Formation of the Nonproteinogenic Amino Acid 2*S*,3*R*-Capreomycidine by VioD from the Viomycin Biosynthesis Pathway

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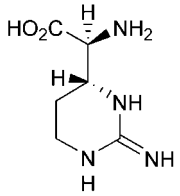
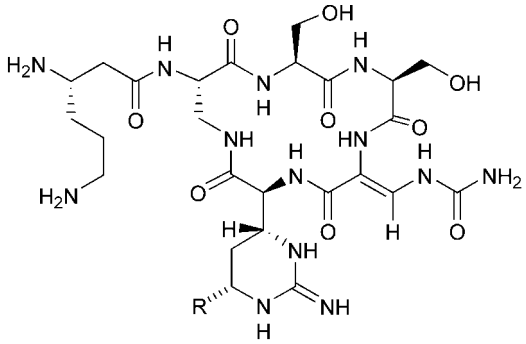


2*S*,3*R*-Capreomycidine (**1**)



Viomycin (Tuberactinomycin B, **2**, R = OH)
Tuberactinomycin O (**3**, R = H)

The arginine-derived, nonproteinogenic amino acid 2*S*,3*R*-capreomycidine (**1**) is a characteristic residue of the tuberactinomycin family of antitubercular peptide antibiotics. These nonribosomal peptides are produced by various actinomycetes and include viomycin (**2**, tuberactinomycin B) and the capreomycins.[1–3] The antibacterial activity is similar to that observed for the aminoglycoside antibiotics and is due to inhibition of bacterial protein biosynthesis by binding to the 16S rRNA in the 30S ribosomal subunit.[4–6] Both viomycin and capreomycin are clinically useful agents for the treatment of tuberculosis but associated toxicity relegates their status as second-line drugs.

Many semisynthetic tuberactinomycins have been prepared in attempts to increase potency and/or reduce toxicity.[7–9] Analogues possessing alterations of the 5-OH-capreomycidine residue of **2** have been generated through alkylation of the hydroxyl group[10] and by treating **2** with trifluoroacetic acid to yield a dehydrated guanidine iminium species, followed by the addition of various nucleophiles.[8] However, opening the cyclic guanidine has a detrimental effect on biological activity. When the capreomycidine residue in tuberactinomycin O (**3**) was replaced with L-Arg or 3-guanidinoalanine, the resulting peptides were inactive or exhibited reduced activity.[11]

The first total syntheses of the tuberactinomycins and analogues relied on capreomycidine isolated from the acid hydrolysate of the natural peptides as starting material, or incorporated amino acids with linear guanidine containing side chains in place of **1**.[12,13] Recently, DeMong and Williams completed an asymmetric synthesis of capreomycin Ib, in which they also prepared 2*S*,3*R*-capreomycidine.[14] Shiba and co-workers first synthesized L-capreomycidine,[15] and we have also reported an asymmetric preparation of ^{13}C-labelled **1** used in studies on the conversion of arginine to the streptolidine core of the streptothricin antibiotics.[16]

Our interest in the biosynthesis of **1** arises from the fact that it is an important element of the tuberactinomycin pharmacophore, is formed by a unique enzymatic process, and represents a valuable building block for combinatorial applications but is not readily available. The viomycin biosynthesis gene cluster has been cloned from *Streptomyces vinaceus* ATCC 11861 and sequenced.[17,18] Our preceding paper demonstrated that the *vioC* gene from this cluster encodes a non-heme iron, α-ketoglutarate-dependent oxygenase that catalyzes the formation of 3*S*-hydroxy-L-Arg from L-Arg.[19] The gene immediately downstream of *vioC* encodes a product, VioD, that exhibits sequence similarity with pyridoxal phosphate (PLP) dependent aminotransferases and was predicted to function as a capreomycidine synthase.[18] We now demonstrate that VioD directs the cyclization of 3*S*-hydroxy-L-Arg to 2*S*,3*R*-capreomycidine (**1**) and represents the first example of a PLP-dependent enzyme that promotes an intramolecular β-replacement reaction.

The *vioD* gene was amplified from cosmid pTOV101 containing a 30 kb segment of the viomycin biosynthesis gene cluster.[17] The amplified DNA fragment was cloned into the *E. coli* expression vector pET28a encoding a *N*-terminal His_6-tag. The resulting plasmid, pET28vioD, was used to transform *E. coli* Rosetta (DE3) cells. Production cultures were grown at 20 °C, and expression was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested, washed, and stored at −80 °C prior to protein isolation. Thawed cells were resuspended and lysed by sonication, and the supernatant was applied to Ni^{2+} or Co^{2+}-chelate chromatography columns. The bound His_6-VioD was eluted with increasing concentrations of imidazole. Several attempts to obtain homogenous His_6-VioD by this method yielded unsatisfactory results as judged by SDS-PAGE analysis. Furthermore, while His_6-VioD was active (vide infra) the majority of the His_6-VioD overexpressed in *E. coli* Rosetta (DE3) was insoluble.

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To address purity and solubility issues, we constructed the plasmid pBADN3vioD in order to overproduce native enzyme. The *vioD* insert from pET28vioD was transferred to the pBADN3 expression vector, and the resulting plasmid, pBADN3vioD, was used to transform *E. coli* Top10 cells. Cells were grown at 20 °C, and expression was induced with L-(+)-arabinose to a final concentration of 1.5 %. Production cultures were maintained at 20 °C for 12 h, then the cells were harvested, washed, and stored at −80 °C. Thawed cells were lysed, and the cleared supernatant was subjected to Q-Sepharose High Performance anion-exchange chromatography. Fractions enriched in VioD were pooled, concentrated, and applied to a Superdex-75 gel filtration column to yield nearly homogenous native VioD (Figure 1). Additionally, the proportion of soluble VioD in the crude lysate was observed to be greater for the native protein than for the His_6-tagged derivative.

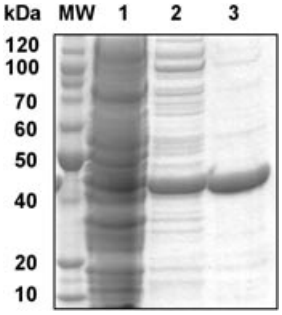


Figure 1. *SDS-PAGE analysis of the expression and purification of VioD in* E. coli. *Lane 1, soluble protein; lane 2, pooled fractions from ion-exchange chromatography; lane 3, purified VioD after gel-filtration chromatography. The calculated MW of VioD is 42.0 kDa.*

VioD assays were conducted in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, and included 1 mM 3*S*-hydroxy-L-Arg, 1 mM PLP, and varying amounts of VioD in a total volume of 200 μL. (Note: the 3*R*- and 3*S*-hydroxy-L-Arg used here are the same materials prepared by Gould et al. in ref. [25] and are deuterated at C-5. For simplicity, the isotope designation is omitted). Reactions were initiated by the addition of enzyme and incubated at 30 °C for 3 to 6 hours. Protein was precipitated with cold ethanol, and the supernatant was decanted and stored at −20 °C. Aliquots were removed, derivatized with dansyl chloride (DNS-Cl) and analyzed by HPLC.[20] Figure 2 illustrates results from representative assays showing the enzyme-dependent appearance of a new compound that is clearly separated from DNS-3*S*-hydroxy-L-Arg (Figure 2 A). The product was not seen in boiled controls (Figure 2 B) or when 3*S*-hydroxy-L-Arg was omitted from the assay. Additionally, VioD was completely stereospecific for 3*S*-hydroxy-L-Arg, as no product formation was observed when 3*R*-hydroxy-L-Arg was evaluated as substrate (Table 1). Neither did L-Arg serve as a substrate. The product of the VioD reaction was confirmed to be 2*S*,3*R*-capreomycidine by coinjecting authentic DNS-**1** with the dansylated assay mixture and by comparing mass spectra of the assay product with authentic **1** (Figure 2 C).[16,21] The partially purified *N*-terminal His_6-VioD was also active under these assay conditions.

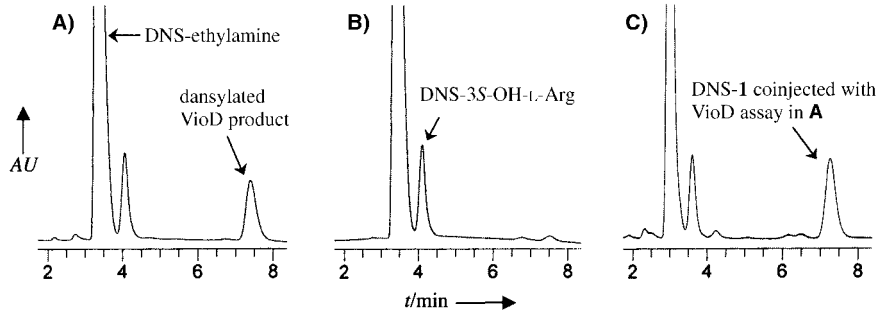


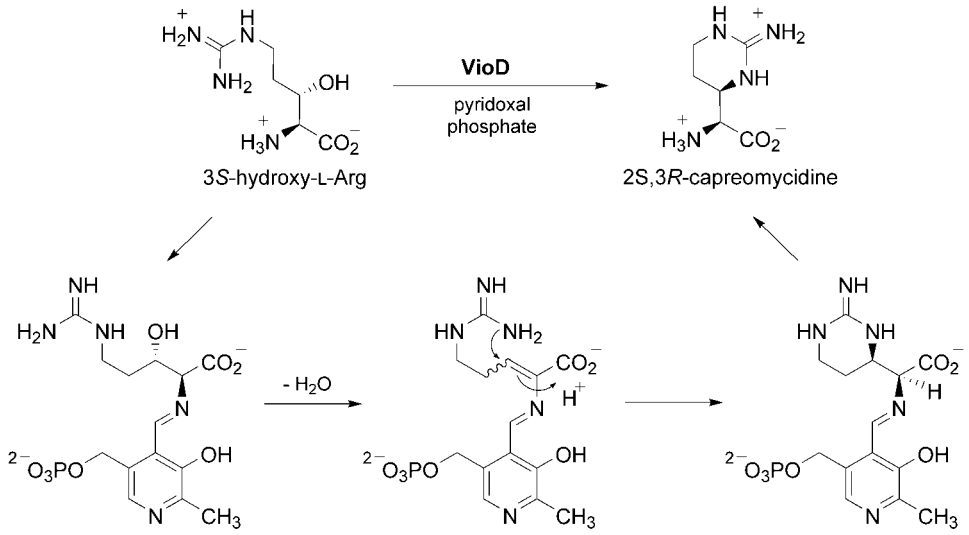
Figure 2. *RP-HPLC chromatograms of dansylated VioD assay mixtures. A) complete VioD assay, B) boiled VioD control, C) coinjection of A and authentic DNS-2S,3R-capreomycidine (DNS-**1**). The mobile phase was an isocratic mixture of MeCN/NH_4OAc (50 mM), pH 5.5, 18:82 for panels A and B and 20:80 for panel C.*

Table 1. *Effect of assay composition on VioD product formation.*

Assay[a]	Product formed
3*S*-OH-L-Arg + PLP	++
boiled VioD	–
3*S*-OH-L-Arg−PLP	+
3*R*-OH-L-Arg + PLP	–
L-Arginine + PLP	–

[a] 6 h incubation, 3 nM VioD.

Scheme 1 illustrates the proposed PLP-dependent elimination/intramolecular β-replacement reaction promoted by VioD. It is not known if the PLP cofactor is covalently bound to VioD through a lysyl imine, as is common with many PLP-dependent enzymes. VioD contains two lysine residues, at positions 29 and 230, and the latter aligns with the active-site lysine found in the consensus sequence of conserved PLP-binding domains, such as pfam00155.11 and COG0436.1 associated with class I or II aminotransferases and aspartate/tyrosine/aromatic aminotransferases, respectively.[22] PLP was typically added to purification buffers because we observed that the enzyme was more stable in the presence of the cofactor. When VioD was purified in the absence of PLP, measurable levels of **1** were produced (Table 1), however we were unable to detect an enzyme-bound PLP cofactor by UV spectroscopy due to the small quantity of enzyme available. The final point regarding the mechanism of VioD is that the configuration of the 2,3-dehydroarginine/PLP adduct is unknown. The more facile *anti* elimination may be expected and would lead to the (*E*)-2,3-dehydroarginine, but the final stereochemical outcome could also arise from the adduct with the *Z* configuration.



Scheme 1. *Proposed mechanism for the VioD-catalyzed formation of* ***1****.*

As noted in our preceding paper on the formation of 3*S*-hydroxy-L-Arg by VioC,[19] the streptolidine moiety of streptothricin F also originates from L-Arg, and **1** probably serves as an intermediate in the rearrangement.[23] The predicted products of the *sttL* and *sttN* genes from the sequenced *Streptomyces rochei* streptothricin F cluster exhibit greater than 40% identity and 51% similarity to VioC and VioD.[24] This lends further support to the intermediacy of capreomycidine in the biosynthesis of the streptothricin antibiotics, even though direct incorporation experiments with labeled **1** and β-hydroxyarginine yielded negative results.[16, 25]

Together with our preceding report on the role of VioC, we have now characterized both enzymes that function in tandem to transform L-arginine to the 2*S*,3*R*-capreomycidine residue of the tuberactinomycins. This will facilitate future in-depth mechanistic and structural studies on the formation of this rare amino acid, while providing the means to produce **1** for both synthetic and combinatorial biosynthesis applications.

Experimental Section

Construction of *E. coli* expression plasmids for *N*-terminal His_6-tagged and native VioD. To construct the expression plasmid pET28vioD, the PCR primers VioDf (5′-TATGAATTCATATGACCGGCCCACTC-3′, *Nde*I site underlined) and VioDr (5′-GCGAAGCTTCATCCCGTCCCCCTTCG-3′) were used to amplify *vioD* by using cosmid pTOV101 as DNA template.[17] The gel-purified PCR product was directly cloned into the pGEM-T Easy vector (Promega), and the purified plasmid was used for sequence confirmation. *vioD* was then cloned into the *Nde*I and *Eco*RI sites of pET28a (Novagen), and the resulting plasmid, designated pET28vioD, was used to produce *N*-terminal His_6-VioD. To produce native VioD, the insert of pET28vioD was excised and cloned into the vector pBADN3; this yielded the expression plasmid pBADN3vioD. The plasmid pBADN3 is a modified form of pBADHisA (Invitrogen) that permits expression of native protein by using an ATG start codon within a *Nde*I site rather than a *Nco*I site, as with the parent vector. pBADN3 was constructed by eliminating the original two *Nde*I sites in pBADHisA by site-directed mutagenesis and then replacing the *Nco*I site in the polylinker with a new *Nde*I site.

Expression and purification of recombinant VioD. Plasmid pET28vioD was used to transform *E. coli* Rosetta (DE3) cells (Novagen) for production of VioD as a *N*-terminal His_6-tagged protein. The expression and purification conditions for *N*-terminal His_6-VioD were the same as described for VioC (preceding paper) with the exception that the final concentration of IPTG was 2 mM. Plasmid pBADN3vioD was used to transform *E. coli* Top10 cells (Invitrogen) for the production of native VioD. Single colonies isolated from fresh transformation plates were grown overnight at 30 °C in LB medium (5 mL) containing ampicillin (100 μg mL^{-1}), and subsequently used to inoculate LB/ampicillin medium (500 mL). Cells were grown at 20 °C until the A_{600} = 0.4–0.6, at which point expression was induced with L-(+)-arabinose to a final concentration of 1.5%. Cells were cultured for an additional 12 h at 20 °C, harvested by centrifugation at 3000 *g* for 15 min at 4 °C, washed with buffer A (50 mM sodium phosphate, pH 8.0, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride) and stored at −80 °C. Frozen cells were thawed on ice, resuspended in buffer A, and lysed by sonication. The cell debris was removed by centrifugation at 35300 *g* for 30 min at 4 °C, and the supernatant was applied to a Q Sepharose High-Performance ion exchange column (3 × 30 cm) previously equilibrated with buffer A containing PLP (1 mM). The column was washed with the same buffer (300 mL) and eluted with a gradient of 0.1–0.6 M NaCl in buffer A (300 mL) containing PLP (1 mM) at a flow rate of 1 mL min^{-1}. Fractions containing VioD activity were pooled and concentrated with a Centricon Plus-20 Biomax-5 (Millipore). Final purification was achieved by using gel-filtration chromatography (Superdex 75, 1.5 × 100 cm) in buffer A at a flow rate of 0.4 mL min^{-1}. Fractions with VioD activity were pooled and concentrated.

VioD assay. Typical assays were conducted in 50 mM MOPS, pH 7.0, and included 3*S*-hydroxy-L-[5,5-$^2H_2$]Arg (1 mM) as substrate, PLP (1 mM), and appropriately diluted VioD in a total volume of 200 μL. Reactions were initiated by the addition of VioD and incubated at 30 °C for various lengths of time. Protein was precipitated with cold ethanol, and the supernatant was decanted and stored at −20 °C prior to derivatization with dansyl chloride (DNS-Cl).[20] Dansylation reactions were conducted by mixing the assay mixture (50 μL) with Li_2CO_3 (50 μL, 80 mM), pH 10, followed by DNS-Cl (50 μL) in MeCN (1.5 mg mL^{-1}). The reactions were mixed and kept at room temperature for 1 h, then quenched with a 2% solution of ethylamine (20 μL). Reverse-phase HPLC analysis (Waters Symmetry® C_{18} column, 5 μm; 3.9 × 50 mm) was performed on a Thermo-Finnigan Surveyor system by using photodiode array detection under isocratic conditions of NH_4OAc (50 mM), pH 5.5, and either 18 or 20% MeCN.

Characterization of the VioD reaction product. The product of the VioD reaction was confirmed to be **1** by HPLC coinjection analysis with authentic **1** that had been similarly derivatized.[16] The

VioD product was further analyzed by ESI-MS with a ThermoFinnigan LCQ Advantage instrument by direct injection. Molecular ions were observed at $m/z = 408.2$ $[M+H]^+$ and 414.4 $[M+Li]^+$, corresponding to dansyl-[5,5-$^2H_2$]capreomycidine. Authentic dansylated-**1** produced ions two mass units lower.

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Keywords: antibiotics · biosynthesis · nonribosomal peptide · tuberactinomycin · viomycin

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