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Mechanistically distinct nonribosomal peptide synthetases assemble the structurally related viomycin and capreomycin antibiotics

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INTRODUCTION

Tuberculosis (TB) is a worldwide burden for human health. More than 1.8 million people succumbed to TB infection in 2008 alone, and as many as two billion people may be passively infected by *Mycobacterium tuberculosis*, the causative agent of TB.[1] One of the challenges facing the successful treatment of *M. tuberculosis* infections is the development of strains that are resistant to many of the antibiotics used in the clinic. Cases of multidrug-resistant TB (MDR-TB), defined as TB caused by a *M. tuberculosis* strain that is resistant to both rifampin and isoniazid, have been identified in nearly every country surveyed.[1, 2] Extensively drug-resistant TB (XDR-TB), defined as MDR-TB that is also resistant to a fluoroquinolone and at least one of three injectables (capreomycin, streptomycin, or amikacin), has spread to more than 45 countries, including the United States.[1, 2] The development and spread of drug-resistant strains of *M. tuberculosis* has put a high priority on the development of new antituberculosis drugs and the generation of derivatives of known drugs that regain their antibiotic activity against resistant strains.

The tuberactinomycin family of antituberculosis drugs are important components of our drug arsenal against drug-resistant *M. tuberculosis*. The most prominent member of this family is capreomycin (CMN), which is a key drug in the treatment of MDR-TB based on its inclusion on the World Health Organization's "Model List of Essential Medicines." [3] Furthermore, if CMN is the injectable that XDR-TB infection is resistant to, it almost guarantees treatment failure.[4] Based on its level of importance in treating drug-resistant forms of TB, it is important that new CMN derivatives be developed that regain activity against resistant *M. tuberculosis* strains. Synthetic procedures to synthesize derivatives have been difficult.[5, 6] More success has been accomplished by semisynthetic approaches,[7–9] but the derivatives are limited by the functional groups present on the cyclic pentapeptide core. Complementing these approaches with metabolic engineering of the enzymology that produces the cyclic pentapeptide core has the potential of enabling further structural diversification. Our focus is on the metabolic engineering aspect of drug development.

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Harnessing the full potential of metabolic engineering to generate new drug derivatives requires a complete understanding of how the targeted drug is biosynthesized by the producing organism. To this end, we have focused on understanding tuberactinomycin biosynthesis at the molecular and biochemical level by using CMN and the structural analog viomycin (VIO) as model systems. Our focus on both CMN and VIO was based on our hypothesis that by studying the biosynthesis of two structurally related molecules (Scheme 1), we would gain considerable insights into how the tuberactinomycins are biosynthesized and structurally modified by the natural enzymology. To date, we have sequenced and annotated the CMN and VIO biosynthesis gene clusters,^[10, 11] reconstituted CMN and VIO production in the heterologous host *Streptomyces lividans*,^[12] biochemically characterized L-capreomycidine formation,^[13] and identified the amino acids activated by each of the adenylation (A) domains of the CMN nonribosomal peptide synthetases (NRPS) and some of the A domains of the VIO NRPS (Scheme 2).^[14] Zabriskie and colleagues have also contributed important genetic and biochemical information that enable more refined models of tuberactinomycin biosynthesis to be developed.^[15–18]

Here we provide additional data concerning the mechanism of peptide assembly by the CMN and VIO NRPSs. Our initial focus was on the addition of the β -lysine moiety into VIO and two of the CMN derivatives (CMN IA and IB, Scheme 1). We show that NRPS enzymology in both systems is involved in β -lysine addition. Surprisingly, our *in vivo* data strongly suggest that β -lysine addition occurs concurrently with cyclic pentapeptide assembly rather than after as initially proposed. Additional work suggests that the VIO NRPS has proofreading capabilities that the CMN NRPS does not have to control the incorporation of only L-serine into the cyclic pentapeptide core rather than L-serine and L-alanine as seen with the CMN NRPS. Finally, all of these data are used to formulate models for CMN and VIO assembly by distinct NRPS mechanisms. Of particular interest is the CMN assembly that likely occurs via an unusual mechanism involving converging NRPSs that generate the branched derivatives CMN IA and IB.

RESULTS AND DISCUSSION

Addition of β -Lysine into CMN IA and IB requires *cmnO* and *cmnM*

We hypothesized that the addition of β -lysine during the biosynthesis of CMN IA and IB was a two-step process whereby the amino acid is first activated and thioesterified to CmnO by the concerted actions of the A and PCP domains of this enzyme and then transferred to the β -amino group of residue 3 of the cyclic pentapeptide core by CmnM, an NRPS C domain (Scheme 2).^[10] Recent *in vitro* data showing that CmnO specifically activates β -lysine^[14] supports the proposed function of CmnO in this process. To continue testing our hypothesis, we constructed CMN biosynthesis gene clusters that lacked either *cmnO* or *cmnM* and assessed whether the production of CMN IA and IB was disrupted when these genes were absent. Production of CMN derivatives was assessed by growing strains of *S. lividans* carrying the modified CMN biosynthesis gene clusters and detecting and identifying the CMN derivatives produced by these strains using HPLC and MS (Figure 1). Consistent with our hypothesis, the absence of either *cmnO* or *cmnM* resulted in a strain that produced CMN IIA and IIB, but failed to produce the β -lysine-containing derivatives CMN IA and IB. Introduction of a plasmid expressing the missing gene restored CMN IA and IB production (data not shown), confirming that the specific loss of *cmnO* or *cmnM* was causative of the observed phenotype. An additional CMN derivative eluting prior to CMN IIA and IIB in the $\Delta cmnO$ strain was detected, but its identity is currently unknown.

Addition of β -Lysine into VIO requires *vioO* and *vioM*

VioO and VioM from the VIO biosynthesis pathway are homologs of CmnO and CmnM, respectively. Based on this homology, it is reasonable to propose that the loss of either VioO or VioM from a VIO-producing strain would result in the production of des- β -lysine VIO (tuberactinamine A). Consistent with this hypothesis, deletion of either *vioO* or *vioM* from the VIO biosynthesis gene cluster resulted in the production of tuberactinamine A rather than VIO based on HPLC and MS analyses (Figure 2). At this time it is not clear why the loss of *vioM* reduced tuberactinamine A production relative to the levels produced by a strain lacking *vioO*. The addition of plasmids expressing *vioO* or *vioM* restored VIO production to the corresponding deletion strain (data not shown). These data, in conjunction with the Δ *cmnO* and Δ *cmnM* data support our hypothesis that β -lysine addition for the tuberactinomycin antibiotic family is catalyzed by NRPSs.

Addition of β -lysine into CMN IA and IB is influenced by the N-terminal “X” domain of CmnA

It is reasonable to hypothesize that differences in the NRPSs that assemble VIO and CMN dictate the subtle structural variation in these antibiotics. The structural differences include: i) the location of β -lysine attachment; ii) L-serine at position 2 of VIO, but L-serine or L-alanine at the same position in CMN; and iii) L-serine at position 3 of VIO, but L-2,3-diaminopropionate in CMN. In relation to these structural differences, our prior *in vitro* work on CmnA determined the A1 of CmnA is specific for L-2,3-diaminopropionate, while the A2 domain activates either L-serine or L-alanine.^[14] These data suggest the mechanistic reason for position 3 being L-serine in VIO but L-2,3-diaminopropionate in CMN is different A domains install the amino acid found at position 3. In the VIO NRPS it is the A2 domain of VioA, while in the CMN NRPS it is the A1 domain of CmnA. This difference also impacts the location of β -lysine attachment because only when L-2,3-diaminopropionate is at position 3 is an amino group available for amide bond formation with β -lysine. A characterization of the remaining differences between CmnA and VioA were expected to provide further insights into how the structural differences between CMN and VIO are generated.

A comparison of CmnA and VioA showed that CmnA contains an ~330 amino acid N-terminal “X” domain that is not present in VioA.^[10] We were interested in determining whether this domain influences CmnA activity and provides a reason for the structural differences between CMN and VIO. We constructed a CMN biosynthesis gene cluster where the DNA coding for the X domain of CmnA was removed by an in-frame deletion. The resulting gene cluster lacked the portion of *cmnA* coding for the X domain, but still coded for all the remaining biosynthetic components. Introduction of this gene cluster into *S. lividans* followed by CMN purification and analysis by HPLC and MS determined that the loss of the X domain did not disrupt CMN IIA and IIB production, but unexpectedly abolished CMN IA and IB production (Figure 3). We conclude from this work that the X domain influences the activity of CmnO and CmnM for addition of the β -lysine moiety.

The finding that a portion of CmnA is essential for β -lysine addition is surprising since this step in biosynthesis was hypothesized to occur after cyclic pentapeptide assembly during both CMN and VIO biosynthesis.^[10, 11] This hypothesis was based on the natural production of CMN IIA and IIB during CMN biosynthesis and our finding that only CMN IIA and IIB are produced in the absence of *cmnO* and *cmnM*. Similarly, tuberactinamine A accumulates when β -lysine biosynthesis^[12] or addition (Figure 2) is disrupted during VIO biosynthesis. These data implied that β -lysine addition occurred after cyclic pentapeptide assembly since disruption of β -lysine addition that was concurrent with cyclic pentapeptide assembly would be expected to abolish production of any peptide based on the assembly line

paradigm of NRPS enzymology. Our finding that the N-terminal domain of CmnA influences β -lysine addition suggests this process may be more complicated than originally proposed. An alternative model is the N-terminal domain of CmnA is a docking domain for CmnO and CmnM, and β -lysine addition is concurrent with cyclic pentapeptide assembly. This is a reasonable hypothesis since the A1 domain adjacent to the N-terminal domain must introduce the L-2,3-diaminopropionate at position 3 of the cyclic pentapeptide core. It is the β -amino group of this residue that β -lysine is attached to; thus, the N-terminal domain of CmnA will be in close proximity to the site of β -lysine attachment. Further support for this model will be discussed below.

Analyzing differences between CmnA and VioA provides insights into both residue 2 incorporation and the timing of β -lysine addition

In addition to playing a role in the structural differences between CMN and VIO at residue 3 and the site of β -lysine addition, CmnA also controls the difference between CMN and VIO at position 2 of the cyclic pentapeptide core. In CMN this residue can be either L-serine or L-alanine, but in VIO this residue is restricted to only L-serine. Our initial hypothesis was that differences in the amino acid specificity codes^[10] of the second A domains of CmnA and VioA would explain the differences in the amino acid composition of residue 2. A comparison of the ten residues making up the proposed amino acid specificity code found a single amino acid difference: leucine at position five of the specificity code of CmnA versus a methionine in VioA. We changed the corresponding leucine codon in *cmnA* to a methionine codon. The resulting gene cluster was introduced into *S. lividans* and CMN production was analyzed. To our surprise, the strain still produced all four derivatives of CMN, including those containing L-alanine at position 2 (Figure 3). We conclude from these data that the difference in the specificity code between the A2 domains of CmnA and VioA does not dictate the amino acid difference in position 2 of the cyclic pentapeptide core.

The failure to change the substrate specificity of the A2 domain of CmnA by amino acid substitution led us to focus on the A domain as a whole. Our hypothesis was that amino acids outside the substrate-binding pocket indirectly influence substrate recognition. If correct, replacing the entire A2 domain of CmnA that catalyzes L-serine/L-alanine incorporation with the corresponding A2 domain of VioA should eliminate the production of CMN IB and IIB, containing only L-alanine at position 2, but not CMN IA or IIA, which contain L-serine at position 2. To test this hypothesis, we constructed a *cmnA-vioA* fusion that coded for a CmnA derivative where the C-A pair from the second module of CmnA was replaced with the corresponding C-A pair from VioA. We modified both domains based on the proposal that C-A pairs co-evolve for substrate recognition and should be moved together when generating hybrid NRPSs.^[19] The modified CMN biosynthesis gene cluster coding for the hybrid NRPS was introduced into *S. lividans* and the production of CMN by this strain was analyzed by HPLC and MS. This strain failed to produce any of the four CMN derivatives (CMN IA, IB, IIA, or IIB). Instead, this strain produced small quantities of three peptides that retained the signature absorbance spectrum of the tuberactinomycins, suggesting they contained the β -ureidodehydroalanine residue.^[20] Analysis of these peptides by MALDI-TOF MS/MS determined they were fragments of CMN (Scheme 3). Two of the peptides were CMN IA/IB lacking residue 1 of the cyclic pentapeptide core, while the third was CMN IA lacking residues 2 and 3. All three peptides contained β -lysine.

These results suggest a number of surprising aspects of CMN and VIO biosynthesis. First, the hybrid NRPS is able to initiate peptide synthesis from either the second or third module. This premature initiation of peptide synthesis by the NRPS is most likely due to the inactivation of the C-domain from VioA when fused into CmnA causing the tethered amino acids on downstream modules to aberrantly initiate peptide synthesis. A control construct reintroducing the original C-A pair into CmnA using the same restriction sites resulted in a

fully functional NRPS (data not shown). This finding suggests that the amino acids changes introduced into the C domain of VioA during the cloning procedure have inactivated this domain. Second, the peptides that contain residues 2–5 of the cyclic pentapeptide core of CMN have either L-serine or L-alanine at the position corresponding to residue 2 of the core. This finding suggests that the corresponding A domain of VioA activates both L-serine and L-alanine even though VIO only has L-serine at this position. Additional analysis of this hypothesis is discussed in the next section. Finally, all three peptides contain β -lysine attached to the β -amino group of the amino acid corresponding to position 3 of the cyclic pentapeptide core. This result was unexpected and suggests the model for the timing of β -lysine addition needs revision.

One model to explain the presence of β -lysine on all three peptides is that CmnM and CmnO are flexible in substrate recognition and are able to amino acylate the cyclic pentapeptide core or shorter, non-cyclic peptides that contain at least the tripeptide L-2,3-diaminopropionyl-L- β -ureidodehydroalanyl-L-capreomycinidine. Alternatively, β -lysine addition occurs earlier in CMN biosynthesis than originally proposed. For example, once L-2,3-diaminopropionate is added to the PCP domain of CmnI or once L-2,3-diaminopropionyl-L-seryl/alanyl-L-2,3-diaminopropionyl-CmnI is formed, the β -amino group of residue 3 is a substrate for β -lysine addition by the concerted actions of CmnO and CmnM. This may explain why the loss of the N-terminal domain of CmnA abolishes β -lysine addition (Figure 3). The A domain adjacent to the N-terminal domain introduces the L-2,3-diaminopropionate residue that becomes acylated with β -lysine; thus, the N-terminal domain is in close proximity to the site of β -lysine addition. If this domain functions as a docking site for CmnO and CmnM, then β -lysine addition to residue 3 may be concurrent with cyclic pentapeptide core assembly. Further biochemical studies will be needed to test this hypothesis.

The A1 domain of VioA activates L-2,3-diaminopropionate while the A2 domain of VioA activates both L-serine and L-alanine

The *in vivo* results discussed above suggest that the A2 domain of VioA activates both L-serine and L-alanine, an unexpected result based on the structure of VIO. To test this hypothesis directly, we cloned the regions of *vioA* that code for the first and second A-PCP pairs of VioA (A1PCP1 and A2PCP2, respectively), overproduced each A-PCP in *E. coli*, and assayed the purified proteins for amino acid activation. To obtain functional enzymes, both A-PCP pairs were co-overproduced in *E. coli* with the MbtH-like protein VioN. We recently showed that this protein is required for VioO activity, and the corresponding protein from the CMN biosynthesis pathway was required for CmnO activity as well as both A-PCP pairs from CmnA.^[14] Consistent with those data, the A-PCP pairs of VioA were more soluble in the presence of VioN, and co-purified with each A-PCP pair (data not shown). Analysis of these enzymes for amino acid activation found A1 from VioA activated L-2,3-diaminopropionate, while A2 from VioA activated both L-serine and L-alanine (Figure 4). The finding that A2 activates both amino acids has significant implications on the model for VIO biosynthesis. First, it suggests that the NRPS naturally incorporates L-serine or L-alanine on the PCP2 domain of VioA and the PCP domain of VioI. Second, since both the 2 and 3 positions of the VIO cyclic pentapeptide core contain only L-serine, there must be a proofreading mechanism within the VIO NRPS to discriminate against L-alanine at either position.

Conclusions

We have presented data that provide significant insights into how the members of the tuberactinomycin family of antibiotics are biosynthesized. The most surprising aspect of these findings is that the homologous NRPSs that assemble the structurally similar VIO and

CMN nonribosomal peptides catalyze peptide assembly by different mechanisms. Hypotheses for how each NRPS catalyzes peptide assembly that results in the structural differences between VIO and CMN are discussed below.

We propose that the VIO NRPS is a six module, non-linear NRPS (Scheme 4). The initiating module will be the A-PCP pair of VioO that introduces the N-terminal β -lysine. The next module will include VioM, a stand-alone C domain, and the A1-PCP1 pair of VioA. Together they will introduce L-2,3-diaminopropionate, and condense this amino acid with the β -lysine from VioO. The adjacent C-A2-PCP2 domains of VioA will function as module 3, with the A2 domain functioning also as the A domain for module 4, which consists of the C-terminal C domain of VioA and the PCP domain of VioI. The classification of the VIO NRPS as a non-linear system is due to the A2 domain functioning in both modules 3 and 4. It was thought that the A2 domain would be specific for L-serine due to the structure of VIO; however, our *in vitro* and *in vivo* data shows this domain is capable of activating both L-serine and L-alanine. This suggests that some component of the VIO NRPS blocks L-alanine from being present in the final product. Since A2 introduces the amino acid onto the PCP domain of both modules 3 and 4, this proofreading capability could occur at a number of locations within the NRPS. For example, the C domain of module 4 may not recognize the tripeptide containing L-alanine at its donor site, nor will it recognize L-alanyl-S-VioI at its acceptor site. Alternatively, the acceptor sites of the C domains from modules 3 and 4 may be selective for L-seryl-S-PCP. Further biochemical studies will be required to discriminate between these possibilities. Module 5 will involve the incorporation of β -ureidoalanine and α,β -desaturation by VioL. The final module introduces L-capreomycin and catalyzes the cyclization of the peptide to form the lariat-like structure of VIO. While functioning as a standard non-linear NRPS, the most unusual aspect of this NRPS is that it efficiently initiates synthesis from module 2 if β -lysine or the enzymes needed for its incorporation (VioO and VioM) are not present. This is surprising since inactivation of upstream modules typically results in cessation of peptide synthesis.^[21]

The CMN NRPS function differs from the VIO NRPS in a few key steps (Scheme 5). First, the addition of β -lysine does not initiate CMN assembly as it does for VIO assembly. Instead the A1PCP1 didomain of CmnA is the initiating module. Second, the A1 domain of the initiating module also functions to introduce the L-2,3-diaminopropionate residue onto the PCP domain of module 3. This domain organization is in contrast to that of the VIO NRPS whereby the A2 domain of VioA introduces the amino acid onto the PCP domain of module 3. Third, β -lysine is added to the β -amino group of residue 3 of the cyclic pentapeptide core rather than the α -amino group of residue 1. Part of the reason for this difference is that CmnA contains an N-terminal domain that influences β -lysine addition. Importantly, VioA lacks this domain. We hypothesized that this domain somehow directs CmnO and CmnM to add β -lysine to a different location than observed with VioO and VioM. Furthermore, our finding that non-cyclic, incomplete peptides contain β -lysine strongly suggests β -lysine addition occurs concurrently with cyclic pentapeptide assembly. This mechanism suggests CMN IA and IB biosynthesis involves a previously unknown class of NRPS, one that is non-linear and capable of initiating peptide synthesis at two independent modules, with eventual convergence to generate a branched nonribosomal peptide. In the end, only introduction of β -ureidoalanine and L-capreomycin followed by peptide cyclization occur by analogous mechanisms when comparing the CMN and VIO NRPSs.

Our findings that the synthesis of VIO and CMN are mechanistically distinct even though the gene clusters are organized in an analogous way, the enzymology is conserved, and the natural products have similar chemical structures, highlight the difficulty in making biosynthetic predictions without genetic and biochemical studies to test these hypotheses.

With an understanding of how the tuberactinomycins are assembled, we have set the stage for metabolic engineering of VIO and CMN biosynthesis to generate new structural derivatives of these medically important natural products.

Experimental Section

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are described in the Supporting Information.

Deletion of *cmnM*, *cmnO*, *vioM*, and *vioO* from their respective clusters

Lambda red mutagenesis^[22] was used to delete *cmnM*, *cmnO*, *vioM*, and *vioO* from their respective gene clusters. The spectinomycin resistance cassette from plasmid pIJ778 was PCR amplified with primer pairs specific for the deletion of *cmnM* (*cmnM*delXba 5'-GTG CTT GAC CTC TCC CCC GCG CAG CGC AGC CTG TGG GTG TCT AGA ATT CCG GGG ATC CGT CGA CC and *cmnM*delNhe 5'-CTA TTC CGC GGT GAT CTC GTC CAG CAC GGC CAG GAA GTC GCT AGC TGT AGG CTG GAG CTG CTT C), *cmnO* (*cmnO*delXba 5'-GTG ACC GCG CTG CAC CGC CTC GAC CAG CTC GCC GGC GCG TCT AGA ATT CCG GGG ATC CGT CGA CC and *cmnO*delNhe 5'-CAC CTC GGG GTC CGG CTG ATC AGG CCC GCC AAC CCG GTC GCT AGC TGT AGG CTG GAG CTG CTT C), *vioM* (*vioM*delNheI 5'-TCA TCG TGT GGG CTC CTC GGG AGA AGG ATC GGA GGA TCG GCT AGC TGT AGG CTG GAG CTG CTT C and *vioM*delXbaI 5'-ATG GGT GGT CAC GAC GTG AGG ACG CGG ATC GCG CTC CGT TCT AGA ATT CCG GGG ATC CGT CGA CC), or *vioO* (*vioO*delNheI 5'-TCA CTG GGG CTC TCC TCG GAA GGG TCC GGA GAC TTC GAC GCT AGC TGT AGG CTG GAG CTG CTT C and *vioO*delXbaI 5'-ATG ACC ACC ATG CCC ACG ACC GGC ACG GCC GGC GAC CGG TCT AGA ATT CCG GGG ATC CGT CGA CC). The resulting products contained the spectinomycin resistance cassette flanked by 39 bp of homology to the 5' and 3' regions of each gene, as well as *XbaI* and *NheI* restriction sites. The PCR products were then electroporated into electrocompetent *E. coli* BW25113 containing the temperature sensitive plasmid pIJ790 and pCMN-P4C8RF or pVIO-P4C3RH as appropriate. Transformants were plated on LB with kanamycin (50 µg mL⁻¹), carbenicillin (100 µg mL⁻¹), and spectinomycin (50 µg mL⁻¹) at 37°C to select for integration of the PCR product into the cosmid and loss of pIJ790.

To remove the resistance cassette, each construct was digested with *XbaI* and *NheI*, ligated together (thus destroying both restriction sites), and electroporated into XL-1Blue MR. Transformants were screened for spectinomycin sensitivity. To confirm loss of the cassette, clones were screened by restriction digest and sequencing. The resulting cosmids, pCMN-P4C8RF-Δ*cmnM*, pCMN-P4C8RF-Δ*cmnO*, pVIO-P4C3RH-Δ*vioM*, and pVIO-P4C3RH-Δ*vioO*, contained a non-polar, in-frame deletion of all but the first and last 13 codons of the deleted gene.

Construction of pCMN-P4C8RF-436-Δ*cmnM* and pCMN-P4C8RF-436-Δ*cmnO* and integration into the *S. lividans* 1326 chromosome

The 6.7-kb *DraI* fragment of pOJ436,^[23] containing the *oriT*, *aac(3)IV* (apramycin resistance), ϕ C31 *attP*, and ϕ C31 *int* genetic information, was cloned into the *HpaI* site of the SuperCos1 backbone of pCMN-P4C8RF-Δ*cmnM* and pCMN-P4C8RF-Δ*cmnO*. The resulting cosmids were capable of integration into the ϕ C31 *attB* site of the *S. lividans* 1326 genome, and selection for this integration was performed using apramycin. *S. lividans* 1326 was transformed with these cosmids by using established polyethylene glycol-assisted protocols for protoplast preparation and transformation.^[24] Transformants were selected by

flooding transformation plates with apramycin ($40 \mu\text{g mL}^{-1}$). Transformants from each plate were streaked for isolation onto R2YE and ISP2^[25] plates supplemented with apramycin ($40 \mu\text{g mL}^{-1}$).

Construction of pVIO-P4C3RH-SVSET- Δ vioM and pVIO-P4C3RH-SVSET- Δ vioO and integration into the *S. lividans* 1326 chromosome

The 4-kb fragment of pSET152^[23] containing the *oriT*, *aac(3)IV* (apramycin resistance), ϕ C31 *attP*, and ϕ C31 *int* genetic information (which we have dubbed the SVSET cassette) was cloned into the SuperCos1 backbone using λ red mutagenesis. Primers were constructed containing 40 bp of sequence flanking the PSV40 site on SuperCos1 and 28 bp of sequence flanking the SVSET cassette. Primers used were SVSETFor (5'- CTG TGG AAT GTG TGT CAG TTA GGG TGT GGA AAG TCC CCA GGG TTC ATG TGC AGC TCC ATC AGC AAA AG) and SVSETRev (5' – TTT GCA AAA GCC TAG GCC TCC AAA AAA GCC TCC TCA CTA CCA GGC TTC CCG GGT GTC TCG CTA CGC CG). The resulting PCR product was electroporated into BW25113/pIJ790 competent cells containing either pVIO-P4C3RH- Δ *vioM* or pVIO-P4C3RH- Δ *vioO* and allowed to recombine. Transformants were selected on LB ampicillin ($50 \mu\text{g mL}^{-1}$) and apramycin ($100 \mu\text{g mL}^{-1}$). The resulting cosmids had the ability to conjugate into *S. lividans* and recombine into the genome at the conserved *attB* site. Each cosmid was transformed into *E. coli* S17-1 for subsequent conjugation into *S. lividans*. Exconjugants were selected for on MS agar supplemented with apramycin ($40 \mu\text{g mL}^{-1}$), and *E. coli* was counter-selected with nalidixic acid ($50 \mu\text{g mL}^{-1}$). Exconjugants were streaked for isolation on ISP2 plates supplemented with apramycin ($100 \mu\text{g mL}^{-1}$).

Construction of pSE34 -*cmnM*, pSE34-*cmnO*, pSE34-*vioM*, and pSE34-*vioO* and transformation of *S. lividans* 1326

Each gene was PCR amplified with 5' *NdeI* and 3' *HindIII* restriction sites. PCR products were subsequently cloned into pET37b or pET28b and subcloned with a ribosome binding site via *XbaI* and *HindIII* into pSE34. The following primers were used: for *cmnM* (*cmnMNdeI* 5'- AAG GGC TTT CAT ATG GTG CTT GAC CTC TCC and *cmnMHindIII* 5'- CCT GTG ACC AAG CTT TAC GTG TCC ACG TTT), for *cmnO* (*cmnONdeI* 5'- GGA GGC CAT ATG ACC GCG CTG CAC CGC CTC GAC and *cmnOHindIII* 5'- GGACAA GCT TGT GCG GCT GGA CGG GCA CCG CGC), for *vioM* (*vioM/NdeI* 5' – ATA ACA TAT GAG GAC GCG GAT CGC GCT CCG and *vioM/SHindIII* 5' – AAT TAA GCT TCT CGT CGT TCA GGA CGA CCT G), and for *vioO* (*vioO/NdeI* 5' – ATA ACA TAT GAC CAC CAT GCC CAC GAC CGG C and *vioO/SHindIII* 5' – TAA TAA GCT TCC ACG AGT TCC CGG CGG CGG TA).

S. lividans 1326 strains were transformed with these plasmids by using established polyethylene glycol-assisted protocols for protoplast preparation and transformation onto R2YE.^[24] Transformants were selected by flooding transformation plates with thiostrepton ($8 \mu\text{g mL}^{-1}$) and/or apramycin ($40 \mu\text{g mL}^{-1}$) as appropriate. Transformants from each plate were streaked for isolation onto ISP2 plates supplemented with thiostrepton ($12.5 \mu\text{g mL}^{-1}$) and apramycin ($100 \mu\text{g mL}^{-1}$).

Analysis of tuberactinomycin production

Single colonies of *S. lividans* constructs were used to inoculate 25 ml of YEME supplemented with apramycin ($40 \mu\text{g mL}^{-1}$) or apramycin and thiostrepton ($12.5 \mu\text{g mL}^{-1}$) as appropriate. Cultures were grown at 30°C at 200 rpm for 8 to 10 days. The cells were subsequently harvested by centrifugation, washed with 10.3% (w/v) sucrose, resuspended in 5 ml of 10.3% (w/v) sucrose, and frozen at -20°C until use.

To test for tuberactinomycin production, 50 μL of the frozen mycelial stock of each strain was used to inoculate 100 mL of VIO production medium,^[11] supplemented with thiostrepton ($12.5 \mu\text{g mL}^{-1}$) when appropriate, in 1-L unbaffled flasks. The cultures were grown at 30°C at 200 rpm for 7–10 days. Any potential tuberactinomycin produced was purified using a previously described protocol for the purification of VIO.^[11] After purification, UV-visible spectrophotometry analysis was performed (Beckman Coulter DU640), and spectra were compared to an authentic CMN or VIO standard. To identify whether one or more of the tuberactinomycin derivatives were produced, the samples were analyzed by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Briefly, purified samples were run on a C18 smallpore column (Grace) on a Beckman Gold HPLC system (with a 126 solvent module and a 168 detector module) with a 1 mL/min flow rate. The separation profile began with 5-min of isocratic development in H_2O –0.1% trifluoroacetic acid (TFA), followed by a 5 min linear gradient up to 20% ACN–0.1% TFA, and finally 10 min in H_2O –0.1% TFA. Elution of metabolites was monitored at 268 nm, the wavelength characteristic of tuberactinomycins.^[20] For MALDI-TOF MS, samples were collected from HPLC elutions, the solvent was evaporated under a vacuum, and the samples were analyzed using a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems). For MALDI-TOF/TOF, peptides were collected from the HPLC and submitted to the University of Wisconsin Biotechnology Center Mass Spectrometry Facility.

Deletion of DNA coding for the N-terminal X domain of CmnA and movement of the resulting CMN gene cluster into *S. lividans* 1326

To determine where the end of the X domain was located, CmnA was aligned with VioA using NCBI's BLASTP.^[26, 27] The subsequent X domain deletion was designed to fuse the start codon of *cmnA* to bp 1021, leaving intact the entire portion of *cmnA* coding for the region of CmnA that aligned with VioA. The spectinomycin resistance cassette from plasmid pIJ778 was PCR amplified with primer pairs specific for the deletion of *cmnA-X* (cmnAtruncXba - 5' ACA TCA GCC GGA GAC GCC ACC ATG ACC GTT GAC CCC ACG TCT AGA ATT CCG GGG ATC CGT CGA CC, cmnAtruncNhe - 5' GGT CGC GTT GGG CGC GAG CAC GAC CCG GCG GTG CTC GTC GCT AGC TGT AGG CTG GAG CTG CTT C). The resulting products contained the *aadA* flanked by 39 bp of homology to the 5' and 3' regions of the truncated *cmnA*, as well as *Xba*I and *Nhe*I restriction sites. The PCR products were then electroporated into electrocompetent *E. coli* BW25113 containing the temperature sensitive plasmid pIJ790 and pCMN-P4C8RF. Transformants were plated on LB with kanamycin ($50 \mu\text{g mL}^{-1}$), carbenicillin ($100 \mu\text{g mL}^{-1}$), and spectinomycin ($50 \mu\text{g mL}^{-1}$) at 37°C to select for integration of the PCR product into the cosmid and loss of pIJ790.

To remove *aadA*, the construct was digested with *Xba*I and *Nhe*I, ligated together (thus destroying both restriction sites), and electroporated into XL-1Blue MR. Transformants were screened for spectinomycin sensitivity. To confirm loss of the cassette, clones were screened by restriction digest or sequencing. The resulting cosmid, pCMN-P4C8RF- $\Delta\text{cmnA-X}$, contained a non-polar, in-frame deletion of the X domain from *cmnA*.

pCMN-P4C8RF- $\Delta\text{cmnA-X}$ was modified with the addition of the SVSET cassette as outlined above for pVIO-P4C3RH-SVSET- ΔvioM . The resulting cosmid had the ability to conjugate into *S. lividans* and recombine into the genome at the conserved *attB* site. Each cosmid was transformed into *E. coli* S17-1 for subsequent conjugation into *S. lividans*. Exconjugants were selected for on MS agar supplemented with apramycin ($40 \mu\text{g mL}^{-1}$), and *E. coli* was counter-selected with nalidixic acid ($50 \mu\text{g mL}^{-1}$). Exconjugants were streaked for isolation on ISP2 plates supplemented with apramycin ($100 \mu\text{g mL}^{-1}$).

Construction of a CmnA-VioA hybrid NRPS

The λ red mutagenesis protocol was used to insert a spectinomycin resistance cassette flanked by *NdeI* and *NheI* restriction sites using primers delC_{Ac}mnAN_{deI} (5' – CGC GAT GGC GGC GCT GCT GTC CCC GCC GTC CCC CGA GCC CCA TAT GAT TCC GGG GAT CCG TCG ACC) and delC_{Ac}mnAN_{heI} (5' – TCC AGC AGG TCG CGC CAG ATC GCG GCC AGC GCC GCC TCG CTA GCT GTA GGC TGG AGC TGC TTC); thus, replacing the DNA coding for the second C-A2 pair in *cmnA* with the spectinomycin resistance cassette. The DNA coding for the corresponding C-A2 pair from *vioA* was cloned with *AseI* and *SpeI* flanking restriction sites using primers C_{Ac}vioAA_{seI} (5' – ATT AAT GAG CTG TCC GCC GCC CAG CAC CGT) and C_{Ac}vioAS_{peI} (5' – ACT AGT CCG GCG GCC GTT CAG TGC CGG TAC). To generate the control strain, the DNA coding the native C-A2 pair from *cmnA* was cloned with the same restriction sites using primers C_{Ac}mnAA_{seI} (5' – ATT AAT GAG GTC TCC CGG GCC GAG CAA CGG) and C_{Ac}mnAS_{peI} (5' – ACT AGT GGT CGC GGT CAG CGG GCG GCT GTC GC). Standard cloning procedures were used to digest out the spectinomycin resistance cassette and replace it with the DNA coding for either C-A2 pair. The resulting cosmid pCMN-P4C8RF-*cmnA*Δ*vioA*-CA₂ and pCMN-p4C8RF-*cmnA*Δ*cmnA*-CA₂ contained an in-frame substitution of the second C-A pair from *vioA* or *cmnA* flanked by *AseI* and *SpeI* restriction sites. The cosmid were modified as described above with the SVSET cassette for conjugation into *S. lividans* 1326.

Construction of *cmnA* L→M

The primers used to PCR amplify the DNA coding for the native C-A2 domains of CmnA were used to introduce this region of *cmnA* into a pGEM vector using standard PCR-based cloning. Mutagenesis of the targeted *cmnA* codon (amino acid 1668 of CmnA) was accomplished using standard quick change PCR protocols. The primers used were: F.CmnA2LtoM (5' - CAC GTC ATG ACC AGC GGC GAG ACG CTG CCC - 3') and R.CmnA2LtoM (5' - GCT GGT CAT GAC GTG CCG CAG GGT CGG GCA - 3'). The resulting clone contained *NdeI* and *SpeI* restriction sites flanking the DNA coding for the CmnA C-A2 domains. The mutated *cmnA* *NdeI*/*SpeI* fragment was used to replace the *NdeI*/*NheI* spectinomycin cassette in *cmnA* as discussed above for construction of the CmnA-VioA hybrid NRPS. The resulting cosmid was modified to contain the SVSET cassette. The pCMN-P4C8RF-SVSET-CMN cosmid was introduced into *S. lividans* 1326 using conjugation. Resulting exconjugants were characterized for CMN production as described in the analysis of tuberactinomycin production (see above).

Overproduction, purification, and characterization of VioA A1PCP1 and A2PCP2

The regions of *cmnA* coding for A1PCP1 and A2PCP2 were PCR amplified and cloned into the *E. coli* overexpression vector pET28b (EMB4 Biosciences). The primers used for amplification were: VioA-APCP1-*NdeI* (5' – CCG CGC GGC AGC CAT ATG GAA GCC AGA GAT GAC GCA CAT – 3') with VioA-APCP1-Stop (5' – GAG TGC GGC CGC AAG CTT TCA GGC GTC GTC GAG CGT GAC GCG 3') and VioA-APCP1-*NdeI* (5' – CCG CGC GGC AGC CAT ATG GGA TCC GCG CTG GAC GCG CTG 3') with VioA-APCP2-Stop (5' – GAG TGC GGC CGC AAG CTT TCA GCC GCC GTC CGG CTC CGG AAG 3'). The resulting plasmids (pET28b-*vioAA1*PCP1 and pET28b-*vioAA2*PCP2) were individually transformed into BL21(DE3)*ybdZ::aac(3)*IV carrying pACYCDuet-*vioN*.^[14] Retention of both plasmids in BL21(DE3)*ybdZ::aac(3)*IV required the presence of chloramphenicol (25 $\mu\text{g mL}^{-1}$) and kanamycin (50 $\mu\text{g mL}^{-1}$).

Overproduction and purification of VioA-A1PCP1 + VioN and VioA-A2PCP2 + VioN followed previously described protocols for the overproduction and purification of similar constructs of the CMN NRPS.^[14] Protein concentrations were determined using BCA assays

with BSA as protein concentration standard. Amino acid-dependent ATP/PP_i exchange reactions were performed as previously described [14] with each containing 1 mM of amino acid with 1 µg (VioA-A1PCP1) or 2 µg (VioA-A2PCP2) of enzyme in a 60 min assay. A no amino acid added negative control was used to define the background level of amino acid-independent ATP/PP_i exchange, and this was subtracted from the activity detected in the amino acid-containing reactions.

Mass spectrometry

MALDI-TOF data on a Voyager-DE Pro Workstation (PerSeptive Biosystems, Applied Biosystems) were obtained at the University of Wisconsin-Madison Biophysics Instrumentation Facility, which was established with support from the University of Wisconsin-Madison and grants BIR-9512577 (NSF) and S10RR13790(NIH). The MALDI-TOF/TOF (MS/MS) analysis of peptides purified from the strain producing the CmnA-VioA hybrid NRPS was performed by the University of Wisconsin Biotechnology Center Mass Spectrometry Facility using an Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF. Equipment in the facility was purchased with funds from the University of Wisconsin, the National Institutes of Health (P50 GM64598, R33 DK070297), and the National Science Foundation (DBI-0520825, DBI-9977525).

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Figure 1. Representative HPLC traces of purified CMN derivatives. Traces from bottom to top: CMN, authentic CMN; Complete, intact CMN biosynthesis gene cluster; $\Delta cmnM$, CMN biosynthesis gene cluster lacking *cmnM*; $\Delta cmnO$, CMN biosynthesis gene cluster lacking *cmnO*. Arrows with labels identify the CMN derivative eluting. Identification of the eluting metabolites was determined by MALDI-TOF MS (Supporting Information). Eluting metabolites were detected by monitoring A_{268} . Traces are offset by 1200 mAu (milliabsorption units).

Figure 2. Representative HPLC traces of purified VIO derivatives. Traces from bottom to top: VIO, authentic VIO; Complete, intact VIO biosynthesis gene cluster; $\Delta vioM$, VIO biosynthesis gene cluster lacking *vioM*; $\Delta vioO$, VIO biosynthesis gene cluster lacking *vioO*. Arrows with labels identify the VIO derivative eluting. Identification of the eluting metabolites was determined by MALDI-TOF MS (Supporting Information). Eluting metabolites were detected by monitoring A_{268} . TMN A, tuberactinamine A (des- β -lysine VIO). Traces are offset by 1500 mAu (milliabsorption units).

Figure 3. Representative HPLC traces of purified CMN derivatives from a strain lacking the N-terminal “X” domain of CmnA ($\Delta X\text{-}cmnA$) or an amino acid substitution in CmnA ($cmnA\ L \rightarrow M$) compared to authentic CMN (CMN). Arrows with labels identify the CMN derivative eluting. Identification of the eluting metabolites was determined by MALDI-TOF MS (Supporting Information). Eluting metabolites were detected by monitoring A_{268} . Traces are offset by 1200 mAu (milliabsorption units).

Figure 4.

Representative results from amino acid-dependent ATP/PP_i exchange assays. A) Activity for VioA A1PCP1; B) Activity for VioA A2PCP2. For each assay, the activity of the reaction having the highest level of ATP/PP_i exchange was set to 100% and the other data was scaled accordingly. Abbreviations: L-DAP, L-2,3-diaminopropionate; Uda, β -ureidoalanine; L-Cam, L-capreomycinidine. Activity from A1PCP1 is shown at the top, while activity from A2PCP2 is shown at the bottom. Triplicate assays determined the rate of L-DAP-dependent ATP/PP_i exchange by A1PCP1 was 10.6 ± 1.0 pmol min⁻¹ μ g protein⁻¹. The rate of L-Ser and L-Ala activation by A2PCP2 was 2.4 ± 0.3 pmol min⁻¹ μ g protein⁻¹ and 0.5 ± 0.2 pmol min⁻¹ μ g protein⁻¹, respectively.

Scheme 1.

Chemical structures of viomycin (VIO), tuberactinamine A (TMN A; des- β -lysine VIO) and the four derivatives that make up capreomycin (CMN). The numbering within the cyclic pentapeptide cores of the antibiotics is used to identify positions noted in the text.

Scheme 2.

Components of the VIO (top) and CMN (bottom) NRPSs. The name of each protein is immediately below each NRPS component. Abbreviations: A, adenylation; C, condensation; A1, first A domain of VioA or CmnA; A2, second A domain of VioA or CmnA; Cy, cyclization domain; X, domain of unknown function. Black ovals are PCP domains. R = OH or H. The order in which the NRPS subunits function is discussed in the text.

Scheme 3.

Chemical structures of the three peptides isolated from a *S. lividans* strain producing the hybrid CmnA-VioA NRPS. HPLC and MS data used to determine the peptide structures are included in the Supporting Information. Numbering in each peptide is used to denote the residues discussed in the text and shown in Scheme 1.

Scheme 4.

Proposed mechanism of the VIO NRPS. VioO is the initiating module to incorporate β -lysine. The arrow connecting A2 to the PCP domain of VioI denotes the amino acylation of VioI by the A2 domain of VioA. Abbreviations and domain notation is as described in Scheme 1, with the addition of DH, dehydrogenase and Hy, hydroxylase.

Scheme 5.

Proposed mechanism of the CMN NRPS. The first A-PCP didomain of CmnA and CmnO are initiating modules to incorporate L-2,3-diaminopropionate and β -lysine, respectively. The arrow connecting A1 to the PCP domain of CmnI denotes the amino acylation of CmnI by the A2 domain of CmnA. Abbreviations and domain notation is as described in Schemes 1 and 2.