Deciphering the Biosynthetic Codes for the Potent Anti-SARS-CoV Cyclodepsipeptide Valinomycin in *Streptomyces tsusimaensis* ATCC 15141

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**Introduction**

Severe acute respiratory syndrome (SARS) was the first pandemic disease of the twenty-first century, which caused significant morbidity, mortality, and economic loss worldwide. [1] The causal agent, a new coronavirus (CoV), is believed to be of animal origin. [2–6] SARS-CoV has a large RNA genome that mutates rapidly. [7,8] The possibility of a SARS resurgence warrants the need for anti-SARS-CoV therapeutics. [9,10] Great efforts have been devoted to the development of effective vaccines and antiviral drugs against SARS-CoV. [11–13] In one study, a cell-based assay was employed to screen a large chemical library and 50 compounds were identified to have anti-SARS-CoV activities. [14] Among those compounds, valinomycin (VLM, C$_{54}$H$_{90}$N$_{6}$O$_{18}$, $\text{MW} = 1111.5$; Scheme 1), a natural product produced by several *Streptomyces* isolates, [15–19] demonstrated the highest potency against SARS-CoV in infected Vero E6 cells with an EC$_{50} = 0.85$ $\mu$M. [14]

VLM’s mode of anti-SARS-CoV action is not yet known, but its antiviral activity is just one of its many inhibitory effects. Previous studies have revealed VLM’s antifungal, [20] insecticidal-nematocidal, [21] and antibacterial activities (moderately against *Mycobacterium tuberculosis* in vitro). [22] It also displays apoptosis-inducing activity in mammalian cell types, inhibition of human NK cell function, [23] and antitumor activities. [18,24] Unfortunately, the cytotoxicity of VLM has so far prevented its clinical use but structural analogues of VLM could provide an alternative.

VLM has a cyclododecadepsipeptide structure that contains a three-repeating sequence of a tetradepsipeptide basic unit, $\alpha$-$\alpha$-hydroxyisovaleryl-$\alpha$-valyl-$\gamma$-lactyl-$\gamma$-valyl, to form a symmetric 36-membered ring molecule. [25–27] Feeding experiments have identified $\alpha$-$\alpha$-hydroxyisovaleric acid ($\alpha$-Hiv), $\gamma$-lactic acid ($\gamma$-Lac) and $\gamma$-valine ($\gamma$-Val) as the biosynthetic precursors in VLM biosynthesis. VLM forms an ionophore that specifically modulates potassium ion transport across biological membranes. [28,29] Many peptide antibiotics act as ion channels. [30]

The cyclic peptide structural features of VLM suggest that it is likely biosynthesized by a nonribosomal peptide synthetase (NRPS) system. [31,32] A previous genetic study claimed to have identified two genetic loci that appeared to be involved in VLM biosynthesis in *S. levoris* A-9, [19] but to date no further results (e.g., gene sequence) have been published or patented. In this study, the gene cluster for VLM biosynthesis (designated as *vlm*) in *S. tsusimaensis* ATCC 15141 has been cloned, sequenced, and partially characterized. Sequence analysis revealed good alignment between modular NRPS organization and the biosynthetic precursor incorporation for VLM biosynthesis. In addition, the molecular basis for nonribosomal peptide intermediate oligomerization mediated by a C-terminal iterative type I thioesterase (TE) is discussed. This work sets the stage for generating VLM analogues through metabolic engineering of the VLM biosynthetic pathway and is aimed at acquiring potent, highly specific, and low-cytotoxic anti-SARS-CoV agents and/or other anti-infective therapeutics.

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Results and Discussion

Identification of a gene fragment essential for valinomycin biosynthesis in *Streptomyces tsusimaensis* ATCC 15141

Based on the accumulated knowledge about the biosynthesis of nonribosomal peptide natural products, particularly of enniatins and related cyclodepsipeptides (Scheme 1), biosynthesis of VLM by a type B NRPS system was predicted as follows. Such an NRPS system would contain four distinctive modules. Each module would be responsible for the selection, activation, epimerization (when necessary), and incorporation of a precursor (also called building block: α-HIV, l-Val, l-Lac, or l-Val) in a linear fashion, to form a tetradepsipeptide basic unit covalently tethered as an ester to the active-site serine of the C-terminal iterative TE domain. Two additional rounds of basic unit buildup would generate a linear 12-building block intermediate stalled on the TE. Subsequently, TE would cleave off the intermediate and catalyze the head-to-tail cyclization to afford the mature VLM product (Scheme 2).

To test whether this candidate *St*-nrps gene is essential for VLM biosynthesis, mutants were created by targeted gene disruption of the *St*-nrps gene in *S. tsusimaensis*. Subsequently VLM production in wild type *S. tsusimaensis* and in four independent mutants was analyzed by fermentation, methanol extraction, solid-phase fractionation, and finally by liquid chromatography–mass spectrometry (LC–MS). VLM gives a very weak UV signal at a wavelength of 210 nm because it does not contain a chromophore, but its positive ion \([\text{VLM} + \text{H}]^+\) has a strong MS signal of 1112.6 (m/z). Wild type *S. tsusimaensis* produced about 0.2 mg mL\(^{-1}\) of VLM under the fermentation con-
It was found that disruption of \textit{St-nrps} did not affect the vegetative growth or sporulation of \textit{S. tsusimaensis} mutants but completely abolished VLM production (data not shown). This experiment unambiguously confirmed that the \textit{St-nrps} gene is essential for VLM biosynthesis in \textit{S. tsusimaensis}.

Cloning, sequencing, and in silico analysis of the VLM biosynthetic gene cluster (\textit{vlm})

Plate1E10 insert DNA was labeled and used as a probe to screen an \textit{S. tsusimaensis} cosmid library and three overlapping cosmids that cover the \textit{vlm} gene cluster region (~80 kb) were identified (Figure 1). End sequencing of cosmid DNAs suggested that cosmid 15 might contain the entire \textit{vlm} gene cluster. Sequencing and assembling of a shotgun library constructed with nebulized cosmid 15 DNA generated a contig with a calculated 5.8-fold coverage. This contig contained a 39,345 bp DNA sequence that included the entire \textit{vlm} gene cluster. Sequence analysis of the contig revealed eighteen open reading frames (ORFs). The overall GC content of this contig is 70.68%; this indicates a typical \textit{Streptomyces} origin.\cite{42}

The function of deduced gene products of individual ORFs was assigned based on similarity to known proteins in the database.
Table 1. Deduced functions for the ORFs in the vlm biosynthetic gene cluster and flanking regions.

| ORF (bp[a] bp) | Size (amino acids) | Protein homologue by accession no. | Proposed function |
|---------------|-------------------|-----------------------------------|-------------------|
| Upstream region: |
| ORF1 (1 < 949[b]) | 316+ | CAA19245 from Streptomyces coelicolor | unknown |
| ORF2 (1336 < 2244) | 292 | NP_767597 from Bradyrhizobium japonicum | unknown |
| ORF3 (2789 < 3520) | 243 | CAB93439 from S. coelicolor | TetR-family regulatory protein |
| ORF4 (3781 < 4410) | 209 | none | unknown |
| ORF5 (4427 < 5272) | 281 | BAC71595 from Streptomyces avermitilis | NS, N10-MTHMP reductase[b] |
| ORF6 (5475 < 6959) | 494 | AAF61246 from Streptomyces griseus | penicillin-binding protein |
| ORF7 (7184 < 8311) | 375 | CAD55532 from S. coelicolor | unknown |
| ORF8 (8509 < 11103) | 864 | Q8Z2V2 of S. avermitilis | FO synthase[b] |
| ORF9 (11670 < 13808) | 712 | BAC70249 from S. avermitilis | unknown |
| ORF10 (13860 < 14288) | 142 | BAC70249 from S. avermitilis | unknown |
| The vlm gene cluster: |
| ORF11 (14516 < 15310) | 264 | NP_631397 from S. coelicolor | necrosis inducing factor |
| ORF12 (15380 < 15877) | 165 | CAC42141 from S. coelicolor | unknown membrane-associated protein |
| ORF13 (15877 < 16086) | 69 | CAB58311 from S. coelicolor | transcriptional regulator |
| ORF14 (16138 < 18186) | 682 | BAC71762 from S. avermitilis | unknown |
| ORF15 (18814 < 19348) | 244 | AAP09421 from Bacillus cereus | discrete type II thioesterase |
| vlm1 (19526 < 29812) | 3428 | AW98629 from B. cereus | NRRP (A–TA–DH2–PCP–C–A–PCP–E–C)[c] |
| vlm2 (29835 < 37802) | 2655 | AW98629 from B. cereus | NRRP (A–DH2–PCP–C–A–PCP–TE)[c] |
| Downstream region: |
| ORF18 (37938 < 39266) | 442 | BAD83980 from Corynebacterium glutamicum | transposase |

[a] < or > indicates the orientation of transcription; [b] incomplete; [c] N5,N10-methylenetetrahydromethanopterin reductase; [d] 7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase; [e] abbreviations for domain names are defined in the text; TA and DH2 domains are hypothetical.

(1) Among the ORFs identified, vlm1 and vlm2 encode NRPSs that constitute an assembly line for VLM biosynthesis (see later for details); vlm1 contains the original sequence tag Plate1E10-T7 (equivalent to the 28911–29719 bp DNA on the contig).

The vlm gene cluster was predicted to encompass seven ORFs, bordered by ORF11 and vlm2, respectively (Figure 1), based on the following observations: 1) downstream of vlm2, ORF18 encodes a putative transposase. Many natural product biosynthetic gene clusters have boundaries flanked by transposable elements, which suggests an origin of horizontal gene transfer; 2) upstream of vlm1, ORF11/12/13 appear to be co-transcribed. ORF13 encodes a transcriptional regulator containing a helix-turn-helix motif, which indicates its DNA-binding property. Regulatory genes are usually present in biosynthetic gene clusters; 3) ORF15 encodes a discrete type II TE, a component often found in biosynthetic pathways for nonribosomal peptides and polyketides. Type II TEs are believed to have a proof-reading function during the cleavage of misprimed thioesters from carrier proteins;[43] 4) ORF9/10 appear to be co-transcribed in an orientation opposite to that of ORF11/12/13; 5) further upstream, ORF8 is a housekeeping gene.

A correlation between the modular organization of the VLM assembly line and structure

NRPSs encoded by vlm1/2 contain sixteen distinctive domains organized into four modules followed by a C-terminal iterative TE (Scheme 2). This modular organization is consistent with the biosynthesis of the tetradepsipeptide basic unit α-5-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl. Module 1 is the initiation module that contains four functional domains, adenylation (A; designated as VLM1A1), hypothetical transaminase (TA), hypothetical dehydrogenase (DH2), and PCP. The presence of these two hypothetical domains in module 1 correlates well with a proposed mechanism for the generation of the biosynthetic precursor D-Hiv (Scheme 3). A study of enniatin biosynthesis in Fusarium sambucinum, α-Hiv was postulated to be derived from L-Val by sequential transamination (catalyzed by an uncharacterized TA) and dehydrogenation (catalyzed by a purified discrete α-Hiv DH2).[54] Generation of precursor α-Hiv in VLM biosynthesis might follow the same route, catalyzed by discrete TA and DH2 enzymes yet to be discovered, or by the integral hypothetical TA and DH2 domains in this module.

Module 2 contains four standard NRPS domains: C, A (designated as VLM1A2), PCP, and epimerase (E). Module 3 contains four domains: C, A (designated as VLM2A3), hypothetical DH2, and PCP. This hypothetical DH2 domain is speculated to act as a L-lactate dehydrogenase to convert pyruvate into L-Lac which is then acylated onto PCP (Scheme 3).[34] Generation of lactate from pyruvate is a common biochemical reaction in many microorganisms that undergo fermentation. However, dedicated L-Lac DH2 has not been characterized from any known natural product biosynthetic pathway. Module 3 is split into two proteins, a phenomenon increasingly found in natural product biosynthetic pathways.[40] Module 4 contains a minimal set of three NRPS domains: C, A (designated as VLM2A4), and PCP.

Collectively, amino acid sequence alignment of four A domains on VLM1/2 revealed that they all contain the ten conserved signature motifs[40] albeit considerable amino-acid substitutions have occurred in VLM1A1 and VLM2A3 (data not shown). Phylogenetic display of the alignment indicates that VLM1A1 and VLM2A3 form a distinctive branch whereas VLM1A2 and VLM2A4 cluster with GsrA, A, which serves as a model A domain with a defined substrate specificity for Phe and a known structure;[43] DhbE, a stand-alone A domain which...
activates the carboxyl acid DBH (2,3-dihydroxybenzoic acid) from the bacillibactin biosynthetic pathway\cite{48}, is distantly related to all of them. At substrate specificity level, VLM1A2 and VLM2A4 contain conserved NRPS codes\cite{49,50} (DALWLGGTFK and DAFWVGGTFK, respectively) that are predicted to specify for L-Val. In contrast, extracted NRPS codes from VLM1A1 or VLM2A3 (AALWIADSGK or VVIWIAEQHK, respectively) do not yield any predictable substrate. Remarkably, both VLM1A1 and VLM2A3 have the highly conserved Asp at position 235 (first position in the NRPS codes; numbered by GrsA_A sequence) replaced by an Ala or Val. Similarly, DhbE also has the conserved Asp residue replaced by an Asn. This finding suggests that VLM1A1 and VLM2A3, together with DhbE, might represent a divergent group of A domains that specify for a class of hydroxyl acid substrates other than proteinogenic amino acids or carboxyl acids. In the proposed model for VLM biosynthesis (Scheme 2) module 1 and 3 are responsible for the incorporation of o-Hiv and i-Lac, respectively. The A domains in these two modules might have adapted themselves to select and activate hydroxyl acids. The fact that o-Hiv and i-Lac were identified by feeding experiments as the biosynthetic precursors for o-Hiv and i-Lac, respectively. Additional biochemical experiments are needed to prove the VLM1A1 and VLM2A3 substrate specificities for o-Hiv and i-Lac, respectively.

Immediately following module 4 is a C-terminal iterative TE domain (designated as VLM2_TE). Terminal TE domains are known to control the termination, release, and cyclization of growing chains in the biosynthetic process of many nonribosomal peptides and polyketides\cite{31,32}. An unsolved mystery regarding the biosynthesis of symmetric cyclo(depsi)peptides is the control of repetition of the basic units. As shown in Scheme 1, VLM contains a three-repeating sequence of a tetradepsipeptide basic unit (designated as a 3×4 mode), whereas montanastatin (C_{36}H_{60}N_{4}O_{12}, M_W = 740.9)\cite{18} contains the exact same tetradepsipeptide basic unit that repeats only twice (a 2×4 mode). This observation raises the question of whether VLM and montanastatin are produced by the same biosynthetic pathway with relaxed control of basic unit repetition, or by two independent pathways, each of which has a stringent control of basic-unit repetition. A low level of montanastatin was detected by LC-MS in the wild-type strain of S. tsusimaensis but not in the St-nrps^- mutants. This result suggests that montanastatin is likely the shunt product of VLM, produced by the same VLM biosynthetic pathway with a relaxed TE control of basic unit repetition. In comparison to VLM and montanastatin, enniatin A has a 3 GDILW 2 mode, whereas cereulide and PF1022A have a 3×4 and 2×4 mode, respectively. Not included in Scheme 1 are additional known symmetric nonribosomal peptide natural products—bacillibactin, enterobactin, and gramicidin S\cite{39}—which have a 3×2, 3×2, or 2×5 mode, respectively. Iterative TE domains in the pathways for the biosynthesis of these symmetric oligomeric products are believed to hold the basic unit until an appropriate number of units are condensed and then proceed with cleavage and cyclization\cite{51}. However, the exact mechanism of controlling the number of repetitions remains unknown. An amino acid sequence alignment (data not shown) of VLM2_TE with DEBS3_TE (accession no. Q03133), SrfA-C_TE (accession no. Q08787), Bs-EntF_TE (accession no. P45745), Ec-EntF_TE (accession no. P11454), CesB_TE (accession no. AY691650), and GrsB_TE (accession no. P14688) showed that VLM2_TE contains a standard catalytic triad, Ser80/His207/Asp107 (residues numbered corresponding to those of SrfA-C_TE) and a conserved substrate-binding motif, Gly-Xxx-Ser-Xxx-Gly, where Xxx stands for any amino acid\cite{31}. No residues stand out to suggest a correlation between con-

Scheme 3. Scheme of generation and incorporation of two precursors in VLM biosynthesis. A) o-Hiv generation, activation, and peptidyl acylation; B) i-Lac generation, activation, and peptidyl acylation.
served amino acid residues, domain, or motif, and the unique oligomerization function of VLM2_TE and other TEs (Bs-EntF_TE, Ec-EntF_TE, CesB_TE, and GrsB_TE) that are from the pathways for the biosynthesis of oligomerized natural products VLM, bacillibactin, enterobactin, cereulide, and gramicidin S, respectively. The biochemical mechanism for the control of the number of basic unit repetitions in the biosynthesis of cyclo- (deps)ipeptides remains of interest for future studies.

In conclusion, the VLM biosynthetic gene cluster (vlm) was cloned from S. tsusimaensis ATCC 15141. This vlm gene cluster contains two biosynthetic genes encoding type B iterative NRPSs. These NRPSs contribute an assembly line that consists of sixteen distinctive domains organized into four modules; each module is expected to incorporate a biosynthetic precursor to form a tetradepsipeptide basic unit. A terminal TE domain might mediate the oligomerization and cyclization of three-repeating sequence of the basic unit to afford the mature VLM product (Scheme 2). The cloning and decoding of VLM biosynthetic machinery offers an opportunity for genetic manipulation of the pathway. Genetic engineering of the VLM biosynthesis pathway serves to complement chemical synthesis to generate novel VLM analogues for screening potent, other anti-infective therapeutics.

Experimental Section

Bacterial strains and culture conditions: E. coli strains DH5α, XL1-Blue MR (Stratagene), and S17-1 [54] were used in this work, according to standard procedures [55]. S. tsusimaensis ATCC 15141, a VLM producer, [19] was purchased from the American Type Culture Collection. S. tsusimaensis was grown at 30 °C in tryptic soy broth (TSB) medium supplemented with sucrose (34 %) and MgCl₂ (25 mM) for mycelia harvesting; on ISP-2 agar for sporulation; on modified ISP-4 agar supplemented with tryptone (0.1 %) and yeast extract (0.05 %) for conjugation between E. coli S17-1 and S. tsusimaensis; and in a composite fermentation medium containing Diaion HP-20 resin (0.05 %) for conjugation between E. coli S17-1 but not in Streptomyces [56] to make a targeted gene disruption construct pYC02-36d. The construct was first transformed into E. coli S17-1 cells and then mobilized into S. tsusimaensis cells by conjugation [56]. Four independent apramycin-resistant conjoints were selected and were verified by Southern blot analysis for the correct integration of plasmid DNA into the host chromosome. Wild-type S. tsusimaensis and the four mutants YC02-38a/b/c/d were then grown in the fermentation medium for 6 days at 30 °C; their mycelia and the HP-20 resin were collected by filtration and the whole mass was lyophilized and extracted twice with two volumes of methanol. Methanol extracts were pooled and a portion was further fractionated by an Oasis® HLB Plus SPE cartridge (Waters, Milford, MA, USA). A fraction eluted by methanol (90 %) was subjected to LC–MS analysis. LC–MS spectra were obtained with an Agilent 1000 LC/MSD Trap SL instrument (Agilent, Palo Alto, CA, USA). The eluents used contained formic acid (0.1 %) in water (eluents A) or in acetonitrile (eluents B). A 15 min gradient from 10 % eluent B to 90 % eluent B was achieved in a 2.1 × 110 mm Zorbax SB-AQ column (Agilent) with a flow rate of 0.5 mL min⁻¹. UVenson LC signal and positive ion MS signal were recorded. The VLM peak appeared at 12.7 min. The VLM standard used in calibration was purchased from Sigma–Aldrich.

DNA manipulations and library construction: Plasmid DNA extraction and DNA fragment recovery from agarose gels were carried out with commercial kits (Qiagen). DNA digestion, agarose gel electrophoresis, ligation, and transformation were done by standard protocols [55]. Total S. tsusimaensis DNA was extracted from mycelia by the Kirby mix procedure [42]. Three genomic DNA libraries were constructed in this work. A genome sampling library [41] was constructed with the 2–4 kb fraction of Mbol partially digested total DNA into the BamHI site of pGEM-3zf (Promega). A cosmid library was constructed with the 35–45 kb fraction of Mbol partially digested total DNA into the BamHI site of SuperCos 1 vector (Stratagene). A shotgun sequencing library was constructed with the 1–2 kb fraction of nebulized cosmid DNA into the pCR®4BluntTOPO vector according to manufacturer’s instructions (Invitrogen). For library screening and Southern blot analysis, digoxigenin labeling of the Plate1E10 probe DNA, hybridization, and detection were performed according to the manufacturer’s protocols (Roche).

DNA sequencing and sequence analysis: For the identification of candidate gene fragments encoding NRPSs, two 96-well plates (192 samples) of the genome sampling library were submitted for end sequencing with the T7 primer to generate random genomic sequence tags (DNA Sequencing Laboratory, University of Wisconsin–Madison, WI, USA). Individual sequencing reads were manually examined and subjected to homology search against GenBank by using the Blastx algorithm [56]. The candidate Str-nrps gene which contained clone Plate1E10, was subjected to additional sequencing by primer walking and mapping. For shotgun sequencing of cosmid 15, eight 96-well plates of the shotgun library (768 samples) were submitted for end-sequencing with T3 and T7 primers at Exagen Co. (Seattle, WA, USA). Contig assembling and sequence polishing were performed by the service company. Sequence quality of the contig containing the vlm gene cluster was examined by PCR amplification with genomic DNA as template and resequencing of any regions of interest on the contig. ORF prediction, DNA translation, and protein sequence alignment were performed by using the Lasergene Package (DNASTar, Madison, WI).

Construction of targeted gene disruption mutants of S. tsusimaensis and examination of VLM production by using LC–MS: A 2.3 kb EcoRl–PstI fragment from Plate1E10 was cloned into a suicide conjugative vector pO260 (replicative in E. coli but not in Streptomyces) [56] to make a targeted gene disruption construct pYC02-36d. The construct was first transformed into E. coli S17-1 cells and then mobilized into S. tsusimaensis cells by conjugation [56]. Four independent apramycin-resistant conjoints were selected and were verified by Southern blot analysis for the correct integration of plasmid DNA into the host chromosome. Wild-type S. tsusimaensis and the four mutants YC02-38a/b/c/d were then grown in the fermentation medium for 6 days at 30 °C; their mycelia and the HP-20 resin were collected by filtration and the whole mass was lyophylized and extracted twice with two volumes of methanol. Methanol extracts were pooled and a portion was further fractionated by an Oasis® HLB Plus SPE cartridge (Waters, Milford, MA, USA). A fraction eluted by methanol (90 %) was subjected to LC–MS analysis. LC–MS spectra were obtained with an Agilent 1000 LC/MSD Trap SL instrument (Agilent, Palo Alto, CA, USA). The eluents used contained formic acid (0.1 %) in water (eluents A) or in acetonitrile (eluents B). A 15 min gradient from 10 % eluent B to 90 % eluent B was achieved in a 2.1 × 110 mm Zorbax SB-AQ column (Agilent) with a flow rate of 0.5 mL min⁻¹. UVenson LC signal and positive ion MS signal were recorded. The VLM peak appeared at 12.7 min. The VLM standard used in calibration was purchased from Sigma–Aldrich.

Nucleotide sequence accession number: The nucleotide sequence reported here has been deposited in the GenBank database under accession no. DQ174261.

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