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Mutagenesis of the thiostrepton precursor peptide at Thr7 impacts both biosynthesis and function

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Abstract

The seventh residue of the highly modified peptide antibiotic thiostrepton is predicted to be critical for antibacterial activity. Substitution of Thr7 in the thiostrepton precursor peptide not only disrupts biological activity but also impacts the successful biosynthesis of thiostrepton analogs.

The thiopeptides are a family of highly modified ribosomally-synthesized peptides, first discovered over sixty years ago.^{1, 2} Thiostrepton A **1** (Fig. 1), produced by *Streptomyces laurentii* ATCC 31255 (*S. laurentii*), is one of the more well-studied thiopeptide antibiotics.³ As established for other thiopeptides, **1** is the product of extensive posttranslational modifications upon a ribosomally-synthesized precursor peptide.⁴⁻⁷ Two macrocycles are harbored within the architecture of **1**: a core macrocycle (Loop 1) common to all thiopeptides, and a second quinaldic-acid containing loop (Loop 2) found only in a subset of thiopeptides.¹ Loop 1 can vary in size and composition, but it is unified by the presence of a six-membered nitrogenous ring.¹ The precursor peptide for **1**, TsrA, contains a 17 amino acid propeptide region that is ultimately incorporated into the final thiostrepton scaffold.⁵ Only three residues in **1** are unaltered by posttranslational modification, including a key threonine residue, Thr7. **1** exerts its antibacterial effect by binding the prokaryotic 50S ribosomal subunit near the GTPase-associated center and disrupting the conformational changes coordinated between the ribosome and its initiation and elongation factors.^{8, 9} When bound to the ribosome, **1** contacts both the 23S rRNA and the ribosomal protein L11, and the Thr7 residue of **1** appears to make critical contacts with the 23S rRNA.¹⁰

We recently reported a platform to produce thiostrepton analogs in *S. laurentii* by site-directed mutagenesis of TsrA, which conceivably permits alterations to Loop 1.¹¹ Despite the implication that the thiostrepton Thr7 plays an important role in antibacterial function, no analogs of **1** with alterations to this residue have yet been generated. Although Ala2Gly and Ala4Gly variants of TsrA were successfully processed to thiostrepton analogs bearing alterations to Loop 2, the expected Thr7Gly analog was not observed.¹¹ It was therefore uncertain whether or not variants of **1** at Thr7 could be successfully engineered. Here, we report further efforts toward alteration of the Thr7 residue of **1** and the antibacterial activities of the isolated thiopeptide analogs.

Two *Escherichia coli* (*E. coli*)-*Streptomyces* shuttle fosmids were constructed bearing Thr7Ser and Thr7Ala substitutions encoded in *tsrA*: int-3A104 and int-3A105, respectively. The two fosmids were individually introduced by intergeneric conjugation into *S. laurentii* NDS1, a *tsrA* deletion mutant, to provide *S. laurentii*/int-3A104 and *S. laurentii*/int-3A105. The culture extracts of the two strains were evaluated for the presence of thiostrepton-related metabolites by both HPLC and HPLC-MS (Fig. 2 and Supplementary Information). Thiostrepton Thr7Ser **2** was detected only by HPLC-MS and was not observable by HPLC analysis, precluding isolation of this variant in a quantity sufficient for biological or structural evaluation. In the case of the extract from *S. laurentii*/int-3A105 (encoding TsrA Thr7Ala), two new metabolites, **3** and **4**, were readily observed by HPLC eluting with a t_R of 18.5 min and 16.9 min, respectively (Fig. 2). Metabolite **3** possessed an UV-visible absorption spectrum similar to **1** and HPLC-MS revealed a mass consistent with that expected for thiostrepton Thr7Ala, with an m/z 817.9 $[M+2H]^{2+}$. On this basis, **3** was purified and its structure verified by one- and two-dimensional NMR and further mass spectrometry (Supplementary Information).

The UV-visible absorption spectrum of **4**, on the other hand, revealed a strong absorbance at 242 nm that is absent in mature thiostrepton analogs (Supplementary Information). Compound **4** was isolated, designated as SL105-1 (Scheme 1), and deduced to have a molecular formula of $C_{62}H_{67}N_{16}O_{15}S_5$ by HR-MALDI-MS (m/z 1435.3575 $[M+H]^+$, calculated m/z 1435.3618). Although initial comparison of the 1H and ^{13}C NMR spectra of **3** and **4** suggested some structural similarities, several distinctions were noted, including differences in the resonances associated with the quinaldic acid residue and the absence of the first three residues in **4**.^{12, 13} Further analysis of **4** by DEPT-135, COSY, HMBC, HSQC and MALDI MS/MS suggested the presence of a 4-(1-hydroxyethyl)quinoline-2-carboxylic acid (HEQ) moiety that, unlike in **1** and **3**, is not connected to the N-terminal amine of the peptide backbone (Supplementary Information). The carbon resonance for Q-2 could not be definitively assigned, and is likely overlapping with another signal among the aromatic resonances of **4**. The presence of the aromatic HEQ structure is, however, supported by COSY and HMBC correlations throughout the ring system, a strong UV absorbance at 242 nm, and spectral data consistent to methyl ester derivatives of HEQ.¹⁴ Furthermore, a MALDI MS/MS spectrum of **4** revealed a fragment ion at m/z 1218.1, an elimination of 217 Da from the parent ion at m/z 1435.4 $[M+H]^+$, lending further support to the presence of HEQ in **4**. HMBC correlations from a methyl group at δ_H 2.14 (Pyr3-3) to a ketone carbonyl at δ_C 196.3 (Pyr3-2) and an upfield-shifted amide carbonyl at δ_C 159.6 (Pyr3-1) suggest the presence of a pyruvyl moiety at the N-terminus of the peptide. Together with additional NMR and MS analyses, these data support the molecular formula suggested by HR-MALDI-MS and the structure proposed for **4** in Scheme 1.

To evaluate the effect of a Thr side chain isostere, we also generated *S. laurentii*/int-3A106, wherein TsrA bears a Thr7Val substitution. HPLC and HPLC-MS analysis of the culture extract from this strain revealed the presence of thiostrepton Thr7Val **5** and SL106-1 **6**, an Val-containing analog of **4** (Fig. 2 and Supplementary Information). Metabolites **5** and **6** were isolated and subjected to high-resolution mass spectrometry and MALDI MS/MS analysis.

Analog **3–6** were evaluated for their antibacterial properties (Supplementary Information). The MIC of each analog was at least 300-fold greater than **1**. The diminished antibacterial activities for **3** and **5** are consistent with the critical role proposed for the Thr7 residue. An unmodified threonine residue in the corresponding position within Loop 1 is highly conserved among ribosome-binding thiopeptides, and this side chain likely participates in critical hydrogen-bond contacts within the thiopeptide binding pocket.¹⁰ The exact interface, however, differs for each individual thiopeptide.¹⁰ Micrococin P₁ is another ribosome-

binding thiopeptide containing an unmodified threonine residue analogous in location to Thr7 of **1**.¹⁰ While Thr7 of **1** appears to contact the 23S rRNA, the corresponding micrococcin P₁ threonine (Thr3) instead approaches the ribosomal protein L11.¹⁰ Micrococcin P₁ derivatives bearing Ala, Asp, and Lys in place of Thr3, like **3** and **5**, were all impaired in their antibacterial activities, whereas a Thr3Ser variant retained activity.^{15, 16}

Although Thr7 substitutions may contribute to the diminished antibacterial efficacy of **4** and **6**, these analogs also lack an intact quinaldic acid-containing loop. Linearization of Loop 2 by cleavage of the ester bond between Thr12 and the quinaldic acid moiety of **1** yields a derivative devoid of antibacterial activity.^{17, 18} The crystal structure of **1** indicates that the hydroxyl groups in the quinaldic acid participate in intramolecular hydrogen bonds, including a hydrogen bond to the Thr7 side chain hydroxyl, that contribute to **1**'s overall conformation.¹⁹ Furthermore, the X-ray crystal structure of **1** bound to the ribosome reveals that the quinaldic acid of **1** is within 3.5 Å of A1067 of the 23S rRNA.¹⁰ The exact parameters influencing the impaired activity in **4** and **6** remain to be determined, however, it is possible that the absence of an intact Loop 2 leads to improper orientation of the quinaldic acid moiety, preventing effectual binding to the ribosome.

HEQ is a discrete intermediate in **1** biosynthesis, and can be activated as the adenylate by *S. laurentii* cell-free extracts.^{14, 20} The exact sequence of events governing formation of Loop 2 in **1** awaits elucidation, and its proposed biosynthetic pathway is included in Scheme 1. First, HEQ is tethered to the Thr12 side chain in **7**. Epoxidation to **8**, proteolysis of the leader peptide, and ultimately a nucleophilic attack from the newly liberated Ile1 N-terminal amine, could then forge the quinaldic acid-containing macrocycle (Scheme 1).^{13, 14} For TsrA Thr7Ala, this process appears to derail at an advanced stage of thiostrepton maturation, likely preceding the proposed epoxidation of the **7** HEQ. Although the HEQ moiety is most likely enzymatically installed even for the shunt product **4**, the adventitious and non-enzymatic attachment of an adenylated HEQ to the Thr12 side chain of an advanced intermediate cannot yet be definitively ruled out. Following peptidolysis of the leader peptide and the two N-terminal proteinogenic amino acids, a dehydroalanine is transiently exposed at the N-terminus in **9** prior to tautomerization and hydrolysis to provide the shunt product **4** (Scheme 1).

The Cys9 configuration (the thiazoline ring) of **4** was not firmly established and this residue is presumed to adopt the D configuration as in **1**. A NOESY analysis of **4** did reveal weak cross-peaks for the Cys9 protons, with relative intensities consistent to those observed in **1** (Supplementary Information).¹⁷ Therefore, we tentatively suggest a D-configured thiazoline ring in **4**. The biosynthetic origin for the D stereochemistry remains obscure, and this position is subject to non-enzymatic epimerization in **1**, favouring the D-configuration.¹⁷ No evidence for epimerization at Cys9 was observed in **4** during our investigations. The Cys9 configuration, if L, could further influence the fate of proposed intermediate **7**.

The Thr7 residue appears to be critical both for **1**'s biological activity and the efficient maturation of TsrA. The precise reason(s) for the inefficient processing of Thr7 variants is(are) unclear, since any one or more of the multiple transformations during thiostrepton maturation could be affected. The transannular hydrogen bond between Thr7 and the quinaldic acid may play a key role during thiostrepton maturation by stabilizing an intermediate's conformation for enzymatic processing. The production level of **1** under is typically $115 \pm 35 \text{ mg L}^{-1}$.¹¹ The mature analogs **3** and **5** were produced at 6 ± 2 and $12 \pm 3 \text{ mg L}^{-1}$, respectively. During posttranslational processing, a notable portion of the TsrA Thr7Ala and Thr7Val precursor peptides are diverted toward shunt products **4** and **6** (observed at 9 ± 3 and $3 \pm 1 \text{ mg L}^{-1}$, respectively).

In summary, we successfully generated variants of TsrA at the biologically critical Thr7 residue. Although impaired in production titers, the thiostrepton variants were subjected to structural characterization and assessment of their antibacterial activities. The Thr7Ala and Thr7Val substitutions impaired biological activity, confirming key interactions between Thr7 of **1** and the ribosome. Furthermore, the identification of **4** grants insight into the late stages of thiostrepton biosynthesis, suggesting that epoxidation of HEQ and quinaldic acid loop closure occur late during thiostrepton maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Bagley MC, Dale JW, Merritt EA, Xiong X. Chem Rev. 2005; 105:685–714. [PubMed: 15700961]
2. Su TL. Br J Exp Pathol. 1948; 29:473–481. [PubMed: 18123292]
3. Trejo WH, Dean LD, Pluscec J, Meyers E, Brown WE. J Antibiot. 1977; 30:639–643. [PubMed: 908663]
4. Brown LCW, Acker MG, Clardy J, Walsh CT, Fischbach MA. Proc Natl Acad Sci USA. 2009; 106:2549–2553. [PubMed: 19196969]
5. Kelly WL, Pan L, Li C. J Am Chem Soc. 2009; 131:4327–4334. [PubMed: 19265401]
6. Liao R, Duan L, Lei C, Pan H, Ding Y, Zhang Q, Chen D, Shen B, Yu Y, Liu W. Chem Biol. 2009; 16:141–147. [PubMed: 19246004]
7. Morris RP, Leeds JA, Naegeli HU, Oberer L, Memmert K, Weber E, LaMarche MJ, Parker CN, Burren N, Esterow S, Hein AE, Schmitt EK, Krastel P. J Am Chem Soc. 2009; 131:5946–5955. [PubMed: 19338336]
8. Brandi L, Marzi S, Fabbretti A, Fleischer C, Hill WE, Gualerzi CO, Stephen Lodmell J. J Mol Biol. 2004; 335:881–894. [PubMed: 14698286]
9. Modolell J, Cabrer B, Parmeggiani A, Vazquez D. Proc Natl Acad Sci USA. 1971; 68:1796–1800. [PubMed: 4331558]
10. Harms JM, Wilson DN, Schlutzen F, Connell SR, Stachelhaus T, Zaborowska Z, Spahn CMT, Fucini P. Mol Cell. 2008; 30:26–38. [PubMed: 18406324]
11. Li C, Zhang F, Kelly WL. Mol Biosyst. 2011; 7:82–90. [PubMed: 21107477]
12. Mocek U, Beale JM, Floss HG. J Antibiot. 1989; 42:1649–1652. [PubMed: 2584149]
13. Mocek U, Zeng Z, O'Hagan D, Zhou P, Fan LDG, Beale JM, Floss HG. J Am Chem Soc. 1993; 115:7992–8001.
14. Priestley ND, Smith TM, Shipley PR, Floss HG. Bioorg Med Chem. 1996; 4:1135–1147. [PubMed: 8831986]
15. Acker MG, Bowers AA, Walsh CT. J Am Chem Soc. 2009; 131:17563–17565. [PubMed: 19911780]
16. Bowers AA, Acker MG, Koglin A, Walsh CT. J Am Chem Soc. 2010; 132:7519–7527. [PubMed: 20455532]
17. Schoof S, Arndt HD. Chem Commun. 2009:7113–7115.
18. Jonker HR, Baumann S, Wolf A, Schoof S, Hiller F, Schulte KW, Kirschner KN, Schwalbe H, Arndt HD. Angew Chem Int Ed Engl. 2011; 50:3308–3312. [PubMed: 21365717]
19. Bond CS, Shaw MP, Alphey MS, Hunter WN. Acta Crystallogr D Biol Crystallogr. 2001; 57:755–758. [PubMed: 11320328]

20. Smith TM, Priestley ND, Knaggs AR, Nguyen T, Floss HG. J Chem Soc, Chem Comm. 1993:1612–1614.

Fig. 1.
Thiostrepton A **1** and analogs to be generated by mutagenesis of TsrA. Thr7 is indicated in blue and the residue numbers are in grey.

Fig. 2.
HPLC analysis of extracts from the cultures of (A) wild-type *S. laurentii*, (B) *S. laurentii* NDS1, (C) *S. laurentii* NDS1/int-3A104 (encoding TsrA Thr7Ser), (D) *S. laurentii* NDS1/int-3A105 (encoding TsrA Thr7Ala), and (E) *S. laurentii* NDS1/int-3A106 (encoding TsrA Thr7Val).

Scheme 1.

Proposed biosynthesis of thioestrepton Thr7Ala **3** and SL105-1 **4**. The positions corresponding to the pyruvyl residue (Pyr3) and HEQ (Q) are shown in red and blue, respectively.