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Capturing Linear Intermediates and C-Terminal Variants during Maturation of the Thiopeptide GE2270

Arianna Tocchetti,[1] Sonia Maffioli,[2] Marianna Iorio,[2] Silke Alt,[2] Emma Mazzei,[2] Cristina Brunati,[2] Margherita Sosio,[1,2,*] and Stefano Donadio[1,2]
[1]KtedoGen Srl, Via Fantoli 16/15, 20138 Milan, Italy
[2]Naicons Scrl, Via Fantoli 16/15, 20138 Milan, Italy
*Correspondence: msosio@naicons.com
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SUMMARY

Thiopeptides are ribosomally synthesized, post-translationally modified peptides with potent activity against Gram-positives. However, only GE2270 has yielded semisynthetic derivatives under clinical investigations. The *pbt* gene cluster from the GE2270 producer *Planobispora rosea* was successfully expressed in the genetically tractable *Nonomuraea* ATCC39727. Gene deletions established that PbtO, PbtM1, PbtM2, PbtM3, and PbtM4 are involved in regiospecific hydroxylation and methylations of GE2270, leading to the generation of various derivatives with altered decorations. Further deletions established that PbtH and PbtG1 are involved in C-terminal amide and oxazoline formation, respectively. Surprisingly, preventing either step resulted in the accumulation of linear precursors in which the pyridine-generated macrocycle failed to form, and only one of the pyridine-forming serine residues had been dehydrated. Often, these linear precursors present a shortened C terminus but retain the full set of methylation and hydroxylation decorations.

INTRODUCTION

Thiopeptides represent a large class of microbial metabolites within the growing family of ribosomally synthesized, posttranslationally modified peptides (Arnison et al., 2013). They arise from a precursor peptide containing an N-terminal leader and a core region, which undergoes the posttranslational modifications (PTMs) that are the hallmark of this family: the characteristic thiazoles (Thz) and oxazoles with a central pyridine/piperidine ring that defines the macrocycle (Arnison et al., 2013; Zhang and Liu, 2013).

Thiopeptides inhibit bacterial protein synthesis by affecting either one of two targets: molecules such as thiostrepton and thiocillins bind to the 23S rRNA/L11 protein interface of the 50S ribosome (Walsh et al., 2010), whereas 29-membered ring thiopeptides, such as GE37468, GE2270, and thiomuracin, bind to elongation factor Tu and block its capacity to chaperone aminoacylated transfer RNAs (tRNAs) to the ribosome (Walsh et al., 2010; Parmeggiani et al., 2006). Many thiopeptides exhibit potent activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* sp., and *Clostridium difficile*. Other thiopeptides inhibit the 20S proteasome, highlighting their potential as antitumor agents (Bhat et al., 2009). However, the poor solubility and pharmacological properties of thiopeptides have limited clinical progress and only derivatives of GE2270 have entered clinical trials for the topical treatment of acne or of *C. difficile*-associated diarrhea (Butler, 2005; Donadio et al., 2010; LaMarche et al., 2012).

GE2270 is produced by the actinomycete *Planobispora rosea* ATCC53733 as a complex of related metabolites differing in the number of methyl groups that decorate residues in the macrocycle, with GE2270A as the main congener carrying a full set of methyl groups (Selva et al., 1995; Tavecchia et al., 1994) (Figure 1). Here, we report the identification of the GE2270 biosynthetic gene cluster from *Planobispora rosea*, highly related to the cluster identified from *Nonomuraea* WU8817 (Morris et al., 2009), and its heterologous expression in *Nonomuraea* sp. Manipulation of the cluster led to the generation of diverse GE2270 derivatives, as well as linear and/or C-terminally truncated forms of this thiopeptide. These results provide important insights into the checkpoints leading to full thiopeptide maturation.

RESULTS

Expressing the *P. rosea* GE2270 Biosynthetic Gene Cluster in *Nonomuraea*

From a draft genome of *P. rosea* ATCC53733, we identified a 21.4 kb segment encoding the predicted 14-aa GE2270 core peptide, preceded by a 39-aa N-terminal leader and followed by a Ser-Ala C-terminal extension (Figure 1). The *P. rosea* GE2270 cluster, named *pbt* (Planobispora thiopeptide), shows high sequence identity (ranging from 64% for PbtR to 93% for PbtA) (Table 1) and complete synteny with the *tpd* cluster from the GE2270-producer *Nonomuraea* WU8817 (Morris et al., 2009). The only exception is represented by *tpdN*, encoding a putative deaminase reductase, which is absent in the *pbt* cluster (Table 1). The structural gene *pbtA* is followed by the six *pbtBCDEFG* genes, predicted to represent the cassette responsible for constructing the pyridine ring and the thiazoles characteristic of the thiopeptide scaffold (Arnison et al., 2013). Other

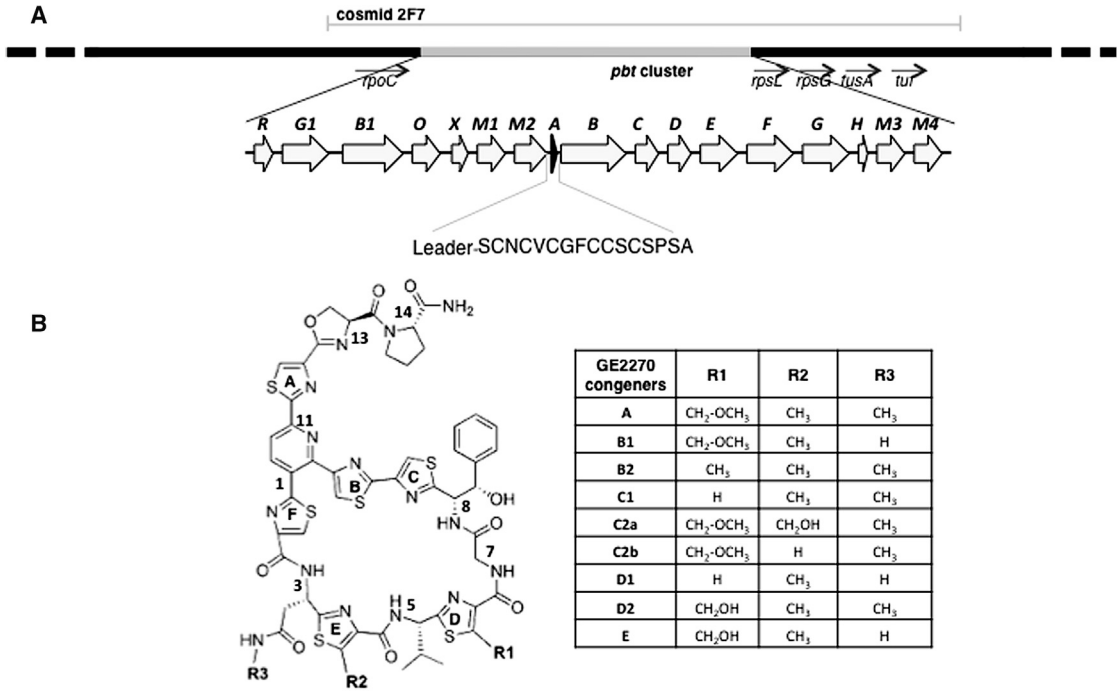

Figure 1. Organization of the *P. rosea pbt* Cluster and the GE2270 Complex

(A) The cluster, flanking regions, and span of cosmid 2F7. The deduced functions of coding DNA sequences are summarized in Table 1. The amino acid sequence of the PbtA core peptide is shown below. Genes *rpoC*, *rpsL*, *rpsG*, *fusA*, and *tuf* designate genes encoding RNA polymerase β′, ribosomal proteins S12 and S7, and elongation factors G and Tu, respectively.

(B) Structure of GE2270 and known congeners. Thiazoles are named A–F as described (Kettenring et al., 1991). Amino acid residues mentioned in the text as numbered. For simplicity, congener T, an analog of congener A carrying an oxidized oxazoline (Selva et al., 1995), has been omitted.

relevant *pbt* genes encode paralogs of PbtB and PbtG, designated PbtB1 and PbtG1, respectively, as well as the predicted P450 monooxygenase PbtO, the methyltransferases PbtM1 through PbtM4, and the NosA homolog PbtH, likely responsible for terminal amide formation (Yu et al., 2010) (Figure 1; Table 1).

As seen with other thiopeptide clusters (Yu et al., 2009), *pbt* is located within a highly conserved region of the genome, with genes coding for DNA-directed RNA polymerase subunits and for part of the translation machinery (elongation factors G and Tu and ribosomal proteins) on the left- and right-hand side of Figure 1A. It is worth noting that the *P. rosea* EF-Tu is insensitive to GE2270 (Sosio et al., 1996).

As no gene transfer systems have been described yet for *P. rosea*, we resorted to heterologous expression to analyze the GE2270 cluster. To this end, we made use of cosmid 2F7, which carries the entire *pbt* gene cluster and flanking regions, including the *tuf* gene (Figure 1A), on a conjugative vector capable of site-specific integration in many actinomycete strains (see Experimental Procedures). Several attempts at GE2270 production in *Streptomyces* spp. (such as *S. lividans*, *S. albus*) proved unsuccessful (data not shown). However, we succeeded with *Nonomuraea* sp. ATCC39727, a microorganism taxonomically related to *P. rosea*, amenable to genetic manipulation (Sosio et al., 2003; Stinchi et al., 2003), and recently used as heterologous host for ribosomally synthesized peptides (Foulston and Bibb, 2010; Sherwood et al., 2013). Methanolic extracts from one of the *Nonomuraea* ex-conjugants carrying cosmid 2F7 (designated strain Nono-2F7) exhibited a high-performance liquid chromatography (HPLC) peak that showed the same retention time and mass spectrometry (MS) profile as authentic GE2270A (Figure 2A). No equivalent peak or MS signal was observed from a *Nonomuraea* exconjugant (designated strain Nono-cos3) carrying the empty vector (Figure 2A). In contrast to *Nonomuraea* WU8817, the genome of the ATCC39727 strain does not contain a GE2270 gene cluster (P. Alifano, personal communication). ^{1}H-NMR analysis of the purified compound confirmed that the metabolite produced by strain Nono-2F7 is indistinguishable from GE2270A as produced by *P. rosea* (data not shown). These results demonstrate that cosmid 2F7 contains all the genes necessary for GE2270 production and suggests that *tdpN* (Morris et al., 2009) is not essential for thiopeptide formation. Depending on experimental conditions, congener C1 was sometimes observed as minor product of Nono-2F7.

Although GE2270 production by Nono-2F7 afforded sufficient GE2270 (~10 mg/l) for characterization, genetic alterations in a cluster usually result in lower yields of the end product. We thus analyzed different growth media for increased GE2270 levels. As shown in Figure 2B, improved media resulted in over 20-fold increase in production, up to 250 mg/L. Interestingly, GE2270 production by Nono-2F7 followed a time course similar to that observed, under nonoptimized conditions with the *P. rosea* native producer, with production starting during the exponential growth phase (Figure 2C).

Because *Nonomuraea* produces the glycopeptide A40926 (Sosio et al., 2003), we made use of a glycopeptide intermediate-resistant *S. aureus* (GISA) strain for analyzing *Nonomuraea*

Table 1. List of Pbt Proteins

CDS	Size (aa)	Tpd Homolog (Identity; %)	Proposed Function	Established Role in GE2270 Biosynthesis
PbtR	237	TpdR (64)	transcriptional regulator	
PbtG1	607	TpdO (83)	YcaO cyclodehydratase	oxazoline formation
PbtB1	792	TpdP (71)	dehydratase	
PbtO	344	TpdQ (80)	P450 monooxygenase	β-hydroxylation of Phe8
PbtX	215	TpdS (80)	hypothetical protein	
PbtM1	372	TpdT (92)	*N*-methyltransferase	*N*-methylation of Asn4
PbtM2	394	TpdU (88)	radical SAM protein	*C*-methylation of ThzE
PbtA	55	TpdA (93)		precursor peptide
PbtB	829	TpdB (73)	lantibiotic dehydratase	
PbtC	302	TpdC (72)	lantibiotic dehydratase	
PbtD	333	TpdD (78)	[4 + 2] cycloaddition	
PbtE	456	TpdE (80)	azoline-synthesizing dehydrogenase	
PbtF	598	TpdF (73)	hypothetical protein	
PbtG	578	TpdG (84)	YcaO cyclodehydratase	essential
PbtH	122	TpdK (86)	hypothetical (NosA)	C-terminal amide formation
PbtM3	388	TpdL (86)	radical SAM protein	*C*-methylation of ThzD
PbtM4	335	TpdM (83)	*O*-methyltransferase	ThzD *O*-methyltransferase

ex-conjugants: a clear inhibition halo was observed with this strain when spotting Nono-2F7 cultures, whereas no effect was seen with cultures from the control strain Nono-cos3, carrying the empty vector (Figure 2D). In contrast, cultures from the Nono-cos3 strain inhibited growth of glycopeptide-sensitive strains, such as *B. subtilis* ATCC6633 ($GE2270^S$) or its derivative G1674 ($GE2270^R$) (Sosio et al., 1996), presumably due to the presence of the glycopeptide A40926 (Figure 2D).

In order to analyze the function of *pbt* genes, we performed targeted gene deletions. This was achieved by λ Red recombineering of cosmid 2F7, replacing selected genes with an antibiotic resistance marker (*cat* or *kan*) flanked by FRT sites (Table S1 available online). In some cases, the resistance marker was excised via flippase (FLP)-mediated recombination (Table S1). The modified cosmids were then transferred by conjugation into *Nonomuraea* ATCC39727 and the resulting metabolites were characterized by HPLC, MS, and nuclear magnetic resonance (NMR) analyses. Routinely, two independent ex-conjugants were analyzed for metabolite content, always with identical results. Identity of compounds was established by a combination of HPLC, MS, and NMR analyses, unless stated otherwise.

Modulating the GE2270 Complex

A distinguishing feature of GE2270 is the pattern of methylations that decorate the Asn (*N*-methyl), ThzE (*C*-methyl), and ThzD (*C*-methylene-*O*-methyl) residues. The presence or lack of these substitutions is reflected in the known GE2270 congeners, which do not, however, encompass all possible permutations at these residues (Figure 1B). As suggested by the predicted functions, *pbtM1* and *pbtM4* are the likely candidates for *N*- and *O*-methylation, respectively, whereas *pbtM2* and *pbtM3* encode radical SAM enzymes expected to be responsible for C-methylation of the thiazoles.

To confirm its function, we replaced *pbtM1* in cosmid 2F7 with *kan*. The resulting ex-conjugant Nono-M1 produced the expected GE2270 B1 congener, lacking the *N*-methyl group, in ~80%, with smaller amounts of the D1 congener, the *C*-desmethyl derivative of B1 on Thz D (Figure 3A; Table S1). Similarly, strain Nono-M4 carries cosmid 2F7 in which *pbtM4* has been replaced by *kan*. This strain yielded a single compound identified as the *O*-desmethyl GE2270 congener D2 (Figure 3B; Table S1). These data confirm the roles of PbtM1 and PbtM4 as the *N*- and *O*-methyltransferases acting on Asn4 and ThzD, respectively (Table 1).

Labeled precursor studies (De Pietro et al., 2001) and the gene encoding the precursor peptide (Morris et al., 2009) indicate that all thiazoles arise from Cys residues and subsequent *C*-methylation of the appropriate residues. After replacing *pbtM3* or *pbtM2* with *cat* in cosmid 2F7, the ex-conjugants Nono-M3 or Nono-M2 were obtained, respectively. The former strain produced a major compound identified as the C1 congener, which carries a desmethylated ThzD moiety (Figure 3C; Table S1). Conversely, strain Nono-M2 produced in 65% yield a compound whose HPLC retention time and MS data were indistinguishable from those of the C2b congener and NMR confirmed the presence of a desmethylated ThzE (Figure 3D; Table S1). Interestingly, strain Nono-M2 produced an additional peak (~35% yield) with m/z of 1,284 $[M + Na]^+$. Further MS and NMR analyses (Table S2) established that this peak corresponds to a previously unreported congener (compound **1**) devoid of methyl groups on ThzE and Asn (Figure 3D). All together, these data demonstrate that PbtM2 and PbtM3 are involved in the regioselective *C*-methylation of ThzE and ThzD, respectively (Table 1).

Upstream to *pbtM1* is *pbtX*, encoding a protein of unknown function with no counterpart in other thiopeptide clusters. Replacing *pbtX* with *cat* in cosmid 2F7 yielded strain Nono-X, which showed an LC-MS profile indistinguishable from that of Nono-M1: the two major peaks were the known congeners B1 and D1 (Table S1). Because we cannot exclude polar effect of the mutations introduced into *pbtX* on *pbtM1* expression, it remains to be determined whether PbtX has any role in methylation of Asp4 together with PbtM1.

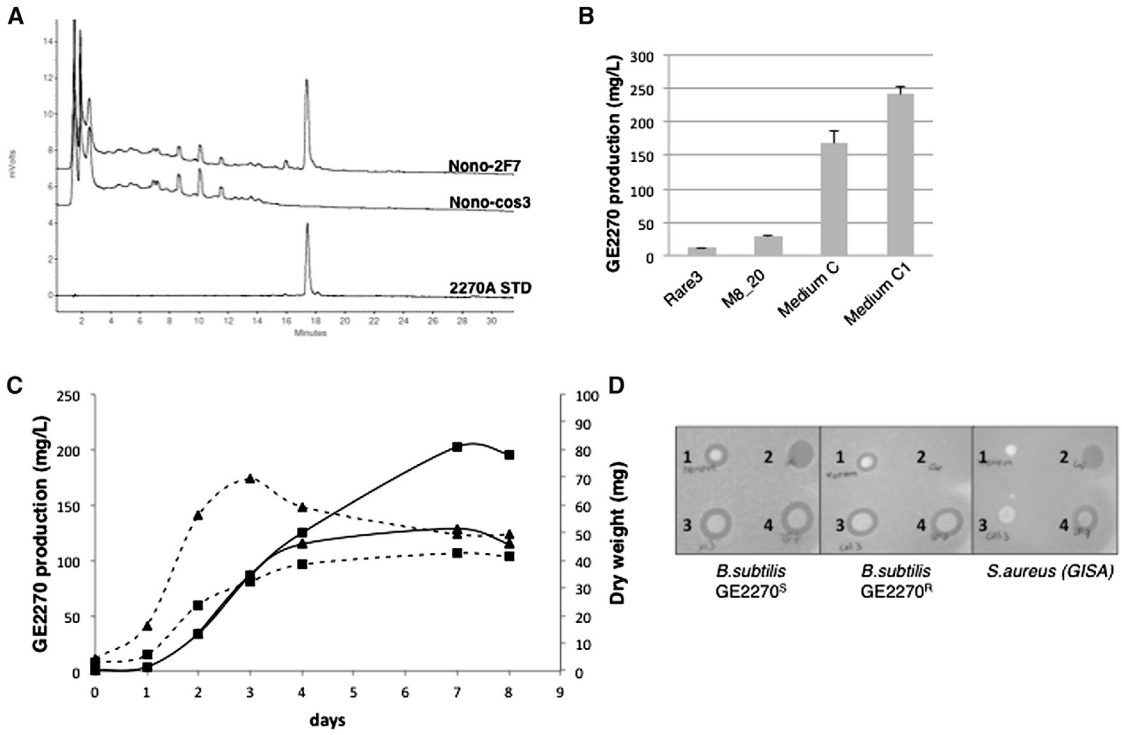

Figure 2. Heterologous Production of GE2270 in *Nonomuraea*

(A) HPLC trace (λ 310 nm) of methanolic extracts from strains carrying cosmid 2F7 (Nono-2F7) or empty vector (Nono-cos3) with respect to a standard of pure GE2270A.

(B) Production of GE2270 by Nono-2F7 in different media. Production levels were established by HPLC after 6 days of growth in each medium, using two replicate flasks. Error bars represent SEM.

(C) Biomass and GE2270 production in native versus heterologous host. Dry weight (dashed lines) and GE2270 levels (solid lines) were determined for *P. rosea* (squares) and Nono-2F7 (triangles).

(D) Antibacterial overlay assay. Samples (10 μl) from cultures of *Nonomuraea* ATCC39727 (spot 1), Nono-cos3 (spot 3), and Nono-2F7 (spot 4) were spotted on MH agar plates inoculated with the indicated microorganism. GE2270A (spot 2) was used as control.

See also Tables S1 and S4.

Generating Additional GE2270 Congeners by Targeted Genetic Intervention

The *pbtO* gene encodes a P450 monooxygenase likely responsible for Phe8 β-hydroxylation. This gene was replaced in cosmid 2F7 by *cat*, which was then excised by the FLP recombinase. The resulting *Nonomuraea* ex-conjugant, designated strain Nono-O, produced two GE2270-related peaks (compounds **2** and **3**) in a 1:1 ratio with positively charged MS signals at m/z 1,260 and 1,274 $[M + H]^+$ (Figure 4A; Table S1), with an MS fragmentation pattern consistent with GE2270 derivatives devoid of the Phe8 hydroxyl. NMR analyses (Figures S1 and S2; Table S2) established that compound **2** corresponds to a GE2270 congener with a full set of methylations on Asn4, ThzD, and ThzE but carrying an unmodified Phe8 residue, whereas **3** is its ThzD-*O*-desmethyl derivative (Figure 4A). These data demonstrate that PbtO is the monooxygenase responsible for β-hydroxylation of Phe8 and suggest that *O*-methylation of ThzD may be less efficient on substrates devoid of a Phe-8 β-hydroxyl.

The ribosomal origin of thiopeptides offers the possibility of altering the amino acid sequence by engineering the structural gene. Such efforts have been applied to produce thiocillin (Acker et al., 2009; Bowers et al., 2010, 2012), thiostrepton (Li et al., 2012), and GE37468 variants (Young and Walsh, 2011). A thorough analysis of replacing most amino acid residues in the core peptide showed that substitutions can be obtained at few residues in GE37468 (Young et al., 2012). To establish whether substitutions are possible in GE2270, we focused on residues Val5 and Gly7, which are not involved in setting the trithiazolylpyridine core in the mature scaffold and thus unlike to perturb the thiopeptide framework. The core peptide variants G7A, G7K, or V5E were constructed and introduced into *Nonomuraea*, generating strains Nono-G7A, Nono-G7K, and Nono-V5E, respectively. Methanolic extracts from Nono-G7A showed an HPLC peak with m/z 1,344 $[M + Na]^+$ compatible with compound **4**, carrying the desired Ala-replacement at position 7 (Figure 4B; Table S1). However, no metabolites were detected from strains Nono-G7K and -V5E, suggesting that the introduced mutations adversely affect thiopeptide formation.

Perturbing Pyridine, Oxazoline, and C-Terminal Amide Formation

Similarly to other thiopeptides, the C terminus of GE2270 is amidated, which implies that the amino acid residues Ser15 and Ala16 present in the precursor peptide are somehow removed during thiopeptide maturation. Recent studies have shown that two divergent strategies are utilized for C-terminal amidation

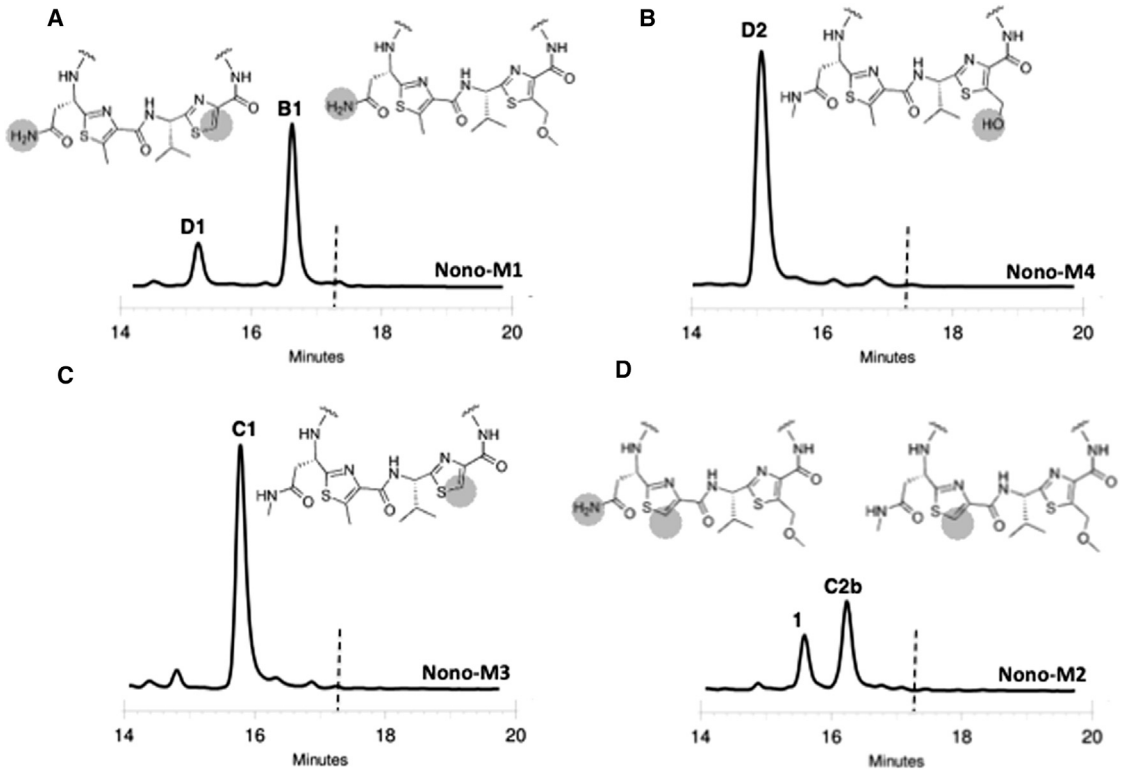

Figure 3. Production of GE2270 Congeners in *Nonomuraea*

HPLC traces (λ 310 nm) of extracts from strains Nono-M1 (A), Nono-M4 (B), Nono-M3 (C), and Nono-M2 (D). Dashed lines indicate the retention time of GE2270A. The known congeners are reported according to their codes (Figure 1B), whereas the identified congener is indicated as "**1**". Structures of the relevant part of GE2270 are also shown for convenience with grey circles indicating the affected position.
See also Tables S1 and S2.

during thiostrepton and nosiheptide biosynthesis (Yu et al., 2010; Liao and Liu, 2011; Liu et al., 2013). Because the *pbt* cluster encodes the NosA-homolog PbtH, we assumed that amide formation in GE2270 results from a mechanism that is similar to the nosiheptide mechanism, in which *nosA* deletion yielded a nosiheptide analog with an additional C-terminal dehydroalanine, resulting from dehydration of the C-terminal Ser in the core peptide (Liu et al., 2013). After replacing *pbtH* with *cat* in cosmid 2F7, the resulting exconjugant Nono-H produced, in a relative ratio of 70:30, compounds **5** and **6** with m/z 1,291 and 1,345 $[M + H]^+$, respectively (Figure 5A; Table S1). NMR analysis established that compound **5** is an analog of congener A, whereas LC-HRMS gave m/z 1,291.2557 $[M + H]^+$ (calculated for $C_{56}H_{55}N_{14}O_{11}S_6$, 1,291.2493) confirming that compound **5** contains a C-terminal carboxylic acid in place of the amide. Quite surprisingly, compound **6** lacked the UV adsorption and MS fragmentation pattern typical of GE2270 congeners. NMR analyses established that **6** is an acyclic precursor of **5** devoid of the pyridine ring, but containing an *N*-terminal methyl ketone (in place of Ser1) and an unmodified Ser11 (Figure S3; Table S3) (LC-HRMS m/z 1,345.2891 $[M + H]^+$; calculated for $C_{56}H_{60}N_{14}O_{14}S_6$, 1,345.2814). Interestingly, compound **6** carries the full set of methylations and hydroxylations seen in the final product (Figure 5A).

In thiopeptides, a YcaO-like cyclodehydratase is proposed to catalyze the nucleophilic addition of each Cys (for thiazole) or Ser (for oxazole) side chain onto the preceding carbonylic group, which is followed by phosphorylation-based dehydration to afford the azoline ring (Dunbar et al., 2012; Arnison et al., 2013). The *pbt* cluster contains two such homologs: *pbtG* in the highly conserved thiopeptide gene cassette, and *pbtG1* upstream to *pbtA* (Figure 1). On the basis of its location and sequence identity, PbtG is likely to be involved in thiazole formation, whereas PbtG1 may catalyze the less efficient oxazoline formation (Belshaw et al., 1998). We thus replaced *pbtG* or *pbtG1* in cosmid 2F7 by marker insertion followed by FLP recombinase excision. The resulting exconjugants, designated Nono-G or Nono-G1, respectively, were analyzed for metabolite content. Although no peak was observed from the Nono-G strain, two peaks in a 70:30 ratio with m/z values of 1,222 $[M + H]^+$ (compound **7**) and 1,266 $[M + H]^+$ (compound **8**) were detected from the Nono-G1 strain (Figure 5B; Table S1). NMR analyses revealed that both **7** and **8** are linear forms in which all the thiazoles are present, but differ from GE2270 for the following features: (1) no oxazoline but intact Ser13, (2) intact Ser11 and methyl ketone (after Ser1) instead of the pyridine ring, and (3) a shorter peptide lacking the C-terminal Pro-amide (Figures 5B and S4–S10; Table S3). Interestingly, compound **8** carries the full set of methylations and hydroxylations, whereas **7** lacks the methoxymethyl on ThzD. All together, these results point to a specific role of PbtG1 in oxazoline formation, a reaction that cannot be substituted for by PbtG, which is, instead,

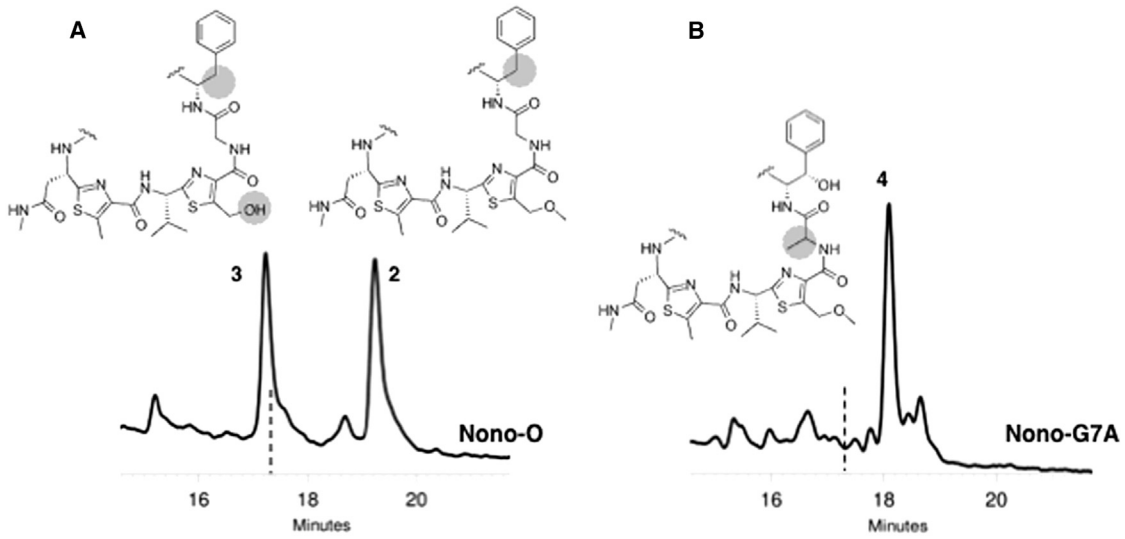

Figure 4. GE2270 Variants

HPLC traces (λ 310 nm) of extracts from strains Nono-O (A) and Nono-G7A (B). Dashed lines indicate the retention time of GE2270A. Structures of the relevant part of GE2270 in compounds **2**, **3**, and **4** are also shown with gray circles indicating the affected position.
See also Figures S1 and S2 and Tables S1 and S2.

essential for early event in thiopeptide maturation consistent with its role in thiazole formation (Table 1).

Next, we investigated the role of the Ser-Ala C terminus in correct C-terminal processing. Cosmid 2F7 was suitably engineered by deleting the last two codon from *pbtA*. The resulting ex-conjugant Nono-AΔ2 presented an HPLC profile with two major peaks in a 80:20 ratio with identical retention times and m/z values to **7** and **8** (Figure 5B; Table S1), as confirmed also by NMR analyses (Table S3). Furthermore, strain Nono-AΔ2 produced also a smaller amount of compound **5**. These data suggest that the presence of Ser15-Ala16 at the C terminus is critical not only for amide formation but also for cyclization of Ser13 into an oxazoline. Consistently, when either of these events is impaired, pyridine formation can no longer occur.

Overall, four additional, fully cyclized GE2270 variants were obtained in the course of this work. Their antibacterial activities, measured as minimum inhibitory concentrations (MIC) against selected strains are shown in Table 2. Although these compounds appear less potent than GE2270A, in particular they lose activity mostly against *Streptococcus* spp., they all retain interesting activities, particularly against *Enterococcus faecalis*.

DISCUSSION

The results presented in this study provide important insights into the biosynthesis of GE2270 and other thiopeptides. A key tool for this work has been heterologous expression in *Nonomuraea* ATCC39727. Foulston and Bibb (2010) have shown that this strain is a suitable host for expressing class I lantibiotics from the related genera *Microbispora* and *Planomonospora* (Sherwood et al., 2013), but have not used it for systematic cluster analysis. Here, we have shown that *Nonomuraea* is a robust host for expression, because each manipulation involved introducing modified cosmids into a "naive" host, followed by analysis of two independent ex-conjugants. We reliably observed comparable levels of metabolites.

After the discovery of GE2270 (Selva et al., 1991), an extensive search for and characterization of the congeners present in the complex was performed (Selva et al., 1995; Colombo et al., 1995). However, details were not reported on the conditions under which the various compounds were obtained and their relative yields. In the work presented here, we have established that five specific enzymes are involved in the four methylations and the hydroxylations that decorate the GE2270 scaffold, but none of these reactions is a requirement for subsequent steps to occur. This is particularly remarkable in the case of PbtO, because we were able to isolate deoxy-Phe congeners (Figure 1). These results suggest that PbtO-mediated hydroxylation occurs quite effectively in vivo, preventing the accumulation of detectable amounts of deoxy-Phe variants; they are also reminiscent of another P450-catalyzed hydroxylation, in which deoxy-erythromycin analogs were discovered only after inactivation of the corresponding gene (Weber et al., 1991). Similarly, the identification of the previously unreported congener **1**, after inactivation of the C-methyltransferase PbtM2, suggests that *N*-methylation in vivo occurs preferentially on a methylated ThzE, although we cannot exclude a polar effect on *pbtM1*. Finally, our results point to the lack of additional *pbt* genes as candidates for installing the oxygen atom on ThzD. Thus, either a host enzyme presents in both *P. rosea* and *Nonomuraea* is responsible for hydroxylation of methyl-ThzD or one of the Pbt enzymes might be able to carry this additional modification. Currently, we are inclined to believe that the GE2270 B2 congener (Figure 1B) represents a shunt product and not a true biosynthetic intermediate.

We have demonstrated that oxazoline formation in GE2270 requires the dedicated YcaO-like cyclodehydratase PbtG1 and that processing of the Pro-Ser-Ala C terminus of the core peptide into Pro-NH_2 requires the NosA-homolog PbtH. An unexpected

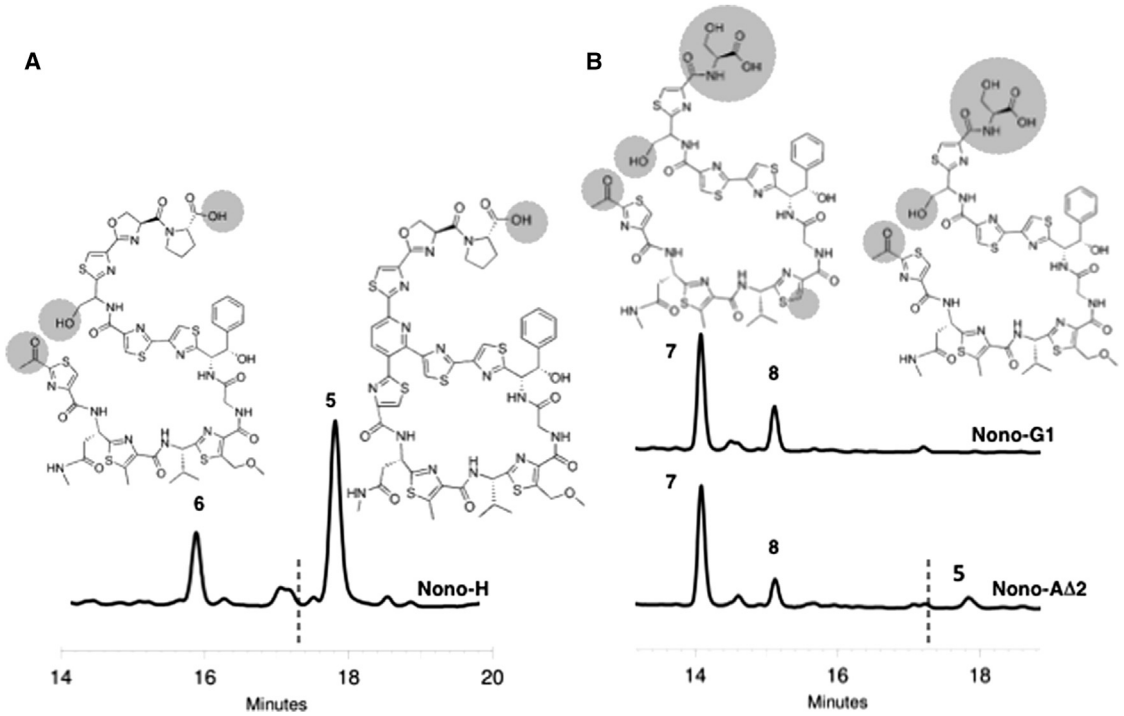

Figure 5. GE2270 Linear Intermediates and C-Terminal Variant

HPLC traces (λ 310 nm) of extracts from strains Nono-H (A) and Nono-G1 and Nono-AΔ2 (B). Dashed lines indicate the retention time of GE2270A. Structures of the relevant part of GE2270 in compounds **5**, **6**, **7**, and **8** are also shown with gray circles indicating the affected position.
See also Figures S3–S10 and Tables S1 and S3.

outcome of these deletions has been the accumulation of the linear variants **6**–**8** devoid of the pyridine ring. In these compounds, Ser13 is still unmodified, whereas Ser1 has yielded the N-terminal methylketone appendage on ThzF, presumably after dehydration and removal of the leader peptide. These results are consistent with and complement the recent observations by Malcomson et al. (2013) in berninamycin biosynthesis: these authors observed the accumulation of linear intermediates in which the pyridine-forming C-terminal Ser is still intact and speculated that a dedicated dehydratase may trigger pyridine ring formation by selectively intervening on that Ser residue after some key events have occurred in thiopeptide maturation. Although Malcomson et al. (2013) observed the berninamycin linear variants after amino acid substitutions in the core peptide that is contained within the final macrocycle, we have shown that oxazoline formation is an essential checkpoint along GE2270 maturation before pyridine ring closure can occur. The presence of a properly processed C terminus seems also an important requisite before macrocyclization can take place, because linear intermediates formed to a significant extent in the Δ*pbtH* strain and exclusively in the *pbtA*-Δ*2* mutant.

Overall, the results presented here allow us to propose a plausible order of biosynthetic reactions (Figure 6). The first step on the precursor peptide maturation is likely to be thiazole formation; this is followed by the PbtG1-catalyzed oxazoline synthesis; subsequent steps are C-terminal amide installment, catalyzed by PbtH; and finally pyridine ring generation, with macrocycle formation. Along this hypothetical scheme, we would like to point out that most linear intermediates observed in this study carry a full set of decorations on Phe8, ThzD, ThzE, and Asn4. This observation indicates that these reactions can occur on linear intermediate(s), presumably after the thiazole rings have been formed (Figure 6).

SIGNIFICANCE

This work provides important insights into the biosynthesis of the thiopeptide GE2270. It shows some flexibility by the synthetic machinery in accepting substitutions in the core peptide. Most importantly, it shows that linear intermediates accumulate when oxazoline formation is prevented and when the C terminus is altered, which suggests these events are important checkpoints before macrocyclization. These linear intermediates retain an intact Ser13 residue, which suggests that regioselective dehydration of this serine is a late event in thiopeptide maturation. We have also demonstrated the role of the methyltransferases and monooxygenases encoded by the *pbt* cluster in the regioselective modification of their cognate residues. Remarkably, these modifications can also occur on linear intermediates. All these observations provide in vivo evidence for the order of steps that lead to thiopeptide formation.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Growth Conditions

All bacterial strains used in this study were from the Naicons collection, except for *Bacillus subtilis* ATCC6633 and *S. aureus* ATCC 29213. *B. subtilis* G1674

Table 2. MICs of GE2270 Congeners Produced in This Study

	MIC (μg/ml)				
Microorganisms	GE2270A	Compound **2**	Compound **3**	Compound **4**	Compound **5**
Staphylococcus aureus ATCC 29213	<0.015	0.50	4	0.07	0.50
Streptococcus pyogenes L49	0.25	>32	>16	12.5	4
Streptococcus pneumoniae L44	<0.08	>32	>16	12.5	4
Enterococcus faecalis L559	<0.004	0.125	0.25	0.015	0.125

has been previously described (Sosio et al., 1996). *Planobispora rosea* ATCC53733 and *Nonomuraea* sp. ATCC39727 were propagated on BTT agar (Sosio et al., 2003) and seed cultures were made in D/seed (Gastaldo and Marinelli, 2003) and RARE3 (Sosio et al., 2003) media, respectively. For GE2270 production, a 72 hr *P. rosea* culture was inoculated (10%) into V6 broth (Binz et al., 2010). For optimized production in *Nonomuraea*, the following media were used: M8_20 (20 g/l dextrose, 20 g/l soluble starch, 4 g/l casein hydrolysate, 2 g/l yeast extract, 2 g/l meat extract, 3 g/l $CaCO_3$, pH 8.0), Medium C (35 g/l soluble starch, 10 g/l dextrose, 5 g/l casein hydrolysate, 3.5 g/l meat extract, 8 g/l yeast extract, 3.5 g/l soybean meal, 2 g/l $CaCO_3$, pH7.2); and Medium C1 (Medium C containing 20 g/l yeast extract and 10 g/l soybean meal). All cultures were incubated at 30°C and 200 rpm on a rotary shaker.

Escherichia coli strains XL1Blue and DH10B were used as general cloning hosts and ET12567/pUB307 as the donor strain in conjugations. The *E. coli* strains were cultured in LB medium at 37°C, with the appropriate selection (50 μg/ml ampicillin, 50 μg/ml apramycin, 12.5 μg/ml tetracycline, 25 μg/ml kanamycin, or 25 μg/ml chloramphenicol).

For dry weight determinations mycelium from 7 ml culture was separated from broth by filtration on a preweighted filter membrane. The mycelium was dried with the filter for 24 hr at 37°C.

Cosmid Library Construction and Identification of *pbt* Cluster

Genomic DNA was extracted from *P. rosea* ATCC53733, grown in seed medium for 3 days, according to described procedures (Kieser et al., 2000). Genomic DNA was digested *Sau*3AI, purified and ligated into the BamHI site of the Supercos3 vector (Figure S11). The cosmid library was constructed using the Gigapack III XL Packaging Extract kit (Stratagene), following the manufacturer's instructions. Pools of cosmid-carrying colonies were PCR-screened with primers specific for *pbtR*, *pbtB*, and *pbtM3*. Cosmid 2F7 resulted positive to all PCR analyses. Sequencing of 2F7 insert ends established that it contains the entire *pbt* cluster. The sequence of the *pbt* cluster is available at GenBank under accession number KF366381.

GE2270 Heterologous Production

Cosmid 2F7 was introduced in *E. coli* ET12567/pUB307, and the resulting strain was used for conjugation with *Nonomuraea* ATCC39727 as described (Stinchi et al., 2003) with minor modifications: during mating experiments, donor and recipient cells were mixed in a 1:1 ratio and plated on MV0.1X agar (2.4 g/l soluble starch, 0.1 g/l dextrose, 0.3 g/l beef extract, 0.5 g/l yeast extract, 0.5 g/l tryptose, 10 mM $MgCl_2$, and 15 g/l agar; pH7.2). After 20 hr at 28°C, each plate was overlaid with 3 ml soft agar containing 200 μg nalidixic acid and 250 μg apramycin and further incubated at 28°C. *Nonomuraea* colonies, which usually appeared after 7–10 days, were streaked on BTT plates containing 25 μg/ml nalidixic acid and 20 μg/ml apramycin. After PCR confirmation of the presence of the *pbt* cluster using primers specific for *pbtR*, *pbtA*, and *pbtM3*, two independent colonies were grown and extracted with 2 volumes of methanol. Following 1 hr at 40°C and centrifugation, 20 μl of the resulting supernatant was analyzed by HPLC on a Shimadzu instrument as described below.

For GE2270 production, 450 μl samples of *P. rosea* and Nono-2F7 cultures were withdrawn every 24 hr and processed as described. Production was quantified by integrating peaks corresponding to GE2270A, using an authentic sample of GE2270A as external standard.

Manipulation of the *pbt* Cluster

Gene inactivations in cosmid 2F7 were performed using the λ Red recombination system (Datsenko and Wanner, 2000). Appropriate DNA fragments were PCR-generated using 70-nt-long primers (Table S4) that included 50-nt extensions to the cognate *pbt* region and 20-nt priming sequences for pKD3 or pKD13, for inclusion of *kan* or *cat* flanked by FRT sites, respectively. After purification, each PCR product was used for electroporation of *E. coli* cells carrying cosmid 2F7 and Red helper plasmid. The resulting colonies were examined for the intended recombination product by PCR analysis using flanking locus-specific primers with the respective *kan*- or *cat*-specific primers.

For the generation of *pbtA* variants, the λ Red recombination system was modified using forward primers carrying the desired mutations in the 70-nt *pbtA* segment (Table S4). The correctness of the PCR products was confirmed by DNA sequencing. Site-specific recombination to remove *kan* or *cat* was performed using the FLP recombinase provided with plasmid 707-FLPe-tet (GeneBridges), according to the manufacture's protocol. The identity of all mutant cosmids was confirmed by PCR.

Antibacterial Assays

Agar diffusion assays were performed by spotting 10 μl of cultures or methanolic extracts on Antibiotic Medium 1 or Müller Hinton (MH) agar plates respectively inoculated with 10^5 CFU/mL of *B. subtilis* or *S. aureus* strains. Plates were incubated for ~16 hr at 37°C.

MICs were determined by the broth microdilution methodology in sterile 96-well microtiter plates according to Clinical and Laboratory Standards Institute guidelines, using MH broth containing 20 mg/l $CaCl_2$ and 10 mg/l $MgCl_2$ for all strains, except for *Streptococcus* spp., which were grown in Todd Hewitt broth (Difco Laboratories). Bacteria were inoculated at 5×10^5 CFU/ ml and incubated for 24 hr at 37°C. The MIC was defined as the lowest drug concentration causing complete suppression of visible growth.

LC-MS Analyses and NMR Spectroscopy

HPLC analyses were performed using an LC 2010A-HT liquid chromatographer (Shimadzu) equipped with a LiChrosphere C18 5 μm, 4.6 × 100 mm column (Merck). Elution was performed at 1 ml/min and 50°C with a linear gradient from 10% to 90% phase B in 30 min. Phase A was 0.1% TFA (v/v) in water and phase B was acetonitrile. UV detection was set at 270 and 310 nm. LC-MS analysis were performed with an Agilent 1100 series liquid chromatographer equipped with a Ascentis express Supelco RP18, 2.7 μ 50 × 4.6 mm column eluted at 1 ml/min at 40°C. Elution was with a 10 min multistep program that consisted of 5%, 95%, 100%, and 5% phase B_1 at 0, 6, 7, and 7.2 min, respectively (phases A_1 and B_1 were 0.05% TFA [v/v] in water and acetonitrile, respectively) with detection at 220 nm. The effluent from the column was split 1:1 into a photodiode array detector and into the ESI interface of a Bruker Esquire 3000 plus ion trap mass spectrometer (Bruker). Mass spectrometry analyses were performed using as sample inlet conditions: sheath gas (N_2) 50 psi, dry gas 10 l/min, capillary heater 365°C; as sample inlet voltage settings: positive polarity, capillary voltage −4,000 V, end plate offset −500 V; as scan conditions: maximum ion time 200 ms, ion time 5 ms, full micro scan 3; as segment: duration 10 min, scan events positive (100–2,400 m/z). LC-HRMS analyses were performed on a Surveyor Accela HPLC system (Thermo Fisher Scientific) connected to an Exactive benchtop mass spectrometer (Thermo Fisher Scientific), equipped with a NSI-ESI ion source. Samples were injected on a C8 reversed phase column (BioBasic C8, 100 × 0.18 mm, 5 μm, 300 Å, Thermo Fisher Scientific) and eluted with the following eluent B_2 gradient: 5% for 3 min; 5% to 65% in 17 min; and 65% to 95% in 5 min (eluents A_2 and B_2 were 0.1% formic acid in water and acetonitrile, respectively); the flow rate was 100 μl/min split in order to achieve a final flux of 2 μl/min.

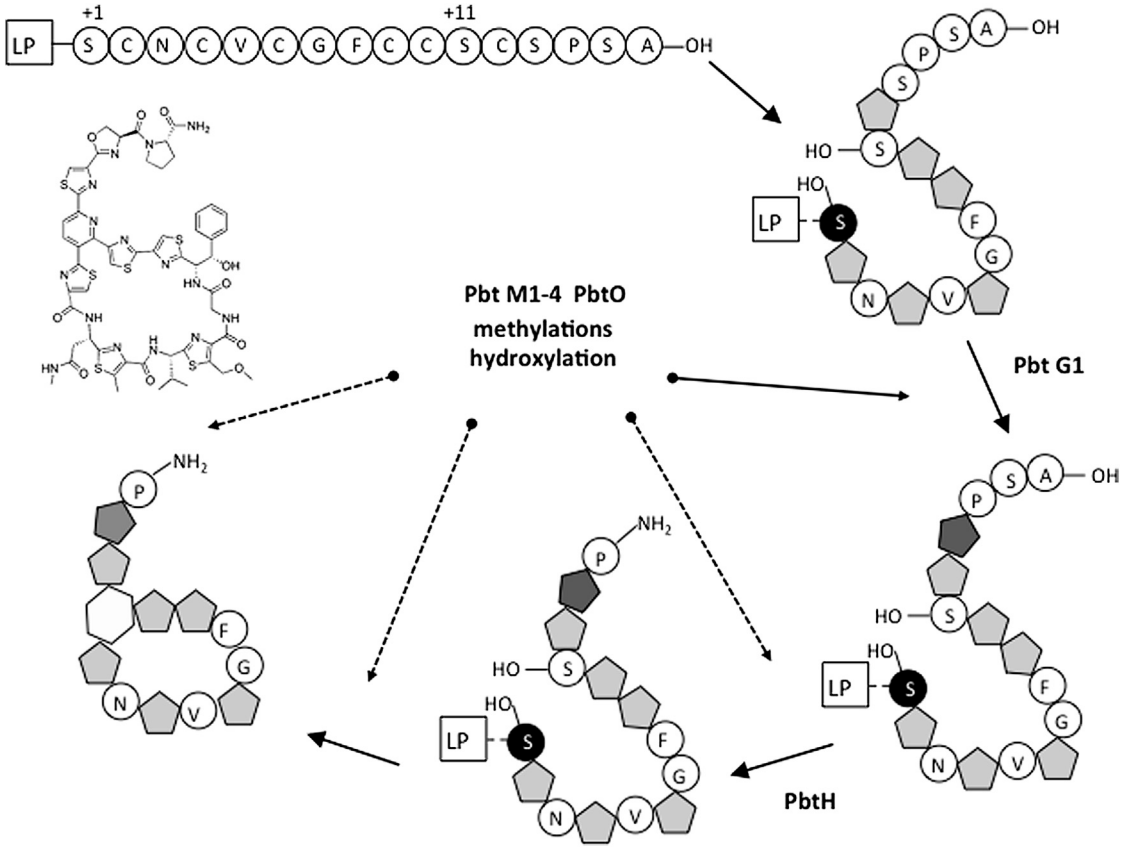

Figure 6. Schematic Representation of Proposed Order of GE2270 Biosynthetic Events

Summary of the experimental evidence that is the likely order of events: thiazole syntheses, oxazoline formation, C-terminal amide installment, and pyridine-mediated macrocyclization. Please note that the timing of leader peptide removal has been arbitrarily placed before pyridine formation. Dashed arrows stemming from PbtM1-4 and PbtO indicate possible additional substrates for these tailoring steps. Thiazoles and oxazoline are symbolized by gray and dark gray pentagons, respectively; and the pyridine ring by a hexagon. The black circle represents Ser1, which is likely to undergo dehydration at some stage before Ser11. Only Ser1 and Ser 11 are numbered in the core peptide. LP indicates the leader peptide.

^{1}H-,^{13}C-, 1D, and 2D NMR spectra (COSY, TOCSY, HSQC, HMBC) were measured in dmso-d_6 or acetone-d_6 at 25°C using an AMX 600 MHz spectrometer (Bruker). Chemical shifts are reported relative to dmso-d_6 (δ 2,50) or acetone-d_6 (δ 2,05).

Production and Isolation of Compounds from *Nonomuraea*

The culture (1 l) was centrifuged at 3,000 rpm for 10 min and the mycelium was extracted overnight with 240 ml methanol on a rotary shaker at room temperature. After centrifugation, the exhausted mycelium was discarded and the methanolic extract was concentrated under vacuum to 20 ml and transferred into a separating funnel, where an equal volume of butanol was added along with few mL H_2O. The resulting butanol phase was evaporated to dryness, dissolved in 2 ml dichloromethane, and purified by medium pressure chromatography on a 12 g normal phase silica Flash RediSep RF column using a CombiFlash RF Teledyne Isco Medium Pressure Chromatography System. The silica was previously conditioned for 2 min at 30 ml/min with dichloromethane, then brought to 50% methanol within 13 min. The fractions containing the desired compound were pooled and concentrated under vacuum. This procedure was used to purify GE2270 congeners A, B1, C1, D1, D2, C2b, and compounds **1** and **4**.

For compounds **2** and **3**, 1 l culture was processed as above up to the methanolic extract, which was evaporated to 50 ml and split into 5 × 10 ml portions, each purified using a 5 g Flash C18 prepacked column (Isolute, Biotage) placed on a Vac Master system (Stepbio). Each loaded solution was washed with 10 ml acetonitrile:water 25:75 and eluted with 15 ml acetonitrile:water 75:25. Fractions were pooled, evaporated to dryness, dissolved in 1 ml acetonitrile:water 75:25 and fractionated by HPLC using a Shimadzu Series 10 system equipped with a LiChrosphere C18 5 μm, 4.6 × 100 mm reversed-phase column (Merck) and a 22 min linear gradient from 10% H_2O to 80% acetonitrile.

For compounds **5** and **6**, cultures were centrifuged at 3,000 rpm for 10 min. The mycelium was processed as above up to the methanolic extract, which was concentrated to 7 mL. This sample was fractionated by medium pressure chromatography on 30 g reversed phase Biotage Snap Cartridge KP-C18-HS by using a CombiFlash RF Teledyne Isco Medium Pressure Chromatography System. The column was previously conditioned for 1 min at 20 ml/min with H_2O, then brought successively to 40% acetonitrile in 5 min, to 60% acetonitrile in 13 min, and finally washed for 2 min with 95% acetonitrile. Fractions containing compound **5** were pooled and concentrated under vacuum and subjected to LC-MS analysis as described above. The supernatant was extracted with BuOH (400 ml, under magnetic stirring overnight at room temperature). After centrifugation (4,000 rpm, 10 min) the buthanolic phase was dried, redissolved in 50 ml MeOH:H_2O 1:1, and resolved by medium pressure chromatography on 30 g of reversed phase Biotage Snap Cartridge KP-C18-HS using a CombiFlash RF Teledyne Isco Medium Pressure Chromatography System. The column was previously conditioned for 1 min at 20 ml/min with pure water, then brought to 40% of acetonitrile in 5 min, successively brought to 60% of acetonitrile in 13 min, and washed with 95% of acetonitrile for 2 min. The fractions containing compound **6** (HPLC retention time 17.83 min) were pooled and concentrated under vacuum.

For compounds **7** and **8**, 400 ml cultures were mixed with 1 vol MeOH and stirred at room temperature for 2 hr. Mycelium was removed by centrifugation

and the hydroalcoholic extract, after 2× concentration under vacuum, was extracted with 350 ml butanol for 30 min at room temperature. The resulting butanolic phase was dried, dissolved in 25 ml H_2O:acetonitrile 75:25, and resolved by medium pressure chromatography on 30 g of reversed phase Biotage Snap Cartridge KP-C18-HS by using a CombiFlash RF Teledyne Isco Medium Pressure Chromatography System. The column was previously conditioned for 1 min at 20 ml/min with H_2O, then brought to 40% of acetonitrile in 5 min, successively brought to 60% acetonitrile in 13 min, and washed with 95% of acetonitrile for 2 min. The fractions containing compound **7** or compound **8** were pooled and concentrated under vacuum.

ACCESSION NUMBERS

The sequence of the *pbt* cluster is available from GenBank under accession number KF366381.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eleven figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.07.005.

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REFERENCES

Acker, M.G., Bowers, A.A., and Walsh, C.T. (2009). Generation of thiocillin variants by prepeptide gene replacement and in vivo processing by Bacillus cereus. J. Am. Chem. Soc. *131*, 17563–17565.

Arnison, P.G., Bibb, M.J., Bierbaum, G., Bowers, A.A., Bugni, T.S., Bulaj, G., Camarero, J.A., Campopiano, D.J., Challis, G.L., Clardy, J., et al. (2013). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. Nat. Prod. Rep. *30*, 108–160.

Bhat, U.G., Halasi, M., and Gartel, A.L. (2009). FoxM1 is a general target for proteasome inhibitors. PLoS ONE *4*, e6593.

Belshaw, P.J., Roy, R.S., Kelleher, N.L., and Walsh, C.T. (1998). Kinetics and regioselectivity of peptide-to-heterocycle conversions by microcin B17 synthetase. Chem. Biol. *5*, 373–384.

Binz, T.M., Maffioli, S.I., Sosio, M., Donadio, S., and Müller, R. (2010). Insights into an unusual nonribosomal peptide synthetase biosynthesis: identification and characterization of the GE81112 biosynthetic gene cluster. J. Biol. Chem. *285*, 32710–32719.

Bowers, A.A., Acker, M.G., Koglin, A., and Walsh, C.T. (2010). Manipulation of thiocillin variants by prepeptide gene replacement: structure, conformation, and activity of heterocycle substitution mutants. J. Am. Chem. Soc. *132*, 7519–7527.

Bowers, A.A., Acker, M.G., Young, T.S., and Walsh, C.T. (2012). Generation of thiocillin ring size variants by prepeptide gene replacement and in vivo processing by Bacillus cereus. J. Am. Chem. Soc. *134*, 10313–10316.

Butler, M.S. (2005). Natural products to drugs: natural product derived compounds in clinical trials. Nat. Prod. Rep. *22*, 162–195.

Colombo, L., Stella, S., and Selva, E. (1995). Contribution of mass spectrometry to the structural confirmation of components of the antibiotic GE2270 complex. Rapid Commun. Mass Spectrom. *9*, 717–722.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

De Pietro, M.T., Marazzi, A., Sosio, M., Donadio, S., and Lancini, G. (2001). Biosynthesis of the thiazolylpeptide antibiotic GE2270. J. Antibiot. (Tokyo) *54*, 1066–1071.

Donadio, S., Maffioli, S., Monciardini, P., Sosio, M., and Jabes, D. (2010). Antibiotic discovery in the twenty-first century: current trends and future perspectives. J. Antibiot. (Tokyo) *63*, 423–430.

Dunbar, K.L., Melby, J.O., and Mitchell, D.A. (2012). YcaO domains use ATP to activate amide backbones during peptide cyclodehydrations. Nat. Chem. Biol. *8*, 569–575.

Foulston, L.C., and Bibb, M.J. (2010). Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes. Proc. Natl. Acad. Sci. USA *107*, 13461–13466.

Gastaldo, L., and Marinelli, F. (2003). Changes in GE2270 antibiotic production in *Planobispora rosea* through modulation of methylation metabolism. Microbiology *149*, 1523–1532.

Kettenring, J., Colombo, L., Ferrari, P., Tavecchia, P., Nebuloni, M., Vékey, K., Gallo, G.G., and Selva, E. (1991). Antibiotic GE2270 a: a novel inhibitor of bacterial protein synthesis. II. Structure elucidation. J. Antibiot. (Tokyo) *44*, 702–715.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics (Norwich, UK: Crowes).

LaMarche, M.J., Leeds, J.A., Amaral, A., Brewer, J.T., Bushell, S.M., Deng, G., Dewhurst, J.M., Ding, J., Dzink-Fox, J., Gamber, G., et al. (2012). Discovery of LFF571: an investigational agent for *Clostridium difficile* infection. J. Med. Chem. *55*, 2376–2387.

Li, C., Zhang, F., and Kelly, W.L. (2012). Mutagenesis of the thiostrepton precursor peptide at Thr7 impacts both biosynthesis and function. Chem. Commun. (Camb.) *48*, 558–560.

Liao, R., and Liu, W. (2011). Thiostrepton maturation involving a deesterification-amidation way to process the C-terminally methylated peptide backbone. J. Am. Chem. Soc. *133*, 2852–2855.

Liu, W., Ma, M., Xue, Y., Liu, N., Wang, S., and Chen, Y. (2013). The C-terminal extended serine residue is absolutely required in nosiheptide maturation. ChemBioChem *14*, 573–576.

Malcomson, S.J., Young, T.S., Ruby, J.G., Skewes-Cox, P., and Walsh, C.T. (2013). The posttranslational modification cascade to the thiopeptide berninamycin generates linear forms and altered macrocyclic scaffolds. Proc. Natl. Acad. Sci. USA *110*, 8483–8488.

Morris, R.P., Leeds, J.A., Naegeli, H.U., Oberer, L., Memmert, K., Weber, E., LaMarche, M.J., Parker, C.N., Burrer, N., Esterow, S., et al. (2009). Ribosomally synthesized thiopeptide antibiotics targeting elongation factor Tu. J. Am. Chem. Soc. *131*, 5946–5955.

Parmeggiani, A., Krab, I.M., Okamura, S., Nielsen, R.C., Nyborg, J., and Nissen, P. (2006). Structural basis of the action of pulvomycin and GE2270 A on elongation factor Tu. Biochemistry *45*, 6846–6857.

Sherwood, E.J., Hesketh, A.R., and Bibb, M.J. (2013). Cloning and analysis of the planosporicin lantibiotic biosynthetic gene cluster of *Planomonospora alba*. J. Bacteriol. *195*, 2309–2321.

Selva, E., Beretta, G., Montanini, N., Saddler, G.S., Gastaldo, L., Ferrari, P., Lorenzetti, R., Landini, P., Ripamonti, F., Goldstein, B.P., et al. (1991). Antibiotic GE2270 a: a novel inhibitor of bacterial protein synthesis. I. Isolation and characterization. J. Antibiot. (Tokyo) *44*, 693–701.

Selva, E., Ferrari, P., Kurz, M., Tavecchia, P., Colombo, L., Stella, S., Restelli, E., Goldstein, B.P., Ripamonti, F., and Denaro, M. (1995). Components of the GE2270 complex produced by *Planobispora rosea* ATCC 53773. J. Antibiot. (Tokyo) *48*, 1039–1042.

Sosio, M., Amati, G., Cappellano, C., Sarubbi, E., Monti, F., and Donadio, S. (1996). An elongation factor Tu (EF-Tu) resistant to the EF-Tu inhibitor GE2270 in the producing organism *Planobispora rosea*. Mol. Microbiol. *22*, 43–51.

Sosio, M., Stinchi, S., Beltrametti, F., Lazzarini, A., and Donadio, S. (2003). The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *nonomuraea* species. Chem. Biol. *10*, 541–549.

Stinchi, S., Azimonti, S., Donadio, S., and Sosio, M. (2003). A gene transfer system for the glycopeptide producer *Nonomuraea* sp. ATCC39727. FEMS Microbiol. Lett. *225*, 53–57.

Tavecchia, P., Gentili, P., Kurz, M., Sottani, C., Bonfichi, R., Lociuro, S., and Selva, E. (1994). Revised structure of the antibiotic GE 2270A. J. Antibiot. (Tokyo) *47*, 1564–1567.

Walsh, C.T., Acker, M.G., and Bowers, A.A. (2010). Thiazolyl peptide antibiotic biosynthesis: a cascade of post-translational modifications on ribosomal nascent proteins. J. Biol. Chem. *285*, 27525–27531.

Weber, J.M., Leung, J.O., Swanson, S.J., Idler, K.B., and McAlpine, J.B. (1991). An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythraea*. Science *252*, 114–117.

Young, T.S., and Walsh, C.T. (2011). Identification of the thiazolyl peptide GE37468 gene cluster from *Streptomyces* ATCC 55365 and heterologous expression in *Streptomyces lividans*. Proc. Natl. Acad. Sci. USA *108*, 13053–13058.

Young, T.S., Dorrestein, P.C., and Walsh, C.T. (2012). Codon randomization for rapid exploration of chemical space in thiopeptide antibiotic variants. Chem. Biol. *19*, 1600–1610.

Yu, Y., Duan, L., Zhang, Q., Liao, R., Ding, Y., Pan, H., Wendt-Pienkowski, E., Tang, G., Shen, B., and Liu, W. (2009). Nosiheptide biosynthesis featuring a unique indole side ring formation on the characteristic thiopeptide framework. ACS Chem. Biol. *4*, 855–864.

Yu, Y., Guo, H., Zhang, Q., Duan, L., Ding, Y., Liao, R., Lei, C., Shen, B., and Liu, W. (2010). NosA catalyzing carboxyl-terminal amide formation in nosiheptide maturation via an enamine dealkylation on the serine-extended precursor peptide. J. Am. Chem. Soc. *132*, 16324–16326.

Zhang, Q., and Liu, W. (2013). Biosynthesis of thiopeptide antibiotics and their pathway engineering. Nat. Prod. Rep. *30*, 218–226.