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Bio-inspired engineering of thiopeptide antibiotics advances the expansion of molecular diversity and utility

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Thiopeptide antibiotics, which are a class of sulfur-rich and highly modified peptide natural products, exhibit a wide variety of important biological properties. These antibiotics are ribosomally synthesized and arise from post-translational modifications, exemplifying a process through which nature develops the structural complexity from Ser/Thr and Cys-rich precursor peptides. Following a brief review of the knowledge gained from nature in terms of the formation of a common thiopeptide scaffold and its specialization to individual members, we highlight the significance of bio-inspired engineering, which has greatly expanded the molecular diversity and utility of thiopeptide antibiotics regarding the search for clinically useful agents, investigation into new mechanisms of action and access to typically 'inaccessible' biosynthetic processes over the past two years.

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Introduction

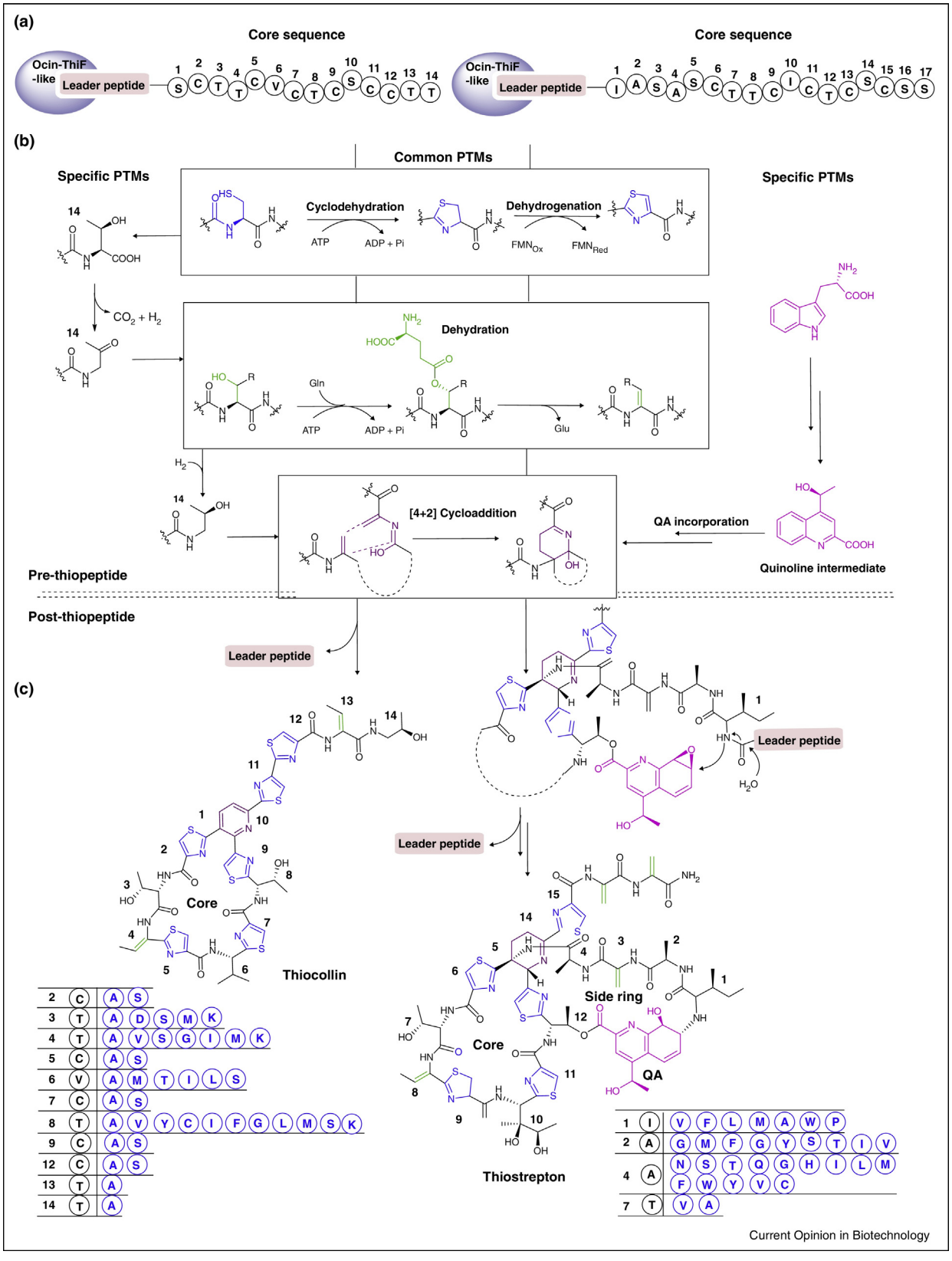
Over the past decades, peptide natural products (NPs) with ribosomal origin have been a focus in the discovery of new biosynthetic mechanisms [1]. Increasing evidence indicates that post-translational modifications (PTMs) of ribosomally synthesized precursor peptides are comparable to non-ribosomal peptide synthetases in terms of the creation of structurally complex molecules [2–4]. A precursor peptide typically consists of an N-terminal leader sequence and a C-terminal core sequence (Figure 1a). A myriad of PTMs can be applied, in a manner either dependent or independent of the former sequence, to transform the latter sequence into mature product(s) (Figure 1b). Although the building blocks are limited to 20 proteinogenic amino acids, in contrast to a much wider array of substrates found in the biosynthesis of non-ribosomal peptide NPs [5], the sequences of precursor peptides and the associated enzyme-processing strategies have been shown to be highly variable and evolvable in the formation of various ribosomal peptide NPs [6].

One example comes from thiopeptide antibiotics [7–10], a growing family of sulfur-rich peptide NPs that are ribosomally synthesized and post-translationally modified (Figure 1c). These antibiotics possess a wide variety of biological properties, e.g., anti-infection, anticancer and immunosuppression, and are beneficial to humans largely because of their highly functionalized unusual architectures, which share a macrocyclic peptidyl core that contains a six-membered heterocycle domain central to multiple azoles and dehydroamino acids [11]. Recent studies revealed a wide distribution of thiopeptide-encoding sequences in the genomes of human microbiota [12], generating interest in the roles played by related products in microbe-host interactions. Derivatization efforts has attracted considerable attention of molecular engineering to further expand the chemical spaces of thiopeptide antibiotics, improve their biological activities and overcome physical disadvantages [13]; however, the accessibility and efficiency of chemical synthesis are often impeded by the structural complexity of these compounds. The ribosomal origin of thiopeptide antibiotics was established in 2009 [14–18], garnering appreciation for the mechanisms that nature employs to develop various PTM strategies and obtain individual thiopeptide members from Cys and Ser/Thr residue-rich precursor peptides. This appreciation has recently motivated rational applications of various technologies for structural diversification (as discussed below), resulting in a number of thiopeptide analogs, either expected or unexpected.

Formation of a common thiopeptide framework and its specialization in nature

Thiopeptide antibiotics structurally appear to be the macrocyclic variants of goadsporin-like NPs, each of which derives a six-membered central heterocycle domain from a linear peptide possessing both azol(in)es and dehydroamino acid residues [19]. The in vitro biosynthesis of the thiopeptide member thiomuracin was successful [20••], benefiting from the recent knowledge regarding

Figure 1


(a)
Core sequence
Ocin-ThiF-like
Leader peptide
Core sequence
Ocin-ThiF-like
Leader peptide
(b)
Common PTMs
Specific PTMs
Cyclodehydration
ATP
ADP + Pi
Dehydrogenation
FMNOx
FMNRed
Specific PTMs
Dehydration
Gln
ATP
ADP + Pi
Glu
[4+2] Cycloaddition
QA incorporation
Quinoline intermediate
Pre-thiopeptide
Post-thiopeptide
Leader peptide
Leader peptide
Leader peptide
(c)
Core
Thiocollin
Side ring
Core
QA
Thiostrepton
Current Opinion in Biotechnology

cyclodehydratases in the biosynthesis of azol(in)e-containing microcins and cyanobactins [21,22], dehydratases in the biosynthesis of dehydroamino acid-involving lantibiotics [23] and, particularly, the enzymes responsible for Diels-Alder-like [4 + 2] cycloaddition reactions [24,25]. Fabricating the structural features of thiopeptides using a logical assembly of the above-mentioned enzymatic activities demonstrates the idea that in addition to the precursor peptide-encoding gene, the biogenesis of a common thiopeptide framework contains a minimum of six conserved PTM genes (Figure 2). These genes code for 1) an Ocin-ThiF-like protein responsible for engaging the precursor peptide [26], 2) a YcaO-like superfamily protein and a flavoprotein for Cys/Ser-residue processing through phosphorylation-based cyclodehydration and subsequent dehydrogenation to produce azoles [27], 3) a pair of proteins with $tRNA^{Glu}$-dependent glutamylation and elimination activities for Ser/Thr-residue dehydration to yield dehydroamino acids [28], and 4) a unique Diels-Alderase-like protein for intramolecular cross-bridging to furnish the central heterocycle domain (Figure 1b) [29]. According to these genetic characteristics, many related biosynthetic gene clusters were mined from the bacterial strains that were previously unknown to be the thiopeptide producers [30].

In addition to common PTMs, the constitution of the thiopeptide family, which includes over 100 natural members, relies on the sequence permutation of precursor peptides and the combination with specific PTMs that are necessary for the individualized treatment of each precursor peptide [31]. A comparative analysis of the currently available biosynthetic gene clusters supports the unifying theme in which nature develops diversity (Figure 2). The incorporation of different specific PTM elements into a minimum of thiopeptide biogenesis results in the variable functionalization of a thiopeptide framework, e.g., the decoration of the central domain and the macrocyclic core system, the fabrication of a side-ring system and the tailoring of the C-terminal extended side chain [32–36]. Intriguingly, the specialization can proceed before or after the formation of a thiopeptide-characteristic scaffold, and many specific PTMs are interdependent on common PTMs [31]. In the pathway of the monomacrocyclic member thiocillin, the oxidative decarboxylation of the C-terminal Thr residue of the precursor peptide is a pre-thiopeptide PTM that immediately follows the formation of thiazol(in)es (Figure 1b). This step is indispensable for Ser/Thr-residue dehydration and subsequent intramolecular cyclization to furnish a thiopeptide framework [37•]. In the pathway of the bi-macrocyclic member thiostrepton, the formation of a quinaldic acid (QA) moiety and its incorporation into the precursor peptide are evidently essential for the construction of the thiopeptide scaffold (Figure 1b). However, the conjugation of QA and the N-terminus of the core peptide sequence was recently confirmed to be a post-thiopeptide PTM during the formation of the side-ring system of thiostrepton [38••].

Diversity-oriented biosynthesis by genetic engineering of precursor peptides

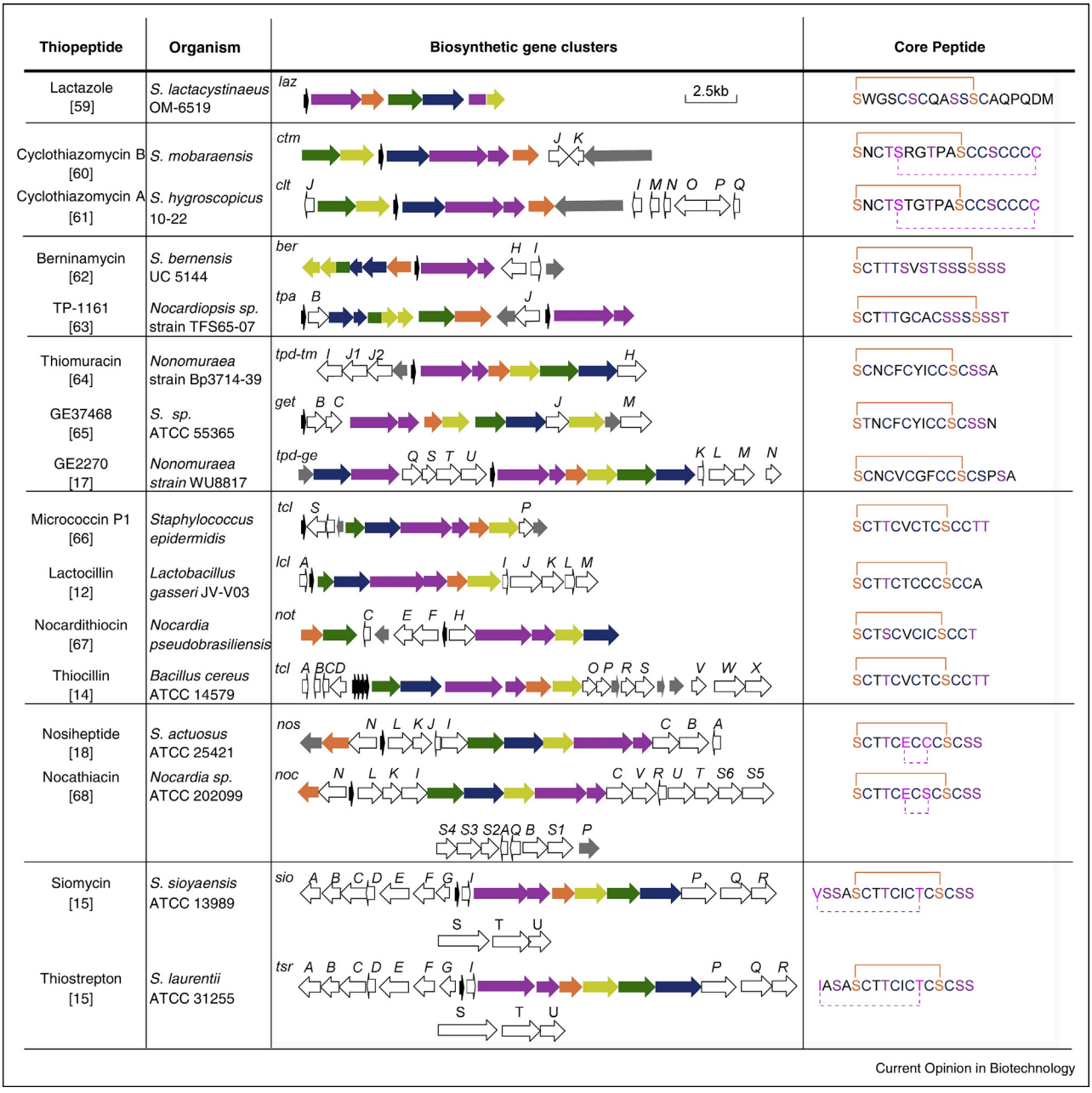
Revealing the ribosomal origin of thiopeptide antibiotics lays the foundation for the structural diversification of peptidyl skeletons by sequence engineering of their precursor peptides using microbial genetics approaches [9]. This diversification, which has produced over hundreds of new analogs, is of significant interest in constructing a thiopeptide-like NP library to search for new drug leads and to evaluate the overall PTM capacity of thiopeptide biosynthetic machineries with respect to the variation of precursor peptides.

Facile preparation of thiopeptide variants considerably simplifies systematic structure-activity (SAR) analysis, which, in fact, is a challenge in current chemical synthesis-based approaches due to the structural complexity of the molecules. Mechanistically different from current chemotherapeutics targeting the bacterial ribosome, many thiopeptides (including thiocillin and thiostrepton) are known to bind within a cleft that is located between the L11 protein and the 23S rRNA of the 50S large ribosomal subunit, thereby hindering translation factor binding and subsequent protein synthesis [39]. A majority of the surface buried by the molecule on the ribosome is attributed to the shared macrocyclic core system; however, in this system, the contribution of residue composition to binding affinity requires evaluation. Recently, saturation mutagenesis of the residues within the macrocyclic core of thiocillin was conducted (Figure 1c) [40••], leading to the production of a number of variants, of which 8 were more active than the parent compound against the test strain *Bacillus subtilis*. These variants, either active or inactive, were then subjected to a comparative analysis by computational modeling, revealing that a side chain substitution changes the ring entropy/conformational flexibility, which has a significant impact on molecular binding, thus affecting antibacterial activity [40••].

A similar diversity-oriented biosynthesis has recently been performed for thiostrepton engineering, with a focus on the residues that conjugate the QA moiety within the

(Figure 1 Legend) Biosynthetic origin, pathways and structures of thiopeptide antibiotics, as exemplified by thiocillin (left) and thiostrepton (right). **(a)** Precursor peptides, whose N-terminal leader sequences (yellow) are bound by pathway-specific Ocin-ThiF-like proteins (blue). **(b)** Common PTMs in the formation of a shared thiopeptide scaffold (middle) and specific PTMs for its specialization toward thiocillin (left) and thiostrepton (right), respectively. **(c)** Chemical structures of thiocillin (left) and thiostrepton (right). The thiopeptide-characteristic hallmarks are highlighted in color. The residues of each precursor peptide and their associated substitutions that produced mature variants are listed [40••,41,42,43••,44•,55–58].

Figure 2



Biogenesis for developing the structural diversity and complexity of thiopeptide antibiotics in nature. The biosynthetic gene clusters are composed of the genes coding for precursor peptides (black), the common PTM genes (i.e., for Ocin-ThiF-like proteins (dark green), YcaO-like superfamily proteins (dark blue), flavoproteins (light green), dehydratase pairs (purple) and Diels-Alderase-like proteins (yellow), various specific PTM genes (white) and the accessory genes involved in self-resistance and regulation (gray). (**b**) Core peptide sequences of associated thiopeptide antibiotics. The shared macrocyclic core systems (solid) and the optional side-ring systems (dashed) are indicated. The residues undergoing PTMs to form various structural characteristics are highlighted in color, e.g., blue for azol(in)e, purple for dehydroamino acids, orange for 6-membered central domain, and purple for side-ring construction [59–68].

27-membered large side-ring system (Figure 1c) [41,42,43••,44•]. Saturation mutagenesis of Ile1, Ala2 and Ala4 at the N-terminus of the core sequence resulted in 6, 8 and 16 new thiostrepton variants, respectively, revealing the discrepancy in the permissibility of PTMs to the changes at these positions. In particular, the double mutation of Ile1Val and Ala2Ser in the thiostrepton-producing *S. laurentii* strain allowed the robust production of siomycin, a naturally occurring analog that is more potent than thiostrepton in immunosuppressive and anticancer activities, thereby possessing great potential for medicinal applications [44•]. This synthetic biology approach is promising and alleviates the concern regarding the production of siomycin in *S. sioyaensis*, the original producer that has been shown to be highly resistant to fermentation optimization and genetic manipulation [45]. The vast majority of resultant thiostrepton variants exhibited in vitro translation-inhibitory activity comparable to that of the parent compound; however, they appeared to be divergent in antibacterial activity against various test strains. The crystallization of the 50S large ribosomal subunit in complex with thiostrepton has revealed that the side-ring residues of the molecule, except for QA, are solvent exposed and are not involved directly in ribosome binding [39]. Whether residue variation at these positions has an effect on the ring entropy/conformation flexibility of the macrocyclic core system (as shown in thiocillin studies) through the side-ring system remains to be determined. In contrast, most thiostrepton variants retained 20S proteasome-inhibitory activities, which were suggested to be related to antimalarial and anticancer properties [42,43••].

Incorporation of naturally unavailable building blocks

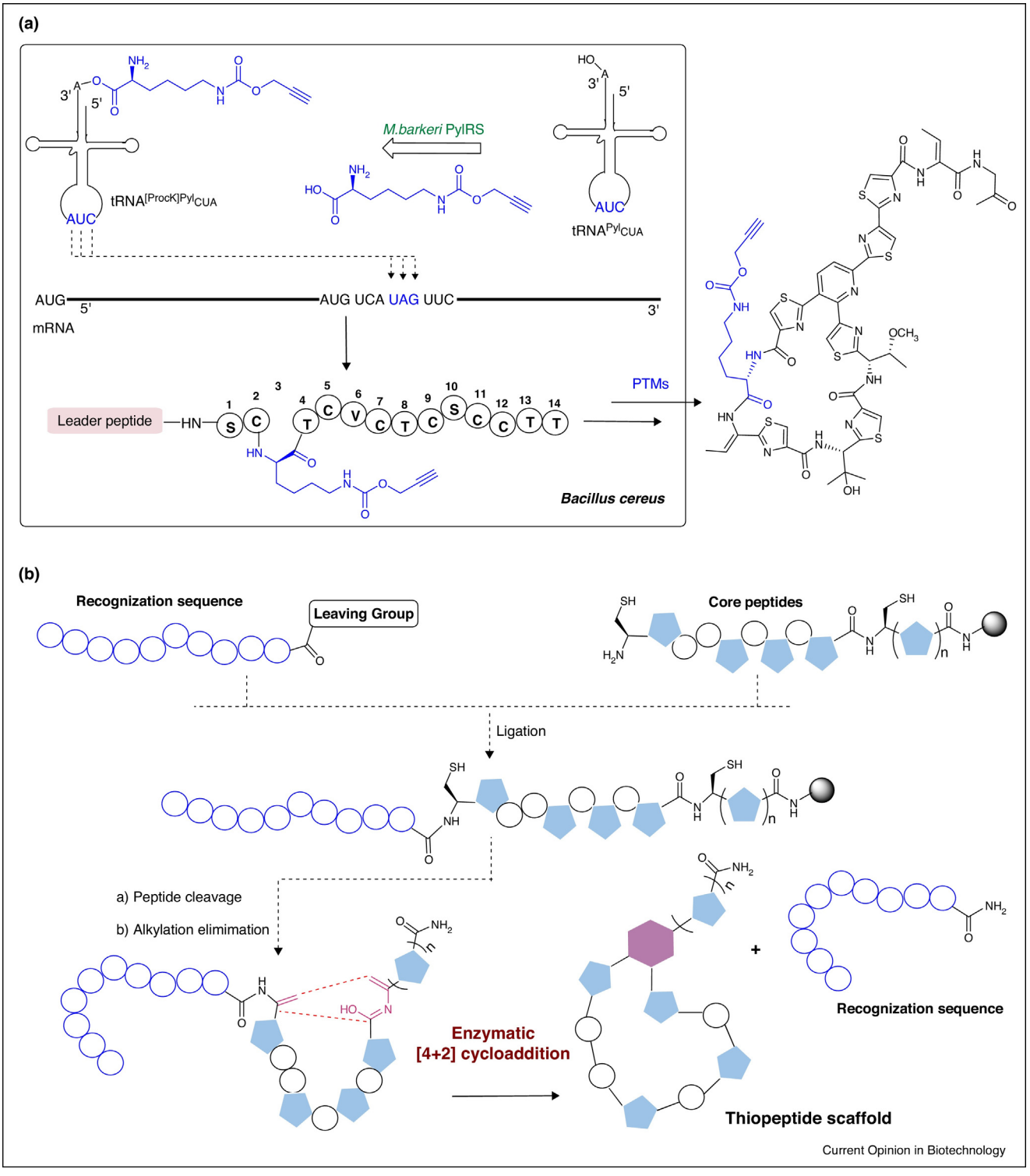
Genetic encoding of noncanonical amino acids (ncAAs) [46], which is an effective approach to expand the building block inventory in bacteria, has been utilized to incorporate naturally unavailable groups into many ribosomally synthesized and post-translationally modified peptide NPs to increase their structural diversity and chemical reactivity. Until recently, thiopeptide engineering using this approach was challenging, because most of the thiopeptide members are produced by Gram-positive bacteria [11], where orthogonal systems composed of nonsense or frameshift suppressor aminoacyl-tRNA synthetases and the associated tRNAs had not been established. The development of an orthogonal amber suppressor aminoacyl-tRNA synthetase/tRNA pair in *Bacillus cereus*, the Gram-positive strain producing thiocillin (Figure 3a), enabled the application of this approach [47••]. Various ncAAs were individually introduced into the sites of the thiocillin precursor peptide that are permissive to substitutions, albeit varying in efficiency, producing tens of new ncAA-bearing mature variants through PTM processing. As expected, the variants with bioorthogonal chemical reactivity were amenable to specific post-biosynthesis modifications, either for further functionalizing the thiocillin scaffold or for developing probes to investigate the biological actions that are less characterized. In fact, genetic incorporation of ncAAs using orthogonal systems has been well established in the Gram-negative host *Escherichia coli*, where reconstituting thiopeptide biosynthetic pathways would be beneficial for the application of this approach to a much wider array of thiopeptide members.

Chemoenzymatic synthesis of thiopeptide variants using the enzymes that catalyze Diels-Alder-like [4 + 2] cycloaddition reactions provides an alternative to introduce synthetic chemical groups into thiopeptide members that share a pyridine-like central domain [48••]. The formation of this heterocycle domain often occurs during the late stages of the biosynthetic pathways [29], thereby allowing for enzymatic cross-bridging of a chemically synthesized and highly modified linear precursor to afford a thiopeptide scaffold. Related Diels-Alder-like enzymes function in the presence of a conserved recognition sequence that is approximately 10 amino acids long, which resides at the C-terminus of the leader part of each precursor peptide. This finding greatly simplifies the process of precursor preparation, which involves the application of solid-phase peptide synthesis (SPPS) to prepare an azol(in)e and dehydroamino acid-containing core peptide and a recognition sequence prior to ligation (Figure 3b). Chemoenzymatic synthesis of thiocillin and thiomuracin variants was examined to test the requirements for the macrocyclization of the core sequences in terms of their composition, size and extent of functionalization [48••]. By exploiting the advances in chemical synthesis, this approach possesses potential for further development to achieve thiopeptide analogs that are not accessible by sole biosynthetic methods.

Target-oriented design and precursor-directed mutational biosynthesis

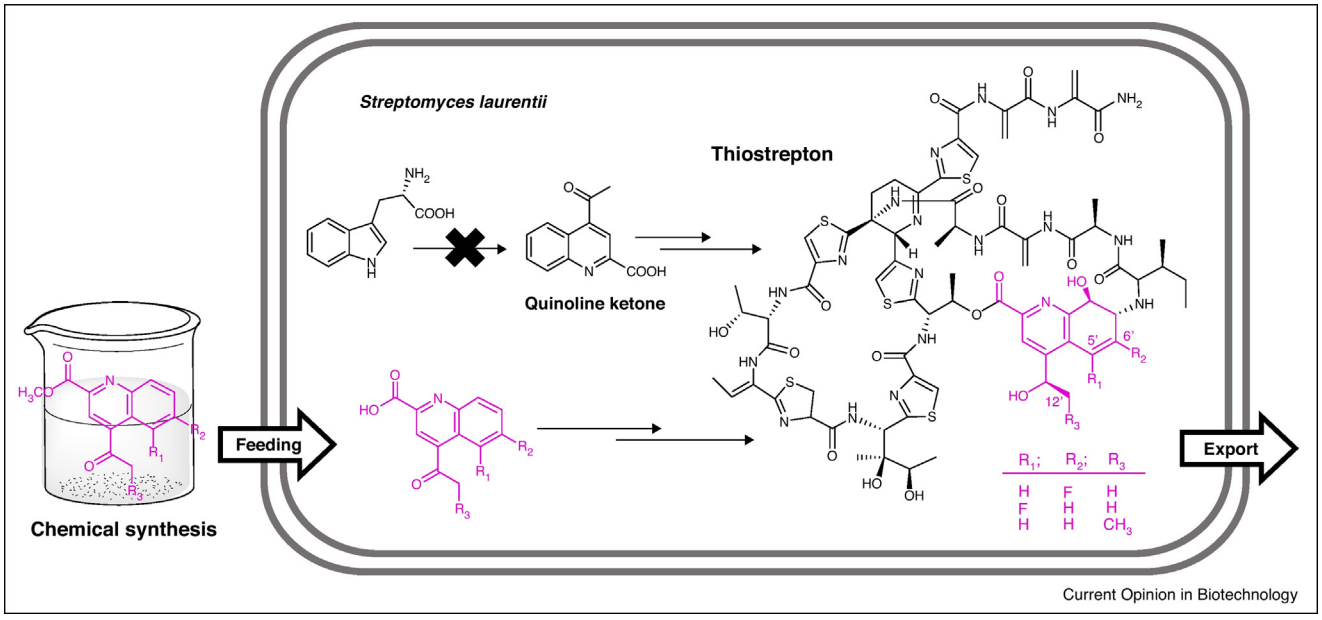
Thiostrepton appears to be unique in the thiopeptide family, primarily because of the QA moiety residing within its side-ring system [39]. QA approaches A1067, a key nucleobase of the 50S large subunit that contributes to ligand interaction and mutation-induced bacterial resistance [49]. Focusing on the selective modification of this biologically relevant moiety, the introduction of pharmaceutical groups (i.e., fluorine and methyl) into thiostrepton was conducted [50••]. The electronic and steric effects resulting from this effort can selectively enhance the interaction of QA and A1067, while the overall binding nature of molecules to the bacterial ribosome, which primarily depends on the thiopeptide core scaffold, is maintainable. Because these designed unnatural variants are structurally complex and difficult to prepare using the current chemical synthesis methods, a precursor-directed mutational biosynthesis approach was developed for their robust production in *S. laurentii*

Figure 3



Bio-inspired incorporation of naturally unavailable building blocks. (**a**) Introduction of ncAAs (e.g., *N*ε-prop-2-ynyloxycarbonyl-l-lysine, for replacing residue Thr3 of the core sequence of the precursor peptide) into thiocillin using the orthogonal system established in the Gram-positive host *Bacillus cereus*. (**b**) Chemoenzymatic route toward of the synthesis of thiopeptide variants. The synthetic steps are indicated by the dashed arrows, in contrast to the enzymatic conversion, which is shown by the solid arrow.

Figure 4



Production of thiostrepton variants that bear a selectively fluorinated or methylated QA moiety (purple) in *S. laurentii*. The ester analogs of the key quinoline ketone intermediate of QA were chemically synthesized and fed individually into a *S. laurentii* mutant strain. This mutant strain, which lacks the first 2-methylation step to initiate QA formation, is incapable of producing thiostrepton. After hydrolysis *in vivo*, each exogenous quinoline ketone analog can surrogate the wild-type intermediate being incorporated, leading to the production of thiostrepton analogs with designed modifications.

by fermentation according to recently uncovered biosynthetic mechanisms in the formation of QA (Figure 4) [35,50••].

The resultant variants are more potent than thiostrepton and control chemotherapeutics, e.g., vancomycin [50••]. The sensitive pathogens included many clinical isolates that are resistant to current drugs, e.g., methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and vancomycin-resistant *enterococci* (VRE), demonstrating the rationale for QA modification. Remarkably, using these variants as chemical probes revealed an unexpected new mechanism of thiostrepton, which is capable of inducing host autophagy during treatment with intracellular pathogens in addition to directly targeting the bacterial ribosome [51••]. This intracellular action, which is unique to thiostrepton-type thiopeptide antibiotics and is sensitive to the modification of the QA group, may inspire future changes in the treatment of intracellular pathogens since the contribution of host cell responses to antimicrobial chemotherapy has been increasingly recognized. Recently, the combination of QA modification and C-terminal tailoring that has improved anti-infectious activity was conducted and showed a synergistic engineering effect [52], as exemplified by the extremely potent methyl ester analog of 5′-fluoro-thiostrepton, which exhibited minimum inhibitory concentrations at <0.125 ng/mL, 0.25–0.5 ng/mL, 0.25–0.5 ng/mL and 0.125–0.5 ng/mL against various PRSP, MRSA, VRE and *Clostridium difficile* clinical isolates, respectively.

Remarkably, 6′-fluorination of QA lowers the reactivity of this moiety and slows the cyclization process for side-ring closure, which rapidly proceeds in *S. laurentii* without this modification, thereby causing the accumulation of an open side-ring epoxy intermediate [38••]. This unexpected finding ultimately revealed the maturation process of thiostrepton, which involves an unusual dual activity of an α/β hydrolase fold protein for cascade endopeptidyl hydrolysis/leader sequence removal and epoxide ring opening/side-chain macrocyclization in the biosynthetic pathway [38••,53]. The endopepeptidase activity of this protein, which is responsible for selective hydrolysis between Met-1 and Ile1 of a wild-type precursor peptide, appears to be promiscuous and tolerates the substitution of either of the residues with nonpolar amino acids. By exploiting the coupled activity for epoxide ring opening and macrocyclization, changing the size of the side-ring system is practical, as evidenced by the results from the mutation of Ala2Ile or Ala2Val, which created a new hydrolytic site between Ile1 and Ile2 or between Ile1 and Val2 of each recombinant precursor peptide, thus allowing the production of an additional thiostrepton variant that bears the contracted QA-containing side-ring system [43••].

Conclusion and perspectives

Following a brief review concerning the generality and specificity of the biosynthesis of thiopeptide antibiotics, we focused on a few recent examples, primarily from the studies on the members thiocillin (mono-marcocyclic) and thiostrepton (bi-macrocyclic), to highlight the approaches used to accelerate the diversification process for the expansion of molecular utility in searching for clinically useful variants, examining new modes of action and accessing the biosynthetic processes that are difficult to realize. As with other ribosomally synthesized and post-translationally modified peptide NPs, thiopeptide antibiotics feature a highly evolvable 'template'-biosynthetic logic that facilitates molecular engineering [54]. This logic has not been fully appreciated to date, and the associated PTMs involve a number of unusual biochemical mechanisms that remain to be determined. A further understanding of these mechanisms would significantly facilitate the design, development and utilization of compatible machineries, synthetic, biosynthetic or both, to expand the chemical spaces of thiopeptide antibiotics and their associated biological functions.

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- •• of outstanding interest

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