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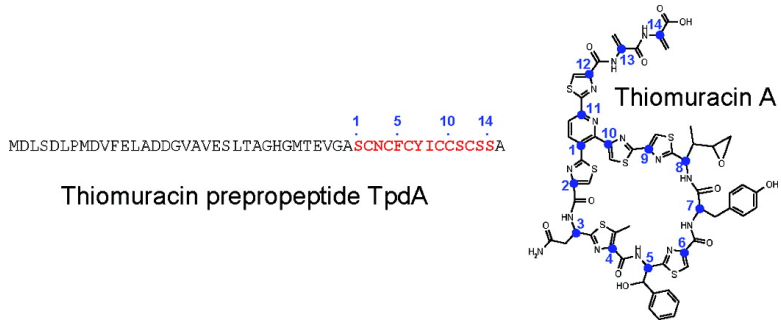

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Ribosomally Synthesized Thiopeptide Antibiotics Targeting Elongation Factor Tu

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Abstract: We identified the thiomuracins, a novel family of thiopeptides produced by a rare-actinomycete bacterium typed as a *Nonomuraea* species, via a screen for inhibition of growth of the bacterial pathogen *Staphylococcus aureus*. Thiopeptides are a class of macrocyclic, highly modified peptides that are decorated by thiazoles and defined by a central six-membered heterocyclic ring system. Mining the genomes of thiopeptide-producing strains revealed the elusive biosynthetic route for this class of antibiotics. The thiopeptides are chromosomally encoded, ribosomally synthesized proteins, and isolation of gene clusters for production of thiomuracin and the related thiopeptide GE2270A revealed the post-translational machinery required for maturation. The target of the thiomuracins was identified as bacterial Elongation Factor Tu (EF-Tu). In addition to potently inhibiting a target that is unexploited by marketed human therapeutics, the thiomuracins have a low propensity for selecting for antibiotic resistance and confer no measurable cross-resistance to antibiotics in clinical use.

Introduction

Many peptidic, microbe-derived natural products are synthesized from proteinogenic and nonproteinogenic amino acids via multienzyme complexes known as nonribosomal peptide synthetases (NRPS). The beta-lactam antibiotics penicillin and cephalosporin are notable examples of peptides of nonribosomal origin. Another clinically relevant NRPS-assembled structure is the immunosuppressive cyclic peptide cyclosporine which is prescribed to prevent graft rejection after organ transplantation.

In contrast to the NRPS biosynthesis route, microorganisms also utilize elaborate biosynthetic strategies to transform ribosomally synthesized short peptides into complex, highly functionalized molecules with diverse biological activities. Unlike NRPS, this process initiates with a chromosomally encoded peptide sequence. Historically, the lantibiotics were the only large group of highly modified peptidic antibiotics known to be ribosomally encoded, with the elucidation of their biosynthetic route over 20 years ago. Lantibiotics are synthesized by Gram-positive bacteria and typically consist of 20−35 amino acids. The lantibiotics are clearly distinguished among the bacterial protein toxins by intramolecular rings formed by the thioether amino acids lanthionine and methyllanthionine. These compounds, typified by nisin from *Lactococcus lactis* and cinnamycin produced by several *Streptomyces* strains, inhibit cell wall biosynthesis of closely related bacterial species.[1−3]

The formation of heterocycles is a strategy used by microorganisms to rigidify flexible peptide chains to shape ribosomal and nonribosomal peptides.[4] Heterocycles incorporated post-translationally are reported for both linear peptides and cyclic peptides synthesized via a ribosomal route. The precursor peptides of the linear peptide toxins microcin B17, a DNA gyrase inhibitor,[5] and streptolysin S[6] are encoded by a chromosomal gene. The mature product is usually obtained through proteolysis after completion of the posttranslational modifications leading to thiazole and oxazole heterocycles. The patellamides from the cyanobacterium *Prochloron* are the first example of cyclic peptides containing heterocycles for which a ribosomal origin could be demonstrated.[7,8] This biosynthetic

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(1) Lubelski, J.; Rink, R.; Khusainov, R.; Moll, G. N.; Kuipers, O. P. *Cell. Mol. Life Sci.* **2008**, *65*, 455–476.
(2) Willey, J. M.; van der Donk, W. A. *Annu. Rev. Microbiol.* **2007**, *61*, 477–501.
(3) Widdick, D. A.; Dodd, H. M.; Barraille, P.; White, J.; Stein, T. H.; Chater, K. F.; Gasson, M. J.; Bibb, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4316–4321.
(4) Walsh, C. T.; Nolan, E. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5655–5656.
(5) Li, Y. M.; Milne, J. C.; Madison, L. L.; Kolter, R.; Walsh, C. T. *Science.* **1996**, *274*, 1188–1193.
(6) Lee, S. W.; Mitchell, D. A.; Markley, A. L.; Hensler, M. E.; Gonzales, D.; Wohlrab, A.; Dorrestein, P. C.; Nizet, V.; Dixon, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5879–5884.
(7) Schmidt, E. W.; Nelson, J. T.; Rasko, D. A.; Sudek, S.; Eisen, J. A.; Haygood, M. G.; Ravel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7315–7320.
(8) Long, P. F.; Dunlap, W. C.; Battershill, C. N.; Jaspars, M. *ChemBioChem* **2005**, *6*, 1760–1765.

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scheme was extended to a new class of cyanobacterial peptides harboring heterocyclized residues called cyanobactins which comprise more than 100 cyclic peptides from symbiotic and free living cyanobacteria.[9] The groups of Hemscheidt and Dittmann recently described the biosynthesis of the microviridin class of tricyclic depsipeptides from the cyanobacteria *Planktothrix*[10] and *Microcystis*.[11] Again, the peptide sequence consists of a leader peptide and a core peptide and is encoded by a structural gene in the biosynthetic gene cluster. Thus, this biosynthetic mechanism seems to be widespread in cyanobacteria and not limited to monocyclic peptides like the cyanobactins.

Thiopeptides are another class of heterocycle-containing cyclic peptides. At present, this class encompasses about 80 structures subdivided into 29 different antibiotic families including the archetypal thiopeptide thiostrepton, which was first isolated more than half a century ago.[12,13] Despite their early discovery and their commercial relevance as a topical veterinary antibiotic (thiostrepton) or as a growth promotant in animal husbandry (nosiheptide), the biosynthetic route for the thiopeptide antibiotics has remained elusive for many decades. GE2270A (**10**) and thiostrepton (**11**) have a tri- and tetrasubstituted pyridine ring at the junction of the macrocycle and the side chain, which distinguishes the thiopeptide antibiotics from other thiazole-containing peptide compounds, such as microcin B17 and streptolysin or the cyanobactins.

The mechanism of antibacterial activity of the thiopeptides is via inhibition of protein synthesis. Nevertheless, despite structural homology within the thiopeptide class of antibiotics, the modes of translation inhibition result from at least two distinct activities: interaction with a region of the 23S rRNA known as the L11 binding domain or interaction with aa-tRNA chaperone elongation factor Tu (EF-Tu). The only thiopeptide that has been in development for human use is a derivative of the EF-Tu inhibitor GE2270A (**10**), which completed a phase 1 clinical trial for the topical treatment of acne.[14]

This report introduces a new family of potent antibacterial thiopeptides, the thiomuracins A−H (**1**−**9**). Based on the central heterocyclic domain, the thiomuracins fall into series *d* of the thiopeptides which is characterized by a trisubstituted pyridine ring system.[13] Also included in this series are the EF-Tu inhibitors GE2270A (**10**)[15] and amythiamicin.[16] The thiomuracins are produced by a strain of *Nonomuraea*[17] and were discovered via a bacterial growth inhibition screen of natural product extracts. The *Nonomuraea* genus belongs to the larger group of G+C rich Gram-positive bacteria referred to as the actinomycetes, best known for being prolific producers of antibacterial compounds including the peptidic antibiotics daptomycin, pristinamycin, and vancomycin. Another *Nonomuraea* strain from the Novartis collection was shown to produce the EF-Tu inhibitor GE2270A (**10**).

We identified the biosynthetic gene cluster for thiomuracin A (**1**) and GE2270A (**10**) in the genomes of the *Nonomuraea* producing strains via a PCR strategy using degenerate primers. Our search was based on sequences of the structural genes that were predicted to encode the precursor peptides. Together with the recently reported thiocillin biosynthesis gene cluster[18] these results offer a significant advancement into our understanding of the biosynthesis of the thiopeptide class of natural products.

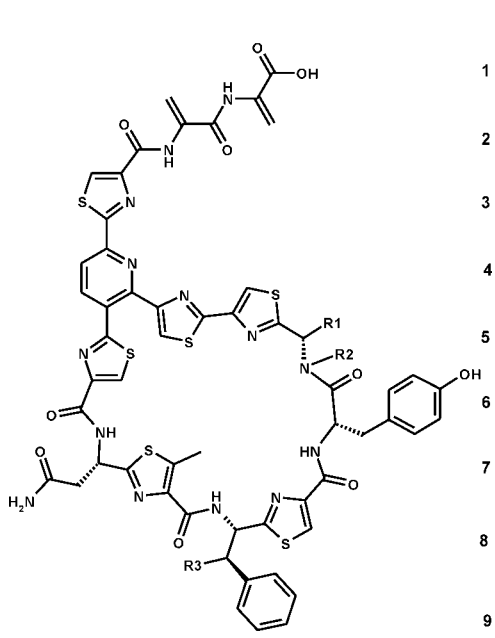
Results

Discovery of New Thiopeptide Antibiotics. A library of pure natural products and microbial extracts was assessed for antibacterial activity via high throughput screening against the difficult-to-treat human pathogen *Staphylococcus aureus*.[19] A bioactive extract from the *Nonomuraea* strain Bp3714-39 was shown by ultraviolet and mass spectral analyses to contain compounds of unknown structure. Comparison of the UV spectrum of one of the newly identified compounds with a library of UV spectra from compounds of known structures revealed a similarity to the antibiotic GE2270A (**10**). The match suggested that the new compounds belonged to the thiopeptide class of molecules. GE2270A (**10**), purified from *Nonomuraea* strain WU8817, was identified as an antibacterial hit in the same assay.

The new compounds were named thiomuracins (Figure 1). Between 2 and 12 mg of thiomuracin B−G (**2**−**7**) and I (**9**) were isolated from a 100 L fermentation which was extracted with ethyl acetate under acidic conditions and purified through a combination of size exclusion and reversed phase chromatography. The main metabolite of the strain, thiomuracin A (**1**), was not isolated directly from the fermentation material because the compound is unstable under acidic conditions and completely decomposed during the acidic extraction. To isolate thiomuracin A (**1**) the broth from a 100 L fermentation was extracted at pH 7 and purified through a two-step normal phase chromatography using dichloromethane and methanol as solvents, which yielded 90 mg of the metabolite. Ultimately, an additional metabolite, thiomuracin H (**8**), was also isolated from a fermentation of *Nonomuraea* Bp3714-39.

Structure Elucidation. High resolution ESI-MS analysis of thiomuracin A (**1**) showed a molecular ion at *m/z* 1339.2123 (M+H), which suggested a corresponding molecular formula $C_{59}H_{50}N_{14}O_{12}S_6$. Comparison of the UV spectrum of thiomuracin A (**1**) with the UV spectrum of GE2270A (**10**) and the presence of a thiazole CH stretching at 3114 cm^{-1} in the infrared spectrum suggested that thiomuracin A (**1**) also belonged to the thiopeptide class of natural products. The chemical constitution of thiomuracin A (**1**) was ultimately determined by homo- and heteronuclear NMR correlations. The ^1H and ^{13}C NMR data of thiomuracin A (**1**) are summarized in Table 1, and observed HMBC correlations are shown in Figure 2. The spin systems of asparagine, tyrosine, β-hydroxy phenylalanine, two dehydroalanines, and a γ-epoxy isoleucine were assigned on the basis of 2D NMR spectroscopy. Besides these amino acids, five

(9) Donia, M. S.; Ravel, J.; Schmidt, E. W. *Nat. Chem. Biol.* **2008**, *4*, 341–343.
(10) Philmus, B.; Christiansen, G.; Yoshida, W. Y.; Hemscheidt, T. K. *ChemBioChem* **2008**, *9*, 3066–3073.
(11) Ziemert, N.; Ishida, K.; Liaimer, A.; Hertweck, C.; Dittmann, E. *Angew. Chem., Int. Ed.* **2008**, *47*, 7756–7759.
(12) Donovick, R.; Pagano, J. F.; Stout, H. A.; Weinstein, M. J. *Antibiot. Annu.* **1955**, *3*, 554–559.
(13) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, X. *Chem. Rev.* **2005**, *105*, 685–714.
(14) Butler, M. S. *Nat. Prod. Rep.* **2008**, *25*, 475–516.
(15) Selva, E.; Beretta, G.; Montanini, N.; Saddler, G. S.; Gastaldo, L.; Ferrari, P.; Lorenzetti, R.; Landini, P.; Ripamonti, F.; Goldstein, B. P.; Berti, M.; Montanaro, L.; Denaro, M. *J. Antibiot. (Tokyo)* **1991**, *44*, 693–701.
(16) Shimanaka, K.; Kinoshita, N.; Iinuma, H.; Hamada, M.; Takeuchi, T. *J. Antibiot. (Tokyo)* **1994**, *47*, 668–674.
(17) Zhang, Z.; Wang, Y.; Ruan, J. *Int. J. Syst. Bacteriol.* **1998**, *48*, 411–422.
(18) Brown, L. C.; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2549–2553.
(19) Schopfer, U.; Engeloch, C.; Höhn, F.; Mees, H.; Leeds, J.; Glickman, F.; Scheel, G.; Ferrand, S.; Fekkes, P.; Pfeifer, M."Hypothesis Driven Screening" Chemogenomics - Methods and Protocols. *Methods in Molecular Biology*; Humana Press: Totowa, in press.



					E. faecalis ATCC29212	*S. aureus* ATCC29213
1	thiomuracin A	R1 =	R2 = H	R3 = OH	0.25	0.5
2	thiomuracin B	R1 =	R2 = H	R3 = OH	0.5	0.5
3	thiomuracin C	R1 =	R2 = H	R3 = OH	1	1
4	thiomuracin D	R1 =	R2 = H	R3 = H	0.5	0.5
5	thiomuracin E	R1 =	R2 = H	R3 = OH	4	16
6	thiomuracin F	R1 =	R2 = H	R3 = OH	0.5	0.5
7	thiomuracin G	R1 =	R2 = H	R3 = OH	0.5	2
8	thiomuracin H	R1 =	R2 = H	R3 = OH	nd	nd
9	thiomuracin I	R1 = R2 =		R3 = OH	0.25	1.0

Figure 1. Structures of thiomuracin A−I (**1**−**9**) and antibacterial activity against *E. faecalis* and *S. aureus* (minimum inhibitory concentrations in μg/mL).

aromatic singlets, two doublets in the aromatic region, and one methyl singlet at 2.7 ppm were present in the ^{1}H spectrum. It was assumed that the singlets and the two doublets in the aromatic region belong to a complex type of structure with a central heteroaromatic cluster, consisting of four thiazoles and one pyridine. An identical cluster is found in the thiopeptide antibiotics GE2270A (**10**)[20] and amythiamicin A.[21] Based on the comparison with published NMR data and the HMBC and ROESY correlations of thiomuracin A (**1**), the sequence of all fragments (6 amino acids, 5 thiazoles, one 5-methyl-thiazole, and a pyridine ring) could be determined and all protons and carbons were assigned. The structure elucidation of thiomuracin B−I (**2**−**9**) was achieved by comparison of the molecular formulas and the NMR data with the data of thiomuracin A (**1**).

For the assignment of the relative and absolute configuration of the thiomuracins, NOE correlations in spectra of thiomuracin I (**9**) were analyzed. In thiomuracin I (**9**) the epoxide side chain has formed a β-methyl-δ-hydroxy-proline ring system. The stereochemistry within the ring system was unambiguously determined by the NOE correlations seen in the ROESY spectrum as shown with arrows in Figure 2. The NOE correlations between 84-H and 60-H allowed the assignment of the relative configuration of the stereocenter in the tyrosine. With regard to the β-hydroxy phenylalanine the coupling constant of 4.4 Hz between H-44 and H-45 indicates that the side chain does not freely rotate. NOEs between H-64/66 of tyrosine and H-49/50/51 of β-hydroxy phenylalanine suggest a neighboring of the two aromatic rings. The ^{1}H shift of OH-52 at 6.4 ppm points to a hydrogen bond of the OH group, probably to the carbonyl group 42. The medium NOE intensity between H-44 and H-45 supports a transoid conformation (with a dihedral angle of 130° to 140° according to the coupling constant 4.4 Hz.). With this information the relative stereochemistry of 6 of the 7 stereocenters could be assigned. In addition, from the relative stereochemistry at the atoms C-44, C-60, and C-81, we concluded that β-hydroxy phenylalanine, tyrosine, and isoleucine amino acids are all either L- or D-amino acids. In analogy to the related thiopeptides GE2270A (**10**) and amythiamicin we assumed that the thiomuracins also possess L-configuration amino acid stereocenters. Therefore the absolute stereochemistry for thiomuracin I (**9**) is proposed as (32S, 44S, 45R, 60S, 81S, 82S, 84S). For thiomuracin A (**1**) our proposed stereochemical assignment (32S, 44S, 45R, 60S, 81S, 82R) unfortunately excludes the epoxide stereocenter, as NMR assignment was not possible.

Antibacterial Activities. Following identification of the antibacterial activity of the *Nonomuraea* strain Bp3714-39 extract, minimum inhibitory concentrations (MIC) of purified thiomuracin analogues were characterized by the standard broth microdilution assay.[22] The results shown in Figure 1 demonstrate the potent antimicrobial activities of the thiomuracins against the Gram-positive species *Enterococcus faecalis* and *S. aureus*. The compounds have no measurable antibacterial activity against the Gram-negative pathogens *Escherichia coli* and *Pseudomonas aeruginosa* (all MIC > 32 μg/mL; data not shown). This lack of antimicrobial activity may reflect poor penetration of the Gram-negative outer membrane by the compounds.

Identification of EF-Tu as the Molecular Target. As a means to identify the target of the thiomuracins, spontaneously resistant mutants of *S. aureus* NCTC 8325-4 were selected on 1 μg/mL (8 × MIC) of thiomuracin I (**9**). Single-step mutants were selected at a frequency of 1.1×10^{-9}, suggesting the presence

(20) Tavecchia, P.; Gentili, P.; Kurz, M.; Sottani, C.; Bonfichi, R.; Lociuro, S.; Selva, E. *J. Antibiot. (Tokyo)* **1994**, *47*, 1564–1567.
(21) Shimanaka, K.; Takahashi, Y.; Iinuma, H.; Naganawa, H.; Takeuchi, T. *J. Antibiot. (Tokyo)* **1994**, *47*, 1153–1159.
(22) [National Committee for Clinical Laboratory Standards]. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically; Approved standard - sixth edition. NCCLS document M7-A6, NCCLS, Wayne, PA, 2003.

Table 1. ^{1}H and ^{13}C NMR Data of Thiomuracin A (**1**)[a]

atom number	group	^{13}C shift (ppm)	^{1}H-NMR ^{1}H shift (ppm)	^{1}H-NMR multiplicity, coupling const.	atom number	group	^{13}C shift (ppm)	^{1}H-NMR ^{1}H shift (ppm)	^{1}H-NMR multiplicity, coupling const.
2	C q	164.71			45	CH	71.63	5.43	m
3	C q	135.2			46	C q	140.07		
4	CH_2	108.36	6.13	s	47	CH	126.28	7.04	m
			5.82	s	48	CH	127.63	7.23	m
5	NH		9.59	s	49	CH	127.31	7.23	m
6	C q	161.75			50	CH	127.63	7.23	m
7	C q	134.72			51	CH	126.28	7.04	m
8	CH_2	104.9	6.47	s	52	OH		6.35	broad
			5.79	s	53	C q	166.43		
9	NH		10.12	s	55	C q	147.15		
10	C q	158.85			56	CH	125.39	8.16	s
14	C q	150.27			58	C q	159.21		
15	CH	128.75	8.63	s	59	NH		7.72	d, 8.1 Hz
17	C q	167.59			60	CH	53.23	4.88	m
19	C q	149.91			61	CH_2	37.98	3.21	m
20	CH	118.66	8.33	d, 8.1 Hz				2.63	m
21	CH	140.96	8.47	d, 8.1 Hz	62	C q	127.03		
22	C q	128.42			63	CH	129.95	6.95	d, 8.1 Hz
23	C q	150.68			64	CH	115.07	6.64	d, 8.1 Hz
25	C q	164.25			65	C q	155.84		
27	CH	126.79	8.49	s	66	CH	115.07	6.64	d, 8.1 Hz
28	C q	149.5			67	CH	129.95	6.95	d, 8.1 Hz
30	C q	160			68	OH		9.12	s, broad
31	NH		8.99	d, 7.3 Hz	69	C q	171.39		
32	CH	48.97	5.19	m	70	NH		9.07	d, broad
33	CH_2	38.31	1.96	m	71	C q	173.06		
			2.76	m	73	CH	116.26	7.47	s
34	C q	171.64			74	C q	147.2		
35	NH_2		7.32	s	76	C q	160.54		
			6.81	s	78	C q	152.99		
36	C q	168.23			79	CH	123.03	8.1	s
38	C q	140.17			81	CH	55.55	5.19	m
39	C q	141.55			82	CH	40.16	1.96	m
41	CH_3	12.03	2.69	s	83	CH	52.96	3.11	m
42	C q	161.42			84	CH_2	44.48	2.73	m
43	NH		8.68	d. 5.9 Hz				2.55	m
44	CH	57.32	5.47	m	85	CH_3	12.55	0.94	d, 7.3 Hz

[a] Proton of carboxy group 1 not visible.

of a gain-of-function point mutation. Several candidate genes were sequenced based upon the similarity of the thiomuracins to thiopeptides of known mode of action (MOA), for example GE2270A (**10**) and thiostrepton (**11**). The selected mutants harbored one of two single nucleotide changes in *tufA* (G826A/C), the gene encoding EF-Tu, within the codon for glycine 275 (*E. coli* numbering). The changes resulted in amino acid substitutions with serine or alanine at that position. Residue 275 is located within EF-Tu domain II, the binding site for the thiopeptide antibiotic GE2270A (**10**).[23]

To investigate whether the phenotype of a thiomuracin I (**9**) resistant mutant of *S. aureus* was specific for the thiopeptides targeting EF-Tu, the parent and a resistant mutant were tested for sensitivity to a panel of antibiotics with a range of mechanisms of action. Table 2 shows the susceptibilities of a thiomuracin I resistant mutant (*tufA*), as well as a thiostrepton resistant mutant (*rplK*), to the selecting compounds, the related thiopeptide GE2270A (**10**), the structurally unrelated protein synthesis inhibitor tetracycline, and unrelated antibiotics of varying MOA. Thiostrepton binds to the 23S rRNA component of the 50S ribosomal subunit, and single-step resistant mutations are typically isolated in *rplK*, the gene encoding ribosomal protein L11.[24] Tetracycline, a microbe-derived natural product inhibitor of bacterial protein synthesis with a structure unrelated to the thiopeptides, binds to the 30S subunit of the ribosome, and resistance in *S. aureus* is typically conferred by a ribosomal protection mechanism via TetM, or by drug efflux via TetK.[25]

(23) Heffron, S. E.; Jurnak, F. *Biochemistry.* **2000**, *39*, 37–45.

(24) Pucci, M. J.; Bronson, J. J.; Barrett, J. F.; DenBleyker, K. L.; Discotto, L. F.; Fung-Tomc, J. C.; Ueda, Y. *Antimicrob. Agents Chemother.* **2004**, *48*, 3697–701.

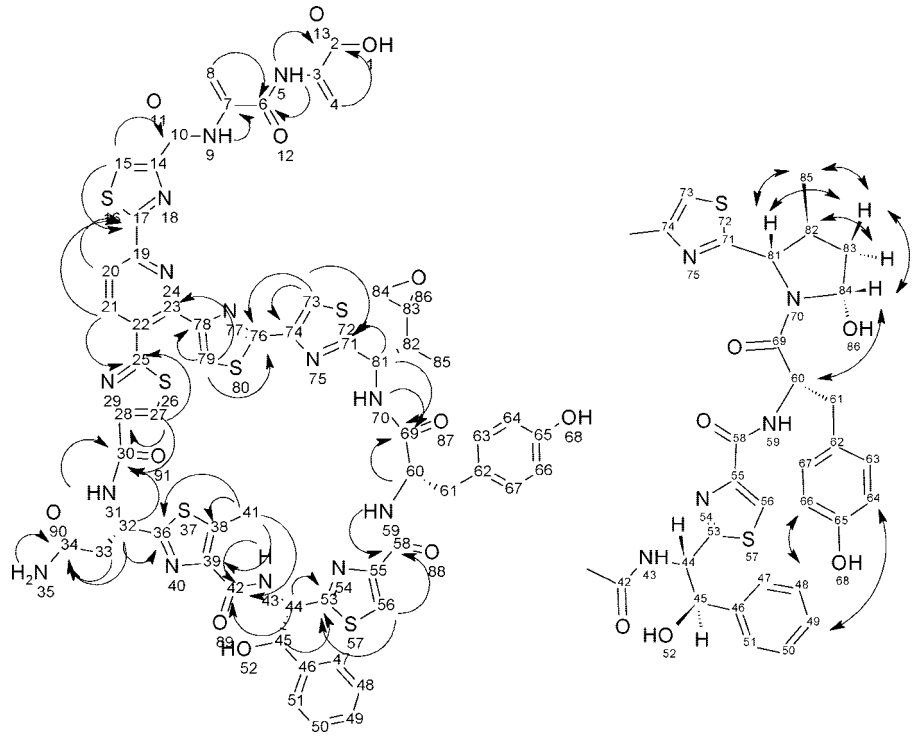


Figure 2. Structure elucidation and relative stereochemistry of thiomuracins. Observed HMBC correlations for thiomuracin A (**1**) and NOE correlations for thiomuracin I (**9**).

Table 2. Microbiological Profile of Thiomuracin

Antibiotic	*S. aureus*		
	Wild type (NCTC 8325−4)	*tufA* G826A (Gly275→Ser)	*rplK* Δ *58−75* *(L11Δ P20-G25)*
Linezolid	1	1	
Daptomycin	≤0.03	≤0.03	
Vancomycin	1	1	
Tetracycline	≤0.25	≤0.25	0.5
Ciprofloxacin	0.25	0.5	
Ampicillin	0.25	≤0.125	
Gentamycin	≤0.25	≤0.25	
Fosfomycin	4	4	
Methicillin	2	1	
Cefotaxime	32	32	
Mupirocin	≤0.25	≤0.25	
Chloramphenicol	4	4	
Erythromycin	≤0.25	≤0.25	
Rifampicin	≤0.25	≤0.25	
Thiomuracin I (**9**)	0.125	4	0.25
GE2270A (**10**)	0.25	>32	0.125
Thiostrepton (**11**)	0.5	0.5	16

Other than resistance to the thiomuracins and GE2270A (**10**), no significant differences in sensitivity of the thiomuracin I (**9**) resistant strain to antibiotics directed against a wide variety of bacterial targets were observed when compared to the parental strain. These data support a target-specific mechanism of resistance to the thiomuracins. Of clinical importance, the thiomuracins did not select for cross-resistance to antibiotics of unrelated structure or mechanisms of action.

To investigate the affinities of thiomuracin analogues for bacterial EF-Tu, the protein was purified from *E. coli* and the binding of thiomuracins for the GDP-bound form of the enzyme was measured using a surface plasmon resonance assay (Biacore). Biotinylated *E. coli* EF-Tu:GDP was immobilized on a streptavidin-coated sensor chip, and association rate constants (k_{on}), dissociation rate constants (k_{off}), and dissociation constants (K_D) for thiomuracin A (**1**) and I (**9**) were measured. The K_D of thiomuracin A (**1**) was 88.8 nM, and that of thiomuracin I (**9**) was 129 nM. For comparison, the K_D of GE2270A (**10**) was determined to be 8.7 nM. The data shown in Table 2 and the affinity measurements described support the hypothesis that thiomuracin I (**9**) and related thiomuracin analogues target EF-Tu, similar to the related thiopeptide GE2270A (**10**).

Ribosomal Biosynthesis. Structure elucidation determined that the amino acid stereoisomeric centers present in thiomuracins and GE2270A (**10**)[26] are in the proteinogenic L-form, which supported a ribosomal synthetic route for these compounds. To test this hypothesis, chromosomal DNA from both thiopeptide-producing *Nonomuraea* strains, Bp3714-39 and WU8817, were mined for sequences that could encode the thiopeptide backbones. A PCR strategy, adapted from a mycobacterial genetic footprinting method,[27] was employed using degenerate oligonucleotides to probe adaptor-ligated genomic libraries. First, primers designed using the amino acid sequence of the thiopeptide macrocycle as a template (Figure 3a) and a primer specific for the adaptor were used to amplify sequences encoding thiopeptide linear precursors. Next, gene specific primers were employed to extend the regions surrounding the thiopeptide encoding sequences. Small homologous open reading frames (ORFs) were identified from both *Nonomuraea* species, which encoded polypeptides with the 14 amino acid primary sequence of the respective thiopeptide backbone toward the C-terminus (Figure 3b). As expected, cysteine and serine residues are in

(25) Chopra, I.; Roberts, M. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260.

(26) Tavecchia, P.; Gentili, P.; Kurz, M.; Sottani, C.; Bonfichi, R.; Selva, E.; Lociuro, S.; Restelli, E.; Ciabatti, R. *Tetrahedron* **1995**, *51*, 4867–4869.

(27) Sassetti, C. M.; Boyd, D. H.; Rubin, E. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12712–12717.

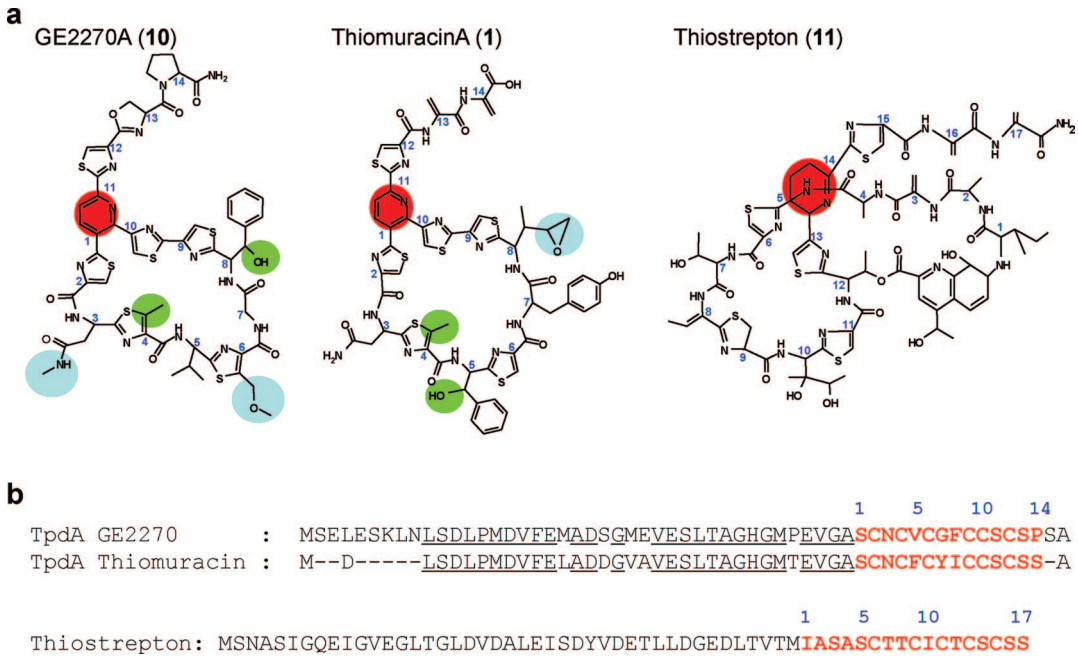


Figure 3. Small genes encode thiopeptide backbones. (a) The thiopeptides thiomuracin A (**1**), GE2270A (**10**), and the archetypal thiopetide thiostrepton (**11**). The thiopeptide canonical substituted heterocyclic ring system (red). Tailoring modifications: shared by GE2270A (**10**) and thiomuracin A (**1**) (green) and compound specific (blue). Amino acid α-carbons (blue numbering). (b) Thiopeptide precursor polypeptides. Thiopeptide backbones (red bold type). Blue numbering according to the molecular trace of α-carbons. Underlined are identical amino acids, and dashes represent gaps in the sequence alignment of leader peptides belonging to GE2270 and thiomuracin.

progenitor positions of the thiazoles and oxazoline heterocycles.[5,28] These propeptide sequences are also in agreement with isotopic feeding experiments performed by De Pietro et al. with the first described GE2270A (**10**) producing strain *Planobispora rosea*.[29] These studies demonstrated that two serine residues are the precursors of the central trisubstituted pyridine ring.[30]

To further test the hypothesis that the thiopetides, as a class, are gene encoded, we also mined the genome of the thiostrepton (**11**) producer *Streptomyces azureus* ETH28555 for a sequence that could encode a thiostrepton (**11**) precursor peptide. A putative structural gene was identified that encodes the 17 amino acid backbone but with a leader peptide with little homology to the thiomuracin and GE2270 encoding prepropeptides. Specific for this class of bicyclic thiopeptides and also belonging to this gene encoded sequence is the four amino acid extension at the N-terminus predicted to fold back and form the intramolecular bridge with quinaldic acid.[13,30] As observed for the proposed thiomuracin and GE2270 biosynthetic routes, two serine codons are found at positions expected to form the central tetra-substituted pyridine ring, and again in agreement with published isotopic feeding experiments.[31]

To exploit the clustering of biosynthetic genes in bacterial genomes, the putative thiomuracin and GE2270 structural genes were used to probe Bacterial Artificial Chromosome (BAC) libraries prepared from the genomic DNA isolated from *Nonomuraea* production strains. BAC clones harboring the structural genes were identified from both libraries. Sequencing demonstrated that the structural genes colocalized within a cluster of genes encoding for the functionalities on the respective thiopeptide structures. The GE2270 and thiomuracin gene clusters were assigned 18 and 12 ORFs, respectively (Figure 4a). The boundaries of the gene clusters were established by comparison of both chromosomal loci and from the deduced functions of the gene products (Table 3).

Although the ORF number and overall organization of the biosynthetic clusters are different, there appears to be a core set of orthologous genes that have a syntenous relationship, now referred to as *tpdA*−*tpdG* (**t**hio**p**epti**d**e). These core genes and their organization are reminiscent of clusters encoding the bacteriocin[6] and cyanobactin[9] biosynthetic machinery, which were recently characterized from other bacteria. The first gene *tpdA* is the structural gene that encodes the propeptide. *tpdA* is upstream from genes *tpdB*−*tpdC*, which have weak similarity to lantibiotic biosynthetic genes[32] and are predicted to be involved in the conversion of serine residues to dehydroalanines. This dehydrative transformation is a prerequisite for forming the pyridine ring (Figure 4b)[13] and supports the original proposed mechanism of Bycroft and Gowland.[33] Intramolecular Aza Diels−Alder cycloaddition between the two dehydroalanine residues formed at position 1 and 11 of the propeptide sequence is distinctive for this class of molecules. Such a reaction results in the elimination of the leader peptide and circumvents the need for a dedicated peptidase, which is necessary for the maturation of the cyanobactin group of cyclic thiazolyl peptides.[9] The genes *tpdE* and *tpdG* share weak similarity with genes involved in thiazole formation found in the patellamide gene cluster (Figure 4a; *patG* and *patD*)[7] and a cluster involved in the production of the streptomycete morphogen goadsporin

(28) Roy, R. S.; Gehring, A. M.; Milne, J. C.; Belshaw, P. J.; Walsh, C. T. *Nat. Prod. Rep.* **1999**, *16*, 249–263.
(29) De Pietro, M. T.; Marazzi, A.; Sosio, M.; Donadio, S.; Lancini, G. *J. Antibiot. (Tokyo)* **2001**, *54*, 1066–1071.
(30) Strohl, W. R.; Floss, H. G. *Biotechnology* **1995**, *28*, 223–238.
(31) Zhou, P.; O'Hagan, D.; Mocek, U.; Zeng, Z.; Yuen, L. D.; Frenzel, T.; Unkefer, C. J.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1989**, *111*, 7274–7276.
(32) Sahl, H. G.; Bierbaum, G. *Annu. Rev. Microbiol.* **1998**, *52*, 41–79.
(33) Bycroft, B. W.; Gowland, M. S. *J. Chem. Soc., Chem. Commun.* **1978**, 256–258.

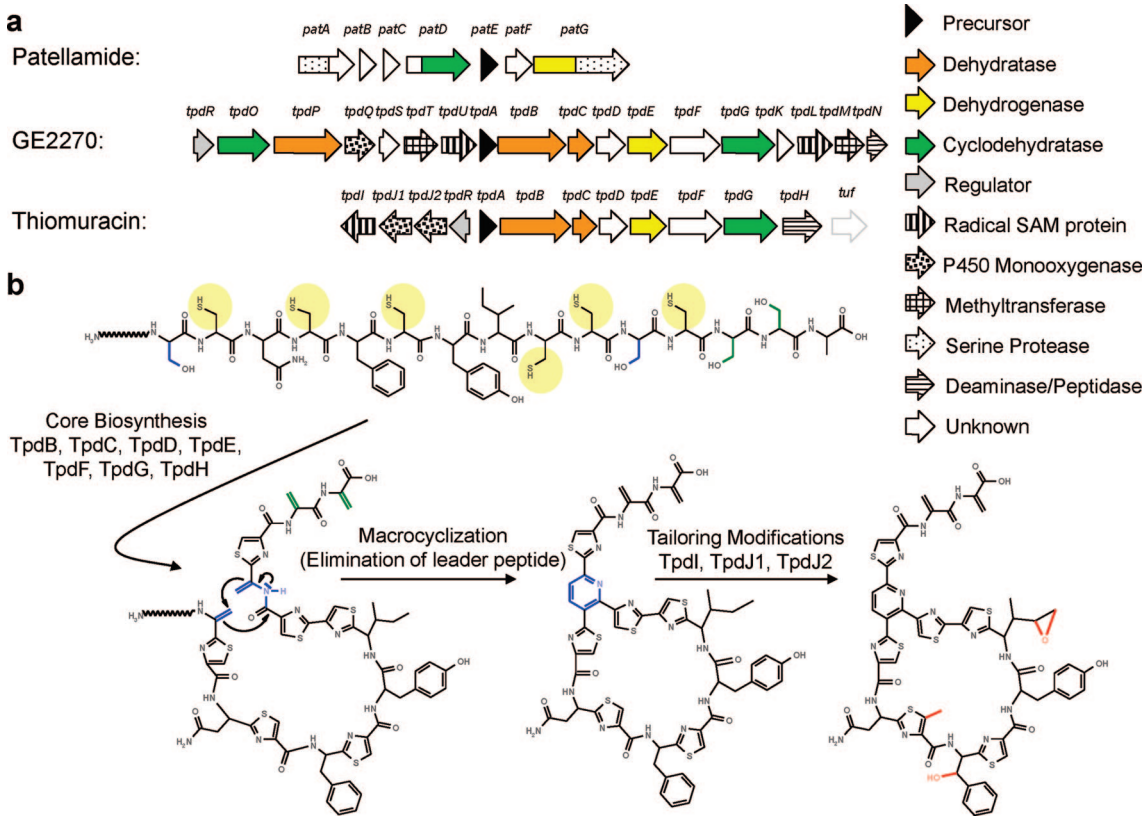


Figure 4. Thiopeptide biosynthesis. (a) Gene organization of the GE2270, thiomuracin and the cyanobacterial patellamide gene clusters. Included in the thiomuracin map is the downstream *tuf* (gray outline) gene encoding an EF-Tu. (b) Proposed thiomuracin biosynthetic route from the *tpdA* encoded linear peptide to the fully modified macrocyclic structure. Highlighted in yellow are the cysteine side chains, which are the precursors for the thiazoles. Serine and dehydroalanine residues forming the pyridine ring (blue bonds and atoms), serine residues forming the dehydroalanine residues found in tail (green bonds and atoms), and tailoring modifications (red bonds and atoms).

(*godE* and *godD*).[34] *tpdF* is predicted to encode a large conserved hypothetical protein that may act as a docking protein. The homology of TpdB, TpdC, TpdE, and TpdG to post-translational modification enzymes suggests that they form a core biosynthetic unit involved in the processing and buildup of the common polythiazole macrocyclic structure shared by both thiopeptide series.

In addition to these core homologous sequences are genes that are predicted to encode tailoring enzymes that introduce modifications common and specific to both molecules (Figure 3a). The ancillary nature of these transformations are represented by their presence and absence on side products isolated from the fermentative broths of Bp3714-39 (Figure 1) and the original GE2270A (**10**) producer.[35] Common to both clusters are genes encoding radical *S*-adenosylmethionine (SAM) proteins,[36] with a significant similarity to the coproporphyrinogen III oxidases, known to catalyze unusual methylations and P450 monooxygenases[37] that could be responsible for the thiazole methylation and the phenylalanine β-hydroxylation, respectively (Figure 3a, blue). Specific for thiomuracin A (**1**) (Figure 3a, green) biosynthesis is the epoxidation of the isoleucine side chain. The presence of this modification can be accounted for by the decomposition of a double hydroxylation performed by an additional P450 monooxygenase (either TpdJ1 or TpdJ2). Alternatively, the epoxide might originate via insertion of molecular oxygen into a terminal olefin derived from a monohydroxylated side chain. Specific to GE2270A (**10**) is the methylation of the asparagine side chain and is expected to involve *tpdT* encoding an *N*-methyl transferase. The unique coupled *tpdL* and *tpdM* encode an additional radical SAM protein and an *O*-methyl transferase that likely catalyze the formation of the methoxyethyl group. Furthermore, the GE2270A (**10**) gene cluster contains paralogous genes encoding an additional cyclodehydratase and dehydratase, *tpdO* and *tpdP*, which may be involved in modifying the tail sequence resulting in the introduction of the oxazoline ring system.[28]

Gene Disruption and Expression Analysis. Two strategies were employed to confirm the involvement of the isolated clusters in thiopeptide biosynthesis. Because the thiomuracin producing *Nonomuraea* strain Bp3714-39 has been genetically intractable thus far, an RT-PCR approach was employed to monitor gene expression of the thiomuracin cluster. The genes chosen for analysis were the transcriptional regulator *tpdR*, the structural gene *tpdA*, and a *tuf* gene, located immediately downstream from the thiomuracin cluster and encoding an elongation factor Tu with an amino acid sequence predicted to confer thiopeptide resistance. The *tuf* gene was not annotated as belonging to the thiomuracin cluster due to its absence in sequences contiguous to the GE2270 cluster. Quantitative RT-PCR demonstrated a strong induction in *tpdA* and *tpdR* gene

(34) Onaka, H.; Nakaho, M.; Hayashi, K.; Igarashi, Y.; Furumai, T. *Microbiology* **2005**, *151*, 3923–3933.
(35) Selva, E.; Ferrari, P.; Kurz, M.; Tavecchia, P.; Colombo, L.; Stella, S.; Restelli, E.; Goldstein, B. P.; Ripamonti, F.; Denaro, M. *J. Antibiot. (Tokyo)* **1995**, *48*, 1039–1042.
(36) Wang, S. C.; Frey, P. A. *Trends Biochem. Sci.* **2007**, *32*, 101–110.
(37) Isin, E. M.; Guengerich, F. P. *Biochim. Biophys. Acta* **2007**, *1770*, 314–329.

Table 3. Thiomuracin and GE2270 Cluster of Genes and Proposed Functions[a]

Protein	Size aa	Homology (Sequence Similarity*)		Protein Family	Predicted Function
Thiomuracin/GE2770					
TpdR	243 237	(31%)	TetR	PF00440	Transcriptional regulation.
TpdA	49 57	(80%)			Prepropeptide.
TpdB	857 827	(41%)	Lantibiotic dehydratase	PF04738	Generation of pyridine ring precursors by dehydration of propeptide serine residues.
TpdC	271 299	(40%)	Lantibiotic biosynthesis		
TpdD	319 333	(40%)	CHP		
TpdE	428 455	(60%)	CHP PatG (54-56%)		Oxidation of thiazoline heterocycles to thiazoles.
TpdF	630 608	(44%)	YcaO-like CHP	PF02624	
TpdG	610 578	(59%)	YcaO-like CHP PatD (38-39%)	PF02624	Cyclodehydration of cysteines to thiazolines.
Thiomuracin					
TpdH	444	Peptidase M20		PF01546	Removal terminal alanine.
TpdI	403	Radical SAM protein		PF04055	Thiazole C-Methylation.
TpdJ1	387	P450Monooxygenase		PF00067	Hydroxylation.
TpdJ2	406	P450Monooxygenase		PF00067	Hydroxylation.
GE2270					
TpdK	122	HP			
TpdL	416	Radical SAM protein		PF04055	C-Methylation.
TpdM	339	O-Methyltransferase		PF00891	Methoxyethyl formation.
TpdN	204	Deaminase reductase		PF01872	Deamination.
TpdO	633	YcaO-like CHP PatD (38%)		PF02624	Cyclodehydration of serine to oxazoline.
TpdP	829	Lantibiotic dehydratase		PF04738	Serine dehydration.
TpdQ	344	P450Monooxygenase		PF00067	Hydroxylation.
TpdS	228	HP			
TpdT	372	N-Methyltransferase		PF05175	Asparagine N-Methylation.
TpdU	414	Radical SAM protein		PF04055	Thiazole C-Methylation.

[a] CHP, Conserved Hypothetical Protein. HP, Hypothetical Protein. *, GAP Pairwise Alignment.

expression that correlated with the onset of thiomuracin production (Figure 5a).

During the process of a classical strain optimization campaign, a mutant that failed to produce thiomuracins was derived via treatment of Bp3714-39 with UV mutagenesis. Sequence analysis of the mutant NS3713 thiomuracin gene cluster demonstrated a fully intact sequence and the absence of any mutation that could explain the nonproducing phenotype. RT-PCR revealed that this strain was defective in the transcriptional induction of the gene cluster (Figure 5b). It is likely that this strain harbors a mutation in an upstream regulatory protein that is essential for induction of gene expression. In addition, a mutant of the GE2270A (**10**) producing strain WU8817 was generated by targeted mutagenesis (Supporting Information). Briefly, a mutant allele was constructed by deleting the GE2270 structural gene *tpdA*, marking the deletion by incorporation of the thiostrepton *tsr* resistance gene, and cloning into an integrase (*int*)-deleted version of the vector pSET152.[38] The resultant suicide plasmid pNE240::*tsr* carrying a mutant *tpdA* allele is able to replicate in *E. coli* and unable to replicate in *Nonomuraea* but carries an origin of transfer for intergeneric conjugation.[39] Selection of *Nonomuraea* ex-conjugants for thiostrepton resistance which marks the *tpdA* deletion and for the plasmid antibiotic markers identified positive clones harboring a plasmid that integrated into the chromosome. An ex-conjugant, named Tpd1, resulting from homologous recombination of pNE240::*tsr* into the GE2270 gene cluster was isolated. This single crossover event was sufficient to disrupt the transcriptional unit resulting in the loss of GE2270A (**10**) production (Figure 5c).

Discussion

The identification of biosynthetic gene clusters for thiomuracin A−I (**1**−**9**) and the related GE2270A (**10**) revealed that both natural products are built up from a linear peptide encoded in the genome of the *Nonomuraea* producing strains. The structural gene *tpdA* comprising the 14 amino acid linear peptide sequence and a leader peptide is central to both biosynthetic gene clusters. Together with *tpdA* the five open reading frames *tpdB*−*G* constitute a core unit predicted to be sufficient for morphing the linear precursor peptide into a heterocycle-rich macrocyclic structure. A putative structural gene encoding the thiostrepton peptide backbone was uncovered in the thiostrepton producer *S. azureus* ETH28555. In an independent study recently published by Fischbach and co-workers, a bioinformatic approach revealed the thiocillin biosynthesis cluster in the genome of *Bacillus cereus* ATCC 14579 as well as the putative berninamycin gene cluster in the *Propionibacterium acnes* KPA171202 genome,[18] thereby supporting the hypothesis that all thiopeptides are biosynthesized via a ribosomal route followed by extensive post-translational modifications.

The core biosynthetic unit displays syntenous genomic organization in the thiomuracin and GE2270 gene clusters, but

(38) Bierman, M.; Logan, R.; O'Brien, K.; Seno, E. T.; Rao, R. N.; Schoner, B. E. *Gene* **1992**, *116*, 43–49.
(39) Mazodier, P.; Petter, R.; Thompson, C. *J. Bacteriol.* **1989**, *171*, 3583–3585.

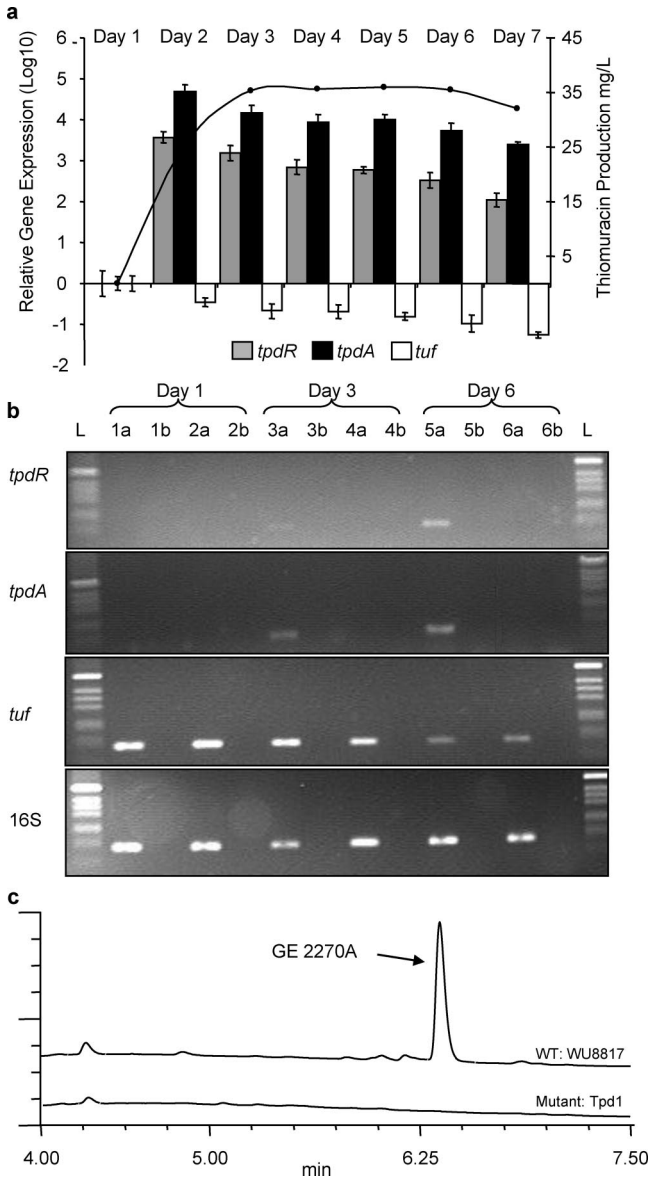


Figure 5. Thiomuracin gene cluster expression analysis. (a) Quantitative RT-PCR: Relative expression of *tpdR*, *tpdA*, and *tuf* over a 7 day production growth curve. All samples were normalized to the copy number of the endogenous 16S rRNA and the sample from day1 was used as the calibrator. Shown is the mean derived from samples run in triplicate (±SD). (b) RT-PCR end-point analysis: Total RNA isolated from both the wild type thiomuracin producing strain (samples 1, 3 and 5) and the nonproducing mutant NS3713 (samples 2, 4 and 6). RNA was isolated from cultures sampled at day 1 (1a, 1b, 2a, and 2b), day 3 (3a, 3b, 4a, and 4b), and day 6 (5a, 5b, 6a, and 6b). Lanes: L, 1kb size ladder; a, RT-PCR; b, reverse transcriptase was omitted. (c) HPLC traces (220 nm) showing the presence and absence of GE2270A (**10**) after 6 day cultivation of the wild type producing strain WU8817 and the gene cluster mutant Tpd1.

with low amino acid sequence identity between orthologue gene pairs. Despite the structural similarity of the compounds and the close taxonomic relationship of the producer strains, the biosynthetic clusters for GE2270 and thiomuracin differ substantially outside of the conserved core unit. This suggests that a detailed analysis of biosynthetic clusters from distantly related thiopeptides like thiostrepton (**11**) or nocathiacin will further increase the diversity of available ancillary enzymes, which can potentially be manipulated to increase the chemical diversity in the thiopeptide class. Some of the amino acid residues in the linear precursor peptides are highly conserved as they are involved in the formation of the thiazole and pyridine heterocycles, while other residues are variable, paving the way to create novel compounds through simple manipulation of the structural gene itself. A similar approach to new structural diversity through pathway engineering of ribosomally encoded peptides was demonstrated for the patellamides[7] and extensively proven for the lantibiotics.[1−3,40]

There is a growing medicinal need for novel antibiotics with clinically unexploited mechanisms of action and nonoverlapping mechanisms of resistance. Future successes in treating serious bacterial infections including those caused by drug-resistant pathogens like MRSA and VRE and other emerging infectious agents require investment in the search for new antibiotics today. Our strategy for success combines an assay for *S. aureus* growth inhibition with a library of pure and complex mixtures of natural products. The discovery of the thiomuracins extends the class of thiopeptide antibiotics and provides new chemical matter targeting the essential bacterial enzyme EF-Tu. This elongation factor, the most abundant protein in bacterial cells, has not been exploited by any of the antibiotics currently approved for use in humans. Thiomuracins are potent antibacterials that do not select for cross-resistance to marketed antibiotics and thereby fulfill many of the criteria for a novel antibacterial lead compound.

Accession codes. The gene cluster DNA sequences and thiostrepton structural gene have been deposited with GenBank (thiomuracin: FJ461360; GE2270: FJ461359; thiostrepton: FJ494914).

Experimental Section

Cultivation. *Nonomuraea* strains Bp3714-39, WU8817, and *Streptomyces azureus* ETH28555 were fermented and propagated at 28 °C as described in the Supporting Information.

Isolation of Thiomuracins. Fermentation broth (100 L) was acidified to pH 4.5 and extracted with 200 L of ethyl acetate. After evaporation of the organic phase the extract was defatted using cyclohexane resulting in 9.3 g of dry extract. For the isolation of thiomuracin A (**1**) the same procedure was used, but prior to extraction the pH of the fermentation broth was set to pH 7.0.

Thiomuracin A (**1**) was isolated from the extract obtained at pH 7.0 using a two-step normal phase chromatography process using dichloromethane/methanol/acetic acid as eluting solvents. The detailed description of purification is described in the Supporting Information. For the isolation of thiomuracin B−I (**2**−**9**) the extract from the acidic extraction was dissolved in methanol and applied to a column packed with Sephadex LH20 and run isocratically with methanol. Fractions containing the compounds were further purified using reversed phase chromatography with water and acetonitrile or methanol as the solvent system yielding 1−12 mg of the different thiomuracins.

Structure Elucidation. The spectroscopic methods used for structure elucidation and spectral data for thiomuracin B−I (**2**−**9**) are shown in the Supporting Information. Thiomuracin A (**1**) IR (KBr pellet): 3380, 3114, 2972, 2928, 1664, 1533, 1516, 1417, 1312, 1230, 1174, 1103, 1063, 1024, 989, 934, 809, 756, 704, 600 cm^{-1}. FT-MS: found, 1339.21225; calcd for $C_{59}H_{50}N_{14}O_{12}S_6$+H, 1339.21296. For NMR data, see Table 1.

Measurement of Antibacterial Activity. *Staphylococcus aureus* NB01001 (ATCC 29213), NB01086 (NCTC 8325-4), NB01086-JAL0210 (*tufA* G826A), NB01086-JAL0107 (*rplK* Δ 58−75), and *Enterococcus faecalis* NB04001 (ATCC 29212) were assayed for

(40) Kuipers, O. P.; Bierbaum, G.; Ottenwälder, B.; Dodd, H. M.; Horn, N.; Metzger, J.; Kupke, T.; Gnau, V.; Bongers, R.; van den Bogaard, P.; Kosters, H.; Rollema, H. S.; de Vos, W. M.; Siezen, R. J.; Jung, G.; Götz, F.; Sahl, H. G.; Gasson, M. J. *Antonie Van Leeuwenhoek* **1996**, *69*, 161–169.

sensitivity to antibacterials via a standard broth microdilution method[22] (CLSI). Briefly, cultures were grown on blood agar plates (Remel) O/N at 37 °C and then used to inoculate tubes containing 5 mL of Mueller Hinton Broth (Difco). After 4 to 5 h of incubation at 37 °C, the culture was adjusted with sterile saline (NaCl 0.86%) to a turbidity of 0.5 McFarland standard. Assays were performed in sterile plastic 96-well microtiter trays with round-bottom wells. The wells of the assay plate were filled with 100 μL of MHB, and 1 μL of a compound dilution series was added to the medium. 1.5 μL of the diluted bacterial culture were added to the wells, and the plates were sealed and incubated for 20 to 24 h at 37 °C in air. MIC values were determined by visual inspection of the plates. The MIC was defined as the lowest drug concentration causing complete suspression of visible bacterial growth.

Measurement of Affinity by Surface Plasmon Resonance. 10 mg/mL of *E. coli* EF-Tu was dialyzed using a Slide-A-Lyzer 10 MWC (Pierce) in 1 L 1x PBS overnight at 4 °C to remove Tris buffer. The protein was then biotinylated at a molar ratio of cross-linking reagent to EF-Tu of 1:0.5 to 1:1 by overnight incubation at 4 °C with Sulfo-NHS-LC-LC-Biotin (Pierce). Biotinylated EF-Tu was immobilized on an SA sensor chip (Biacore), and compounds were assayed using a Biacore T100. The data were globally analyzed by Biacore Evaluation Software Version 1.1 to give the association rate constant (k_{on}), dissociation rate constant (k_{off}), and dissociation constant ($K_D = k_{off}/k_{on}$).

Selection of Thiopeptide-Resistant Strains of *S. aureus*. Trypticase soy agar plates containing 4x MIC against *S. aureus* of each of the compounds were prepared by adding an appropriate amount of compound to molten Tryptic Soy Agar. 10^{10} colony forming units of *S. aureus* NB01086 (NCTC 8325-4) were plated and incubated at 37 °C for 24 to 48 h. Colonies that appeared on selection plates were phenotypically characterized, and representatives were restreaked for isolated colonies onto BAPs and selective TSA plates. Resistance frequencies were determined by calculating the number of colonies arising on selective plates divided by the total CFU plated. Cells that grew upon restreaking in the presence of the selection compound were frozen at −80 °C in 15% glycerol.

Identification of Structural Genes Encoding Thiopeptide Precursor Proteins. *Nar*I digested chromosomal DNA was purified by passage over a QiaQuick DNA cleanup column (QiagenAG). An adaptor-ligated library was generated by ligating to the fragmented DNA a 500-fold molar excess of the following adapter: 5′-CGACCACGACCA (phosphorylated on the 5′terminus and includes a 3′ C6-TFA-amino modification) and 5′-AGTCTCGCA-GATGATAAGGTGGTCGTGGT.[27] Thiopeptide structural genes were amplified from respective genomic libraries by using the adapter primer (5′-GTCCAGTCTCGCAGATGATAAGG) and degenerate primer designed on the thiopeptide macrocycle. For GE2270: Inosine was included in the oligonucleotide to reduce the degeneracy (CysPheGlyCysValCysAsnCys: 5′-CARAAICCRCAI-ACRCARTTRCA). For thiomuracin and thiostrepton: The degenerate primers were designed based on the CODEHOP principles with a 5′ consensus clamp and a 3′ degenerate core[41] (CysAsnCysPhe-CysTyrIleCysCys: 5′-CAGCAGATGTARCARAARCARTTRCA. CysThrThrCysIleCysThrCys: 5′-CACGTGCAGATR CANGT-NGTRCA-3′). Cycling conditions were as follows: 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and 72 °C for 10 min.

Cloning and Annotation of GE2270 and Thiomuracin Gene Clusters. 1536 clone BAC libraries were constructed by Amplicon Express (Pullman, WA) by partially digesting genomic DNA with *Hind*III and ligating into pEC1BAC cleaved with *Hind*III. The libraries belonging to the GE2270 producer (AWU) and thiomuracin producer (ABP) had average insert sizes of 100kb and 90kb, respectively. BAC DNA isolated from two positive clones, AWU002-A16 and ABP003-G24, were sequenced to completion with an accuracy >99.99% (Phred 20 trim_cutoff = 0.01, QiagenAG). Coding regions were identified by using Vector NTI (Invitrogen, version10.0.1).

Gene Cluster Expression Analysis. Cell pellets were suspended in 1 mL of Trizol (GIBCO BRL) and transferred to 2-mL screw cap tubes containing 0.5 mL of 0.1-mm diameter zirconia/silica beads (BioSpec Products). Three 40-s pulses in a bead beater disrupted cells. RNA was isolated from the Trizol reagent, treated twice with TURBO DNase I (Ambion), and purified using RNeasy (QiagenAG). cDNA was prepared using a QuantiTect (QiagenAG) reverse transcription kit. Real-time PCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystems), and samples were analyzed in triplicates in 384-well plates using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen). Cycling conditions and primer sets were as described in the Supporting Information.

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Note Added in Proof. Two papers have recently been published describing thiostrepton biosynthesis.[42,43]

Supporting Information Available: Scheme representing insertional inactivation of GE2270 gene cluster, ^{1}H NMR spectrum (600 MHz, d_6-DMSO) of thiomuracin A (**1**), supplementary methods, and spectral data for thiomuracin B−I (**2**−**9**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(41) Rose, T. M.; Henikoff, J. G.; Henikoff, S. *Nucleic Acids Res.* **2003**, *31*, 3763–3766.

(42) Liao, R.; Duan, L.; Pan, H.; Ding, Y.; Zhang, Q.; Chen, D.; Shen, B.; Yu, Y.; Liu, W. *Chem. Biol.* **2009**, *16*, 141–147.

(43) Kelly, W. L.; Pan, L.; Li, C. *J. Am. Chem. Soc.* **2009**, *131*, 4327–4334.