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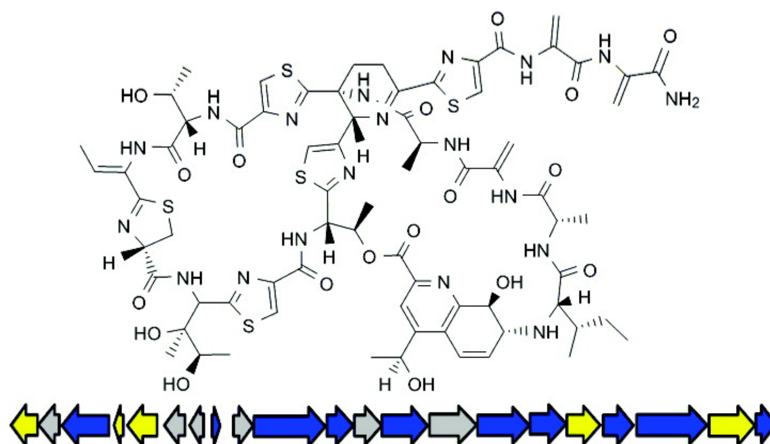

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Thiostrepton Biosynthesis: Prototype for a New Family of Bacteriocins

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Abstract: Thiopeptide antibiotics are a group of highly modified peptide metabolites. The defining scaffold for the thiopeptides is a macrocycle containing a dehydropiperidine or pyridine ring, dehydrated amino acids, and multiple thiazole or oxazole rings. Some members of the thiopeptides, such as thiostrepton, also contain either a quinaldic acid or indolic acid substituent derived from tryptophan. Although the amino acid precursors of these metabolites are well-established, the biogenesis of these complex peptides has remained elusive. Whole-genome scanning of *Streptomyces laurentii* permitted identification of a thiostrepton prepeptide, TsrA, and involvement of TsrA in thiostrepton biosynthesis was confirmed by mutagenesis. A gene cluster responsible for thiostrepton biosynthesis is reported, and the encoded gene products are discussed. The disruption of a gene encoding an amidotransferase, *tsrT*, led to the loss of thiostrepton production and the detection of a new metabolite, contributing further support to the identification of the *tsr* cluster. The *tsr* locus also appears to possess the gene products needed to convert tryptophan to the quinaldic acid moiety, and an aminotransferase was found to catalyze an early step in this pathway. This work establishes that the thiopeptides are a type of bacteriocin, a family of genetically encoded antimicrobial peptides, and are subjected to extensive posttranslational modification during maturation of the prepeptide.

Introduction

Thiopeptides are widely distributed metabolites isolated from Gram-positive bacteria of both terrestrial and marine origin.[1−3] Thiostrepton A **1** (Figure 1), considered the prototype of this family, was first isolated from *Streptomyces azureus* ATCC 14921 in the 1950s and later from *Streptomyces laurentii* ATCC 31255 (*S. laurentii*) in 1977.[1,4,5] In both the solution and crystal structures of thiostrepton **1**, the molecule adopts a globular conformation, with the two large ring systems of the peptide backbone folded upon one another.[6,7] The highly modified thiopeptides are characterized by a macrocycle possessing a central pyridine, hydroxypyridine, or dehydropiperidine (Figure 1).[1] This core scaffold typically presents several dehydrated amino acids and multiple thiazole or oxazole rings. Some thiopeptides, such as thiostrepton **1** and nosiheptide **2**, are further modified by a quinaldic acid or indolic acid appendage, respectively.[6,8]

Thiostrepton **1** and the other thiopeptides are potent antibacterial agents against Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, for which the available chemotherapeutic options are severely limited.[9,10] The thiopeptides exert their antibiotic effect by inhibiting elongation during prokaryotic translation by one of two general mechanisms. One group of thiopeptides, including thiostrepton **1** and nosiheptide **2**, binds directly to the large ribosomal subunit in the region that interacts with elongation factor G and interferes with translocation along the mRNA transcript.[11−13] The second mechanism attributed to certain thiopeptides, such as GE2270A **3**, is to bind elongation factor Tu (EF-Tu). Upon binding of GE2270A **3** to EF-Tu, formation of the EF-Tu·GTP·aminoacyl-tRNA complex is prevented, and efficient delivery of an incoming aminoacyl-tRNA to the ribosome is impaired.[14,15] The development of the thiopeptides into clinically useful antibacterial agents has been limited by the lipophilicity of these metabolites. There is therefore interest

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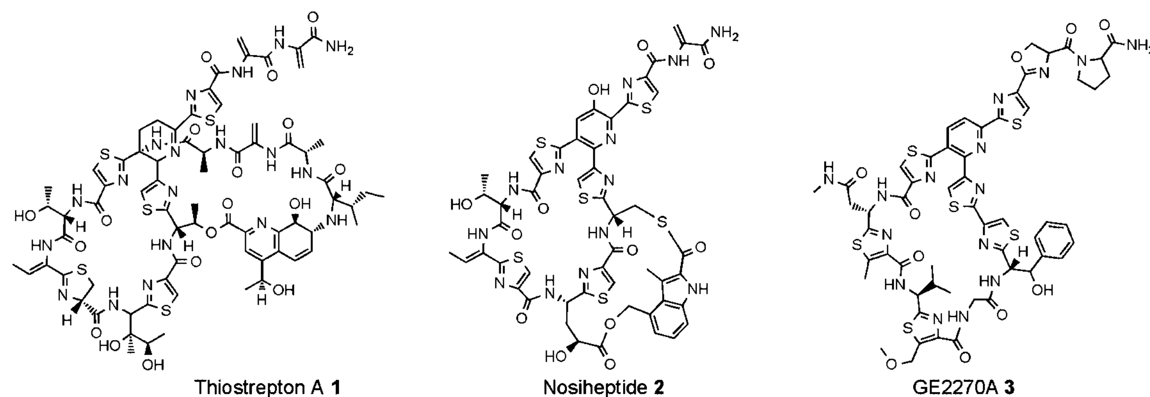

Figure 1. Examples of thiopeptide antibiotics.

in the generation of thiopeptide analogues of improved water solubility by the means of biosynthetic engineering, synthesis, or semisynthesis.[9,16]

The amino acid origins of the thiopeptides are well-established,[17−22] but an answer to the question of whether the peptide backbone arises from a nonribosomal peptide synthetase or by the posttranslational modification of a ribosomally synthesized peptide has remained elusive. Most microbial metabolites arise from loci on the chromosome possessing genes encoding both biosynthetic enzymes and those for self-resistance. This is not the case for the thiopeptides.[23,24] Since this limits the options available for biosynthetic gene cluster identification, we adopted a genome-scanning strategy to pinpoint the genes required for thiostrepton biosynthesis in *S. laurentii*. In this work, we establish that thiopeptides do, in fact, arise from a genetically encoded peptide and constitute a newly identified class of bacteriocin, a ribosomally synthesized antimicrobial peptide.

Experimental Section

General. All chemicals and solvents were reagent grade, purchased from VWR or Sigma-Aldrich, and used without further purification unless otherwise indicated. Pyrosequencing of the *S. laurentii* ATCC 31255 genome and individual sequencing reactions were performed by Eurofins MWG Operon (Huntsville, AL), and sequencing of the shotgun fosmid libraries was performed at Functional Biosciences, Inc. (Madison, WI). Protein-coding regions of DNA sequences were predicted using FramePlot 4.0Beta (http://nocardia.nih.go.jp/fp4/).[25] Protein sequences were analyzed by BLAST (Basic Local Alignment Search Tool).[26] Authentic thiostrepton was purchased from Sigma-Aldrich. 2-Methyltryptophan was prepared as described previously.[27] High-performance liquid chromatography (HPLC) was performed on a Beckman Coulter System Gold equipped with a diode-array detector. Mass spectroscopy was performed by the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility with a Micromass Quattro LC Mass Spectrometer.

Bacterial Strains, Plasmids, and Media. Tables of strains and plasmids used in this work are provided in the Supporting Information. *S. laurentii* was obtained from American Type Culture Collection (ATCC). pGM160 was kindly provided by Dr. Muth at University of Tübingen, Germany.[28] All *Escherichia coli* (*E. coli*) strains were grown in Luria−Bertani liquid medium or on solid medium supplemented with the appropriate antibiotic(s). Kanamycin (50 μg/mL), apramycin (50 μg/mL), nalidixic acid (25 μg/mL), and chloramphenicol (30 μg/mL) were used for the selective growth of *E. coli* or *S. laurentii*. pGM160K was constructed from pGM160, replacing the thiostrepton resistance gene with that for kanamycin resistance. ISP-3 agar medium was used for the growth and sporulation of *S. laurentii*. MS agar was prepared as described previously and used for intergeneric conjugation.[4] The seed and fermentation media used for thiostrepton production by *S. laurentii* were the same as described previously.[29] Unless specified, restriction enzymes, DNA ligase, and other materials for recombinant DNA procedures were purchased from standard commercial sources and used as provided.

Growth of *S. laurentii* and Production of Thiostrepton. Growth of *S. laurentii* and production of thiostrepton were performed as described previously with only minor modifications.[29] Briefly, the fermentation was carried out in a two-step process. First, 100 mL of seed medium in a 500-mL flask was inoculated with 0.1 mL of a 24 h *S. laurentii* culture in tryptic soy broth. After 48 h at 28 °C and 200 rpm, 5 mL of this seed culture was used to inoculate 100 mL of fermentation medium in a 500-mL flask. The resulting fermentation culture was incubated at 28 °C and 200 rpm for 5 days. The whole culture was extracted twice with an equal volume of chloroform. The chloroform layers were pooled together and solvent removed in vacuo. The solid residue was dissolved in 1 mL of chloroform for HPLC and HPLC-MS analysis.

HPLC was performed using a Phenomenex Jupiter Proteo C18 column (4 μm, 250 mm × 4.60 mm). The column was developed with a linear gradient of 0−100% of acetonitrile in water over 30 min at 1 mL/min, monitoring absorbance at 254 nm. Thiostrepton eluted with a t_R of about 22.5 min. HPLC-MS was performed with a Phenomenex Syngeri RP column (250 mm × 2 mm) and developed with 20% Buffer B in Buffer A for 8 min followed by

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a gradient from 20−100% Buffer B over 35 min (Buffer A, 5% acetonitrile and 0.1% formic acid; Buffer B, 95% acetonitrile and 0.1% formic acid) at 0.25 mL/min. Under these conditions, thiostrepton elutes at a t_R of about 28.4 min providing ions at m/z of 1664.4 $[M + H]^+$ and m/z of 832.9 $[M + 2H]^{2+}$. The predominant ion for thiostrepton was the $[M + 2H]^{2+}$, while the $[M + H]^+$ was only a minor species.

Construction of *S. laurentii* Fosmid Library and Screening for Selected Sequences. A genomic fosmid library of *S. laurentii* was constructed according to the instructions of the Epicenter Copy Control Fosmid Library Production Kit (Madison, WI). *S. laurentii* genomic DNA was prepared according to protocol.[30] Clones were isolated and stored according to kit instructions. The *S. laurentii* fosmid library was screened by PCR for the desired sequence. Four rows from each 96-well plate of the fosmid library (48 clones) were combined and the fosmid pool isolated. The two primer pairs used in the screening were TSRA3-F/TSRA3-R and TSRA4-F/TSRA4-R. Individual rows (containing a pool of 12 clones) from each plate and individual clones were identified by iterations of PCR. The PCR products were cloned into pCR-Blunt II-TOPO vector and confirmed by sequence analysis.

Inactivation of *tsrA*. Inactivation of *tsrA* was accomplished by PCR-targeted gene replacement[31] using pGM160K. The *tsrA* gene and flanking regions were amplified as a 1.8 kb fragment from genomic DNA of *S. laurentii* using the primers TSRA1-F and TSRA1-R. The resulting PCR product was cloned using the Zero Blunt TOPO Cloning Kit, yielding pJP11 and was confirmed by DNA sequencing. The plasmid pJP11 was digested with *Hind*III, and the resulting 1.8 kb fragment ligated into pGM160K, yielding pCL60. A 1.4 kb apramycin resistance cassette containing the *aac*(3)*IV* resistance gene and *oriT* was amplified from pIJ773 using the TSRA2-F and TSRA2-R. The resulting PCR product was used for in-frame replacement of the *tsrA* gene in pJP60 by λ RED-mediated recombination,[31] generating pCL41.

Following transformation into *E. coli* ET12567/pUZ8002, pCL41 was introduced into *S. laurentii* by conjugation.[31] A colony resistant to apramycin and sensitive to kanamycin was selected following homologous recombination between pCL41 and chromosomal DNA and designated as *tsrA*$^-$. The allelic replacement of *tsrA* by the apramycin-resistance gene and *oriT* in *tsrA*$^-$ was confirmed by PCR (Figure S2) with the primers TSRA3-F and TSRA3-R. A 1.1 kb PCR product was expected for wild-type *tsrA* versus a 2.4 kb PCR product for *tsrA* mutant. The PCR products were cloned into pSC-B-amp/kan and confirmed by sequence analysis.

Inactivation of *tsrT*. TsrT was inactivated by a strategy similar to that used for the inactivation of *tsrA*. A 1.4 kb apramycin resistance cassette containing the *aac*(3)*IV* resistance gene and *oriT* was amplified from pIJ773 using the primers TSRT1-F and TSRT1-R. The resulting PCR product was used for in-frame replacement of *tsrT* in JA8H9, generating pTSRT1. The apramycin resistance cassette and its flanking region was amplified as a 3.5 kb fragment from pTSRT1 using the primers TSRT2-F and TSRT2-R. The resulting PCR product was cloned using the Zero Blunt TOPO Cloning Kit, generating pTSRT2 and confirmed by DNA sequence analysis. Digestion with *Hind*III, provided a 3.5 kb fragment, which was then ligated into pGM160K to yield pTSRT3.

Following transformation into *E.coli* ET12567/pUZ8002, pTSRT3 was introduced into *S. laurentii* by conjugation. A colony resistant to apramycin and sensitive to kanamycin was selected following homologous recombination between pTSRT3 and chromosomal DNA and designated *S. laurentii* LP3*tsrT*$^-$. The allelic replacement of *tsrT* by the apramycin resistance gene and *oriT* in *tsrT*$^-$ was confirmed by PCR with the primers TSRT3-F and TSRT3-R (Figure S4). A 2.3 kb PCR product was expected for wild-type *tsrT* versus a 1.8 kb PCR product for the *tsrT* mutant. The PCR products were cloned into pSC-B-amp/kan and confirmed by sequence analysis.

Expression and Purification of TsrV. The gene encoding TsrV was amplified by PCR using primers TSRV1-F and TSRV1-R. The product was digested with *Nde*I and *Xho*I, ligated into pET28b(+) to provide pTSRV1, and confirmed by sequence analysis. *E. coli* BL21(DE3) containing pTSRV1 was incubated at 37 °C overnight in 2 × 50 mL Luria−Bertani medium supplemented with 50 μg/mL kanamycin. The following morning, 6 × 10 mL of the overnight culture was used to inoculate 6 × 1 L of Luria−Bertani medium supplemented with 50 μg/mL kanamycin, and the resulting cultures were grown at 37 °C until $OD_{600} = 0.4$. At this time, the temperature was reduced to 15 °C and protein expression induced with the addition of 0.04 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for an additional 24 h. Harvested cells were resuspended in 40 mL of lysis buffer [20 mM Tris (pH 8.0), 300 mM NaCl, 2 mM imidazole, 10% glycerol, 1 mg/mL lysozyme, 1 mM PMSF, 200 μM pyridoxal 5′-phosphate (PLP)]. The cells were disrupted by sonication (10 10-s pulses with a 30 s pause). The lysate was clarified by centrifugation (at 18 459*g*, 4 °C, 30 min). The cell-free extract was incubated with 2 mL of Ni-NTA resin (Qiagen) for 1 h. The slurry was loaded onto a column, and the resin was washed with 20 mL lysis buffer and then 40 mL wash buffer [20 mM Tris (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10% glycerol, 20 μM PLP]. Protein was eluted with 12 mL of elution buffer [wash buffer containing 300 mM imidazole], collecting 3 mL fractions. Fractions containing the protein were pooled together and dialyzed against 2 × 1 L storage buffer [20 mM Tris (pH 8.0), 50 mM NaCl, 10% glycerol, 20 μM PLP, and 1 mM DTT]. The protein was concentrated with a Millipore-0.5 centrifugal filter. Protein concentration was determined by the method of Bradford, using bovine serum albumin as a standard.[32] The protein was flash-frozen in liquid nitrogen and stored at −86 °C.

TsrV-Dependent Aminotransferase Activity Assays. Reaction mixtures (100 μL) for detection of 2-methyl-indolepyruvate formation were prepared using 1 mM 2-methyltryptophan, 1 mM indole-3-pyruvic acid, 100 μM PLP, and 1.5 μM TsrV in 100 mM KH_2PO_4 (pH 7.8). The reactions were incubated at 24 °C for 30 min. After 30 min, the reaction mixtures were frozen in liquid nitrogen and stored at −80 °C, until ready for use. Samples were analyzed by HPLC-MS using a Phenomenex Gemini C18 column (150 mm × 2 mm). The column was developed at 0.2 mL/min by 100% Buffer A for 5 min, then a gradient of 0−100% Buffer B over 40 min, followed by 100% Buffer B for 5 min (Buffer A, 5% acetonitrile and 0.1% formic acid in water; Buffer B, 95% acetonitrile and 0.1% formic acid in water).

Nucleotide Sequence Accession Numbers. The sequences reported here have been deposited into the GenBank database under the accession number FJ652572.

Results and Discussion

Cloning and Sequencing of the Thiostrepton Biosynthetic Gene Cluster in *S. laurentii*. Whole genome scanning was employed to locate the genes required for **1** biogenesis. The *S. laurentii* genome was partially sequenced to 7.26 Mb on over 3600 contiguous fragments. Sequence analysis permitted the identification of gene products with weak similarity to lantibiotic dehydratases. More interestingly, a gene was identified that encoded a bacteriocin prepeptide, TsrA. Following the leader peptide of TsrA, the 17 amino acid sequence at the C-terminal end is identical to that predicted for a prepeptide of thiostrepton (Figure 2).

A PCR screen of a *S. laurentii* genomic fosmid library established the colocalization of *tsrA* and one of the genes to a

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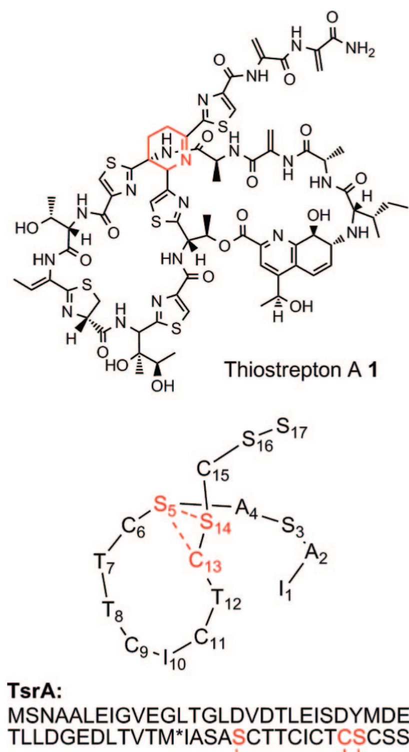

Figure 2. Thiostrepton and TsrA.

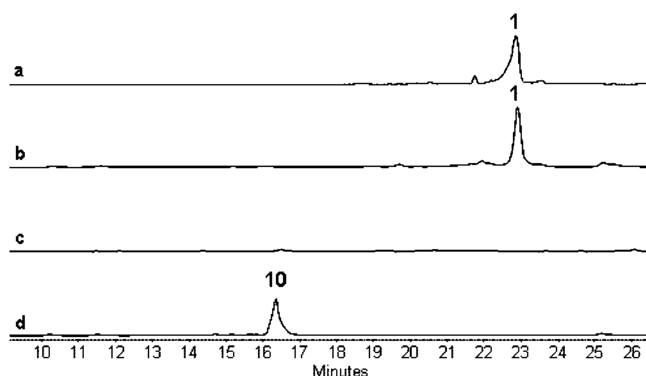

Figure 3. HPLC analysis of (a) thiostrepton and extracts from the cultures of (b) wild-type *S. laurentii*, (c) *S. laurentii tsrA*$^{-}$, and (d) *S. laurentii* LP3*tsrT*$^{-}$.

lantibiotic dehydratase, suggestive of a biosynthetic operon for a bacteriocin-containing dehydroalanine or dehydrobutyrine residues. To establish the requirement of the prepeptide for formation of **1**, we constructed a mutant replacing *tsrA* with an apramycin resistance cassette. The *S. laurentii tsrA* mutant abrogated **1** production (Figure 3), confirming its involvement in thiostrepton biosynthesis. The region surrounding *tsrA* was then sequenced to nearly 30 kb from two overlapping fosmids, JA8H9 and JA11E10, and found to contain 21 potential open reading frames (Figure 4 and Table 1).

Posttranslational Modifications of TsrA. The proteins encoded in this cluster are consistent with earlier feeding studies[22,29,33−35] and the expectation that dehydration and heterocyclization occur during TsrA maturation. Two proteins encoded in the *tsr* locus, TsrC and TsrL, were identified having similarity to the LanB lantibiotic dehydratases, and either one could impart the dehydroalanine and dehydrobutyrine residues present in **1**.[36,37] Each cysteine residue in **1** is subjected to cyclodehydration upon the peptide backbone. Microcin B17, the cyanobactins (e.g., patellamides A and C), and goadsporin are all examples of genetically encoded peptides for which multiple thiazole rings result from posttranslational modification.[38−41] The thiazole and oxazole rings in the *E. coli* metabolite microcin B17 result from the action of the McbBCD complex.[39] In goadsporin, these heterocycles are thought to result from the combined action of GodD and GodE.[40,42] Likewise, PatD and PatG are proposed to produce the aromatic heterocycles in patellamides A and C.[41] GodD and PatD are presumed to be functional analogues of the cyclodehydration catalyst McbB and the docking protein McbD, fused into a single polypeptide chain.[40−42] Dehydrogenation to the aromatic heterocycle is mediated by the FMN-dependent McbC for microcin B17, and likely GodE and PatG in the case of goadsporin and the patellamides, respectively.[39−41,43] It therefore seems likely that TsrH (36% similarity to GodD) is the cyclodehydratase for the thiostrepton system and TsrF, predicted to bind FMN, then oxidizes the dehydroheterocycle to the aromatic species.[40,42,44]

The D-thiazoline at C9 is unique to dehydropiperidine thiopeptides, and an epimerization of C9 must occur during TsrA maturation.[1,22] One possible explanation for this stereochemistry is that heterocyclization and oxidation of all the cysteine residues precedes introduction of the D-configuration at C9. In this scenario, the C9 D-thiazoline is introduced following reduction of the thiazole. This route, however, has been excluded. Both deuterium atoms from L-[3-^{13}C,^{2}H]-serine remain in the C9 thiazoline of **1**, suggesting that epimerization likely occurs prior to cyclodehydration.[22] Furthermore, there is precedent for the posttranslational epimerization of an amino acid side chain in both animal peptides and lantibiotics.[45,46] Typically, only one dehydratase is needed for the dehydration of serine and threonine residues during lantibiotic maturation.[45] If this is true for **1** as well, then either TsrL or TsrC, similar to enzymes that abstract a peptide's α-protons, could operate as an epimerase rather than a dehydratase. When introduced prior to cyclodehydration, the C9 D configuration could prohibit subsequent dehydrogenation

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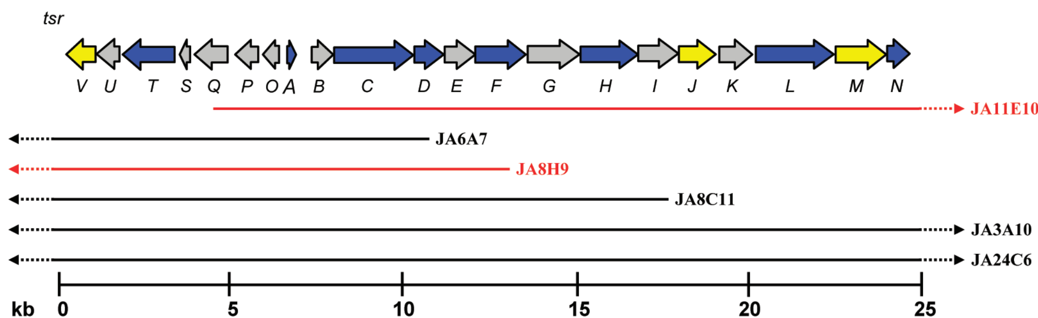

Figure 4. Organization of the *tsr* cluster.

Table 1. Deduced Functions of the Open Reading Frames in the Thiostrepton Biosynthetic Gene Cluster

gene	size[a]	protein homologue and origin[b]	identity/similarity %	proposed function
tsrV	362	putative aminotransferase (YP_871797.1); *Acidothermus cellulolyticus* 11B	47/62	aminotransferase
tsrU	276	putative lipase (NP_631032.1); *Streptomyces coelicolor* A3(2)	50/62	unknown
tsrT	618	hypothetical protein AsnH (YP_001615582.1), *Sorangium cellulosum* 'So ce 56′	57/69	amidotransferase
tsrS	135	ester cyclase-like (ZP_01388898.1); *Geobacter* sp. FRC-32	36/56	unknown
tsrQ	409	acyl-CoA dehydrogenase, short-chain specific (YP_076186.1); *Symbiobacterium thermophilum* IAM 14863	34/50	oxygenase
tsrP	311	putative methyltransferase (YP_001535414.1) *Salinispora arenicola* CNS-205	40/56	putative methyltransferase
tsrO	183	hypothetical protein MchlDRAFT_5537 (ZP_02058450.1); *Methylobacterium chloromethanicum* CM4	29/45	unknown
tsrA	58	putative lantibiotic precursor peptide (NP_834755.1); *Bacillus cereus* ATCC 14579	42/61	thiostrepton precursor peptide
tsrB	263	putative hydrolase (YP_289599.1); *Thermobifida fusca* YX	29/39	unknown
tsrC	933	lanthionine biosynthesis protein (NP_834752.1); *Bacillus cereus* ATCC 14579	21/40	lantibiotic dehydratase
tsrD	346	lantibiotic biosynthesis protein (NP_834751.1); *Bacillus cereus* ATCC 14579	24/47	dihydropyridine synthase
tsrE	368	unknown (ABC02780.1); *Actinomadura melliaura*	25/41	unknown
tsrF	589	conserved hypothetical protein (ZP_02912150.1); *Geobacillus* sp. WCH70	29/42	thiazoline dehydrogenase
tsrG	612	hypothetical protein SGR_4409 (YP_001825921.1); *Streptomyces griseus* subsp. *griseus*	25/35	unknown
tsrH	681	hypothetical protein (ABC02784.1); *Actinomadura melliaura*	37/44	cyclodehydratase/docking protein
tsrI	474	cytochrome P450-like enzyme (YP_001102921.1); *Saccharopolyspora erythraea* NRRL 2338	29/44	oxygenase
tsrJ	437	putative nonribosomal peptide synthetase, adenylation domain (ABI22132.1); *Streptomyces lavendulae*	28/39	adenylation enzyme
tsrK	396	cytochrome P450 (YP_001275131.1); *Roseiflexus* sp. RS-1	31/48	oxygenase
tsrL	912	hypothetical protein SGR_4412 (YP_001825924); *Streptomyces griseus* subsp. *griseus*	27/36	unknown/lantibiotic dehydratase/ epimerase
tsrM	599	Fe−S oxidoreductase (EAY55855.1) *Leptospirillium* sp. Group II UBA	26/39	methyltransferase
tsrN	272	short-chain dehydrogenase/reductase SDR (YP_001362890.1); *Kineococcus radiotolerans* SRS30216	41/52	dihydropyridine reductase

[a] Numbers are in amino acids. [b] NCBI accession numbers are given in parentheses.

by TsrF, since the L stereochemistry would likely be required of its substrate.

A defining feature of the thiopeptides is the pyridine (e.g., **2** and **3**) or dehydropiperidine (e.g., **1**) ring resulting from the condensation of two serine residues at their β-carbons and the α-carboxyl carbon an adjacent cysteine residue (Scheme 1).[17−22] This likely follows generation of the dehydroalanine residues, as suggested by Bycroft, Floss, and others.[17−22,47] In **1**, the dehydropiperidine arises from S5, C13, and S14. One proposal for the introduction of this central scaffold of the thiopeptides suggests what is formally a hetero-Diels−Alder cyclization, though not necessarily proceeding as a concerted cycloaddition (Scheme 1A).[20,21,47,48] A hetero-Diels−Alder strategy has, in fact, been employed in the syntheses of this core feature of the thiopeptides, including those for thiostrepton, GE2270A, and other model systems.[49−54] Each of these synthetic approaches, however, necessitated modifications to the proposed biosynthetic

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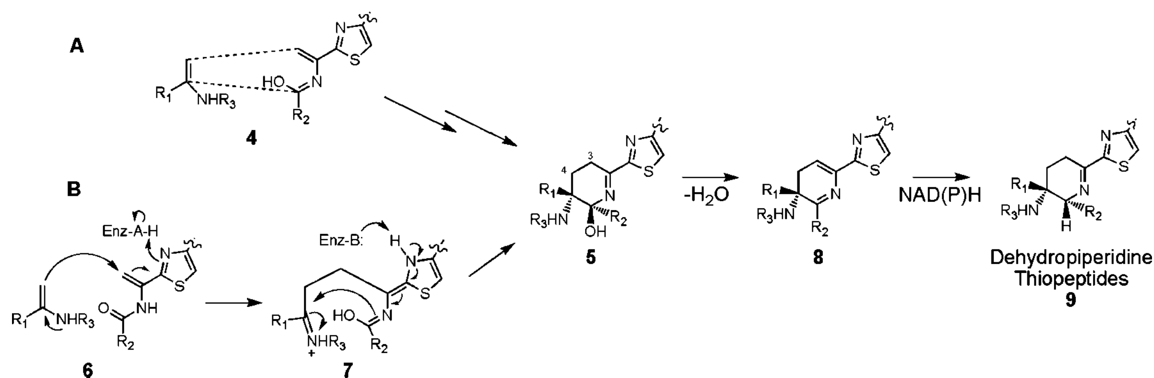
Scheme 1. Proposed Biosynthesis of the Dehydropiperidine Thiopeptides



cyclization substrate in order to present an appropriate partner diene and dienophile for cycloaddition. These syntheses of the pyridine and dehydropiperidine thiopeptide scaffolds suggest that a suitable biosynthetic intermediate for a hetero-Diels−Alder cyclization would need to be produced as a free metabolite or sequestered within the active site of the cyclization enzyme.

As an alternative to a hetero-Diels−Alder cycloaddition, the cyclase facilitating formation of the dihydropyridine ring of **5** could operate by acid and base catalysis with the strategic utilization of the adjacent thiazole as an electron sink, comparable to thiamine diphosphate-dependent transformations (Scheme 1B). TsrD bears weak similarity to the C-terminal region of lantibiotic dehydratases and could be harnessed for this cyclization, although alternative candidates cannot yet be ruled out. Dehydration of **5** then leads to the dihydropyridine **8** (Scheme 1). For **1** and the reduced thiopeptides, an NAD(P)H-dependent reductase, such as TsrN, acting upon **8** would restore the dehydropiperidine.

Two additional modifications of the TsrA backbone are required during thiostrepton maturation: carboxyl-terminal amidation and the dihydroxylation of I10. The latter can be explained by the action of one or both of the cytochrome P450s TsrI and TsrK. Most carboxyl-terminal amides that are present in peptides are introduced by the oxidative cleavage of the carboxyl-terminal residue.[55,56] This is not the case for thiostrepton since no additional residues follow S17 of TsrA. Instead, an amidotransferase would be required to transmit ammonia from glutamine to an activated carboxylic acid. To our knowledge, utilization of an amidotransferase is a novel strategy to introduce a carboxyl-terminal amide into a peptide. TsrT, a protein similar to asparagine synthetases, could fulfill this role.

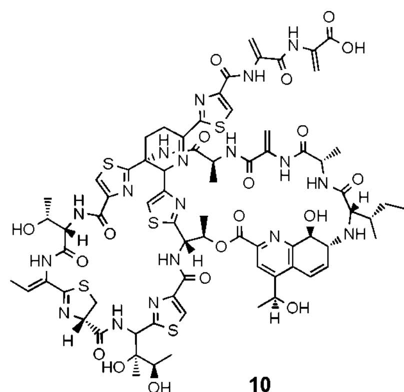
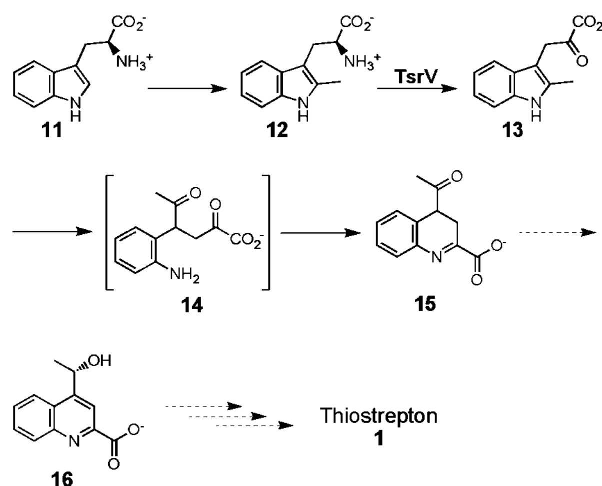


Figure 5. Proposed structure of **10**.

Scheme 2. Proposed Biosynthesis of 4-(1-Hydroxyethyl)quinoline-2-carboxylic Acid



To address the function of the putative amidotransferase in thiostrepton biosynthesis, *tsrT* was inactivated in *S. laurentii*. As anticipated, this strain lost the ability to produce **1**, while accumulating a new metabolite with a similar ultraviolet−visible absorption spectrum to thiostrepton **1** (Figure 3 and Supporting Information). Further analysis by HPLC-MS revealed a $[M + 2H]^{2+}$ ion at *m*/*z* 833.4, relative to that at 832.9 for **1**, indicating an increase in molecular weight from 1664.4 to 1665.4. This is consistent with generation of a thiostrepton analog, **10**, possessing the free carboxylic acid at the C-terminus (Figure 5).

Biosynthesis of the Quinaldic Acid Moiety. The quinaldic acid moiety observed in **1** originates from tryptophan **11** (Scheme 2).[22] There are several consistencies in the *tsr* cluster with earlier studies.[22,29,33,35] An early step in the conversion of tryptophan to the free intermediate 4-(1-hydroxyethyl)quinoline-2-carboxylate **16** is a methylation to provide 2-methyltryptophan **12**.[33,35] Unlike a majority of *S*-adenosylmethionine (SAM)-dependent methyltransferase reactions, this methylation was shown to proceed with net retention of the methyl group configuration.[35] Similar methylations, thought to be facilitated by radical SAM-dependent enzymes, have been implicated in the biosyntheses

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of the β-lactam thienamycin and the aminocoumarin clorobiocin.[57−59] One putative methyltransferase in the *tsr* locus, TsrP, fails to account for the stereochemical requirements needed for the formation of 2-methyltryptophan **12**. On the other hand, TsrM, with similarity to radical-SAM-dependent proteins, is a more appropriate candidate to accommodate the methylation of **11** with retention of the methyl group stereochemistry.

The next step proposed for the biosynthesis of **16** is the generation of 3-(2-methylindolyl)pyruvic acid **13**, occurring prior to ring expansion to the quinaldic acid. The α-keto acid could be introduced either by a PLP-dependent aminotransferase, a FAD-dependent amino acid oxidase, or an NAD^+-dependent dehydrogenase, as observed in the biosyntheses of indolmycin, rebeccamyin, and scytonemin, respectively.[60−62] One protein in the *tsr* locus, TsrV, is similar to a number of putative PLP-dependent aminotransferases and, on the basis of sequence comparison, contains the active site lysine residue needed to covalently bind the cofactor.[63] TsrV was heterologously expressed from *E. coli* BL21(DE3) with an N-terminal hexahistidine affinity tag. In the presence of 2-methyltryptophan **12** and the cosubstrate 3-indolylpyruvic acid **17**, TsrV catalyzed the formation of L-tryptophan **11** and 3-(2-methylindolyl)pyruvic acid **13**, as detected by HPLC-MS (Figures 6 and Supporting Information). Further characterization of TsrV will address not only whether 3-indolylpyruvic acid is truly the preferred α-keto acid cosubstrate but also whether the aminotransferase is tolerant of additional tryptophan derivatives.

It is not yet clear which gene products are responsible for the latter steps needed to generate thiostrepton's quinaldic acid moiety. Expansion of the pyrrole ring could occur by a pathway depicted in Scheme 2, leading to the quinoline ring system of **16** by a series of rearrangements and isomerizations, reminiscent of proposals for quinoline alkaloid biosynthesis.[29,64] It is interesting to note that a distinct mechanism to cleave the C-2/C-3 bond of tryptophan is likely utilized for the thiostrepton quinaldic acid relative to that observed for other bacterial quinaldic acid metabolites and the early steps of tryptophan catabolism. Xanthurenic acid, quinoxaline-2-carboxylic acid, and 3-hydroxyquinaldic acid are constituents of quinolobactin, echinomycin, and thiocoraline, respectively.[65−68] In contrast to the pathway proposed for the quinaldic acid **16**, these latter three tryptophan derivatives require the oxidative cleavage of the indole between C-2 and C-3 by a heme-dependent dioxygenase.[65,67,68] Prior to introduction of the quinoxaline or quinoline ring system, the C2 carbon is lost as formate, whereas the C2 carbon is retained in **16**.[22,65,67,68]

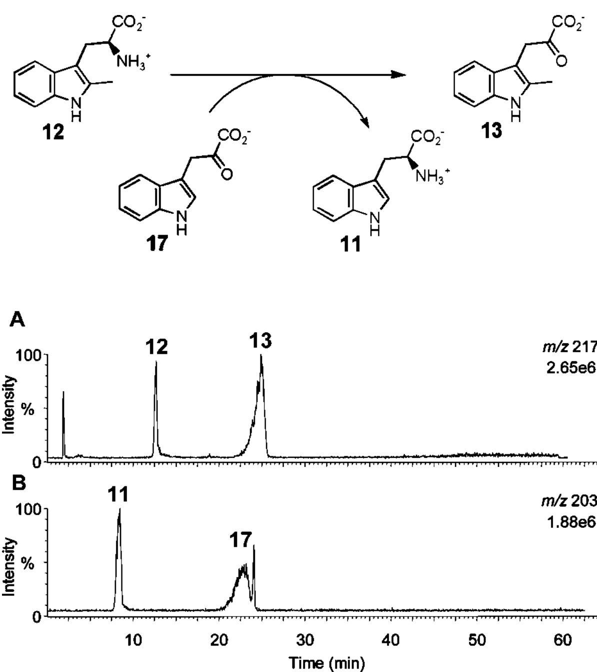

Figure 6. Reaction catalyzed by TsrV and HPLC-MS analysis of TsrV activity with 2-methyltryptophan. The chromatograms show the negative ions extracted for (A) *m/z* 217 and (B) *m/z* 203. Retention times and molecular ions are as follows. 2-Methyltryptophan **12** at $t_R = 12.7$ min and a $[M - H]^-$ ion at *m/z* 216.9; 3-(2-methylindolyl)pyruvate **13** at $t_R = 24.9$ min and a $[M - H]^-$ ion at *m/z* 215.8; tryptophan **11** at $t_R = 8.5$ min and a $[M - H]^-$ ion at *m/z* 202.8; 3-indolylpyruvate **17** at $t_R = 22.8$ min and a $[M - H]^-$ ion at *m/z* 201.8.

Floss et al. demonstrated the ATP-dependent activation of **16** in a cell-free extract, and this a logical function for TsrJ, which has similarity to adenylating enzymes.[29] Following attachment of the quinaldic acid to the T12 side chain, epoxidation by either TsrQ (a flavin-dependent enzyme) or one of the cluster's cytochrome P450s (TsrI or TsrK) would then provide a suitable substrate for nucleophilic attack by the N-terminal amine of I1 to complete the attachment of this moiety and the generation thiostrepton's second macrocycle. Whether introduction of the quinaldic acid appendage in thiostrepton **1** precedes or follows any of the other posttranslational modifications of the prepeptide backbone is unknown at this time.

Prepeptide Processing and Self-Resistance. There is no obvious gene product from the *tsr* locus that would be a suitable candidate to liberate the TsrA leader peptide. TsrU and TsrB are both similar to the α/β-hydrolase superfamily but are either more similar to lipases than proteases or lack the conserved motif for the nucleophilic serine, respectively.[69] It is certainly conceivable, however, that the putative lipase TsrU could be utilized as a protease rather than a lipase. Both lantibiotics and microcins can recruit proteases encoded from outside their biosynthetic gene clusters; therefore, the lack of a dedicated TsrA peptidase for thiostrepton would not be particularly troubling.[45,70−72] On a similar note, there is no evident gene

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product conferring a mechanism for self-resistance to **1** in the *tsr* genetic locus. Previous work by Floss and co-workers demonstrated that an rRNA methyltransferase encoded outside the limits of a thiopeptide biosynthetic cluster imparts resistance to thiostrepton in *S. laurentii*.[24] The self-resistance gene for nosiheptide **2** in the producing bacterium, *Streptomyces actuosus*, was also found to reside outside a biosynthetic gene cluster, suggesting that this may be common for thiopeptide antibiotics.[73]

Conclusion

We have established that thiostrepton is derived from TsrA, a simple, genetically encoded peptide. Disruption of *tsrA* and *tsrT* in *S. laurentii* abolish production of thiostrepton, and the *tsrT* mutant led to the accumulation of a thiostrepton derivative. Initial characterization of TsrV established that it is an aminotransferase catalyzing a step in the conversion of tryptophan to 4-(1-hydroxyethyl)quinoline-2-carboxylate, a substituent attached to thiostrepton. Additional investigations will establish the temporal mechanisms guiding the formation of this extensively modified peptide.

Several cryptic biosynthetic clusters were recently identified to harbor enzymatic machinery comparable to that observed here for **1**.[42] Both **1** and goadsporin are peptides formed in actinomycetes that are processed by dehydration and heterocyclization, and similarities between their maturation enzymes are observed.[40] These similarities extend to other peptide metabolites identified by Lee et al., particularly with TsrD homologues, suggesting a widespread occurrence of thiopeptide metabolites across genera.[42] Therefore, this report establishes the thiopeptide antibiotics as a new class of bacteriocin.

Despite potent in vitro activity, development of a clinically useful thiopeptide antibiotic is hampered by the poor solubility of these agents in water. The looming crisis in antimicrobial chemotherapy has contributed to a renewed interest in the discovery and development of thiopeptide analogs with improved water solubility. The complex architecture of thiopeptides is a significant challenge toward de novo synthesis. Biosynthetic engineering of thiopeptide analogues presents an attractive alternative that can be coupled with semisynthetic modifications. This will be facilitated by a detailed understanding of the biosynthetic machinery required to construct the complex thiopeptide scaffold, and it is now possible to query the production of variants through site-directed mutagenesis of TsrA.

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Supporting Information Available: Additional materials and methods, tables of strains, plasmids, and primers, and supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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