite similar positioning of Thz15-Py1/16-Thz17 in the complexes, the relative strand orientations are inverted. In TP15, the amino acid interacting with P + 1 pocket (Ile4) is located on the N-terminal strand, while for TP1, the analogous residue (Leu9) is on the C-terminal side. Altogether, these data indicate that v.t.2 library thiopeptides can access a conformationally diverse space, aided by post-translationally installed non-proteinogenic elements.

**Cellular Activity of the Discovered Thiopeptides.** All natural thiopeptides with a confirmed mode of action invariably inhibit intracellular targets.\(^{19,22}\) In the last series of experiments, we ascertained whether our compounds could also access the intracellular target protein (TNIK is localized to the cytosol and the cell nucleus) in human cell models. First, we prepared four chloroalkane-tagged thiopeptide derivatives (TP1-ct, TP8-ct, TP14-ct, and TP15-ct) and performed a chloroalkane penetration assay to measure the extent of their cytosolic uptake (Supporting Information S2.11).\(^{53}\) We found that three out of four tested compounds (TP1-ct, TP14-ct, and TP15-ct) internalized into HEK293H cells at single-digit µM concentrations, which is comparable to Tat, a well-known cell-penetrating peptide (Figure 5a).\(^{56,57}\) Based on these results, we decided to study the cellular inhibition of TNIK, focusing on the most active thiopeptide, TP15. As judged by immunoblotting (Supporting Information S2.12), a 24 h treatment of HCT116 colon carcinoma cells with 20 µM TP15 decreased the level of TNIK, pSer764, the product of TNIK autophosphorylation,\(^{39,42}\) similar to a small molecule inhibitor control, NCB0846 (Figure 5b).\(^{52}\) At the same time, while NCB0846 downregulated the overall expression of TNIK both at the protein and mRNA levels (as measured by RT-qPCR; Figures 5b, S28), TP15 did not, and it only affected TNIK pSer764. Consistent with the TNIK inhibition by NCB0846,
the RT-qPCR experiment (Supporting Information S2.13) also found that the treatment of HCT116 cells with TP15 suppressed the transcription of AXIN2 and MYC mRNA, two classic targets of the Wnt signaling pathway, \(^{58,59}\) in a concentration-dependent manner (Figure 5c). The protein levels of c-Myc and Axin2 also decreased following a 24 h incubation with 20 \( \mu M \) TP15 (Figure 5b). These results suggest that de novo discovered thiopeptides, similar to their natural counterparts, show promise for targeting intracellular proteins.

### CONCLUSIONS

The nature of RiPP biosynthesis suggests a plethora of combinatorial bioactivity reprogramming approaches so long as the underlying biosynthetic machinery is catalytically promiscuous. Accordingly, the degree of substrate tolerance for RiPP enzymes frequently serves as the main criterion when determining a pathway’s amenability for reengineering. We argue that the issue of biosynthesis integrity—as determined by the pathway’s ability to produce well-defined compounds— is equally important. Formation of complex mixtures from a single precursor peptide limits the practical utility of RiPP bioengineering because product heterogeneity complicates the annotation of active species, their purification, and structural determination. In fact, screening of combinatorial libraries of RiPP analogs frequently leads to the identification of product mixtures as active species, \(^{14,17}\) and to the best of our knowledge, no such effort has produced fully characterized compounds. These issues evidently become exacerbated with increasing the number of participating enzymes, as indicated by the past thiopeptide reengineering reports. \(^{23-31}\) Our study undertakes thiopeptide activity reprogramming using five biosynthetic enzymes, LazBCDEF, the most complex pathway reengineered in this manner to date. Despite the complexity, we largely avoided the aforementioned issues: the full control over the course of lactazol biosynthesis granted by the FIT-Laz system enabled its further reengineering to alleviate the formation of product mixtures, and the chemical synthesis of thiopeptides circumvented the purification and structural characterization challenges. As a result, our effort produced 10 structurally unambiguous kinase inhibitors from the 15 candidates identified in the selection. Altogether, we believe that this approach (namely, in vitro biosynthesis reconstitution, reengineering, and selection combined with chemical synthesis) can be applied to greatly expand the repertoire of RiPPs amenable to combinatorial workflows. For lactazol bioengineering, further natural product likeness and hence the molecular complexity of compounds may be attained by combining FIT-Laz with promiscuous enzymes from other pathways or by incorporating additional noncanonical amino acids via genetic code reprogramming.

In terms of the compound diversity, the thiopeptide library constructed here is the largest of its kind, not only among RiPPs but also among other natural product analogs. Likely related to the library size, the identified compounds are considerably more potent than common with other bioactivity reprogramming approaches (with an obvious caveat that the target proteins are different). The privileged nature of thiopeptide PTMs may also contribute to the success of the selection: as our structural analysis indicates, for both TP1 and TP15, the Thz15-Py1/16-Thz17 moiety plays a dual role by interacting with the protein and by acting as the template to promote peptide folding. The high potency of TP15 combined with its intriguing mechanism of inhibition suggests that it may be utilized for the development of therapeutic kinase inhibitors. Notably, TP15 achieved these properties without structural optimization—the compound was studied as identified from the selection—and the TNIK-AMPPNP-TP15 structure provides ample opportunities for further structure-guided design.

Efficient cytosolic internalization of TP1, 14, and 15 at concentrations comparable to that of Tat suggests that de novo-discovered lactazol-like thiopeptides may be employed to target intracellular proteins. At present, the exact mechanism of cell entry for thiopeptides (both the natural products and our compounds) remains unknown. Perhaps, the structural rigidity and hydrophobicity conferred to the structures by the heterocyclic core play a role in the process. Although additional studies will be required to substantiate this discussion, our results suggest further exploration of pseudo-natural thiopeptides for drug discovery applications.
Experimental procedures and results are summarized in Supporting Information and Supplementary Tables. X-ray crystal structures were uploaded to Protein Data Bank (pdb entries 7xzq [TNIK-TP1] and 7xsr [TNIK-AMPPNP-TP15]). Other data are available from the corresponding authors upon reasonable request.

**ACKNOWLEDGMENTS**

This work was supported by KAKENHI (JP20K15407 to A.V.; JP16H06444 to Y.G., H.O., and H.S.; Specially Promoted Research, JP20H05618 to H.S. and T.S.; JP19K22243, JP20H02866 to Y.G.; JP21H04712 to H.O.) from the Japan Society for the Promotion of Science (JSPS). The X-ray structural analysis was supported by Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from Japan Agency for Medical Research and Development (AMED) under grant number JP19am0101070 (support number 1698). We are grateful to Prof. Joshua Kritzer for sharing the expertise on the chloroalkane penetration assay and to Prof. Kakimi and Prof. Nagaoka for assistance with flow cytometry. We also thank the beamline staff at BL32XU of SPring-8 for their automatic X-ray diffraction data collection and analysis.

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**Notes**

The authors declare the following competing financial interest(s): The authors declare the following competing interests: A.V., Y.Z., J.C., Y.G. and H.S. are listed as co-inventors on a provisional patent application related to this work. A.V., Y.G., H.O. and H.S. are also listed as co-inventors on the PCT/JP2019/038431 patent pertaining to lactazole engineering. Other authors declare no competing interests.
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