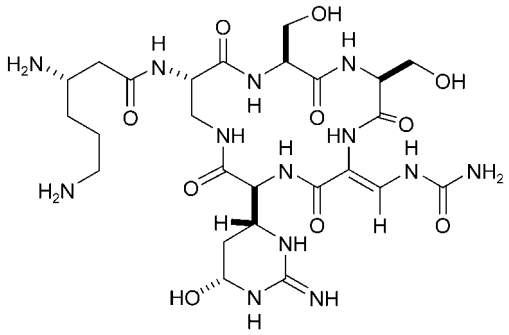
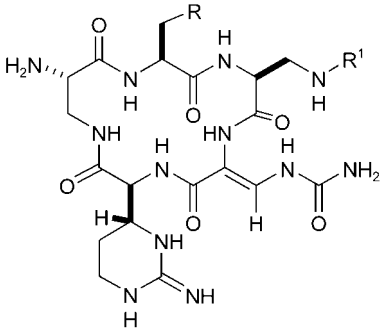
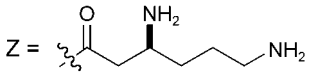


Viomycin (Tuberactinomycin B, **1**)



Capreomycin IA (**2a**): R = OH; R^1 = Z
Capreomycin IB (**2b**): R = H; R^1 = Z
Capreomycin IIA (**2c**): R = OH; R^1 = H
Capreomycin IIB (**2d**): R = H; R^1 = H

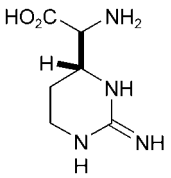


VioC is a Non-Heme Iron, α-Ketoglutarate-Dependent Oxygenase that Catalyzes the Formation of 3*S*-Hydroxy-L-Arginine during Viomycin Biosynthesis

Xihou Yin[a] and T. Mark Zabriskie*[a, b]

The tuberactinomycins (Tbms) are nonribosomal peptide antibiotics produced by various species of *Nocardia* and *Streptomyces* and include viomycin (**1**, tuberactinomycin B) and the capreomycins (**2**).[1–3] These highly basic compounds exhibit a range of biological activities that center on their ability to bind RNA and, most importantly, disturb bacterial protein biosynthesis.[4–6] Both **1** and **2** show potent activity against *Mycobacterium tuberculosis* but associated ototoxicity and nephrotoxicity limit their clinical use to the treatment of recurring or multidrug-resistant *M. tuberculosis* infections.

To gain a better understanding of Tbm biosynthesis, particularly the formation of the nonproteinogenic amino acid residues, and to facilitate genetic and combinatorial-biosynthesis approaches to generate less toxic Tbm analogues, we recently cloned the gene cluster that directs viomycin biosynthesis from *Streptomyces vinaceus* ATCC 11861.[7] During our initial analysis of the viomycin gene cluster, we identified an open reading frame predicted to encode a non-heme iron, α-ketoglutarate-dependent oxygenase and, through gene-disruption experiments, correlated the function of this gene with the production of **1**. Sequence comparison of the *S. vinaceus* oxygenase with enzymes of proven function revealed a close similarity with clavaminate synthase (CS), a trifunctional oxygenase involved in the conversion of the arginine derivative deoxyguanidinoproclavaminic acid to the β-lactamase inhibitor clavulanic acid.[8, 9] This suggested that the *S. vinaceus* enzyme may function in the oxidative transformation of L-Arg to capreomycidine (**3**), the characteristic arginine-derived residue present in all Tbms. Thomas et al. have also identified and sequenced the entire viomycin cluster and proposed that this oxygenase gene, termed *vioC*, encodes an arginine β-hydroxylase.[10]



capreomycidine (**3**)

Because CS catalyzes a β-hydroxylation, an oxidative cyclization, and a dehydrogenation, it seemed plausible that VioC would promote one or more of these reactions in the formation of **3**. Previous isotope-incorporation experiments in vivo with the capreomycin producer established that H-2 and one H-3 of arginine are lost in the conversion to **3**.[11]

[a] *Dr. X. Yin, Prof. T. M. Zabriskie*
Department of Pharmaceutical Sciences, Oregon State University
Corvallis, Oregon 97331-3507 (USA)
E-mail: mark.zabriskie@oregonstate.edu

[b] *Prof. T. M. Zabriskie*
Program in Molecular and Cellular Biology, Oregon State University
Corvallis, Oregon 97331-3507 (USA)
Fax: (+1) 541-737-3999

  DOI: 10.1002/cbic.200400082

This finding is most consistent with the intermediacy of a 2,3-dehydroarginine species that could arise by either a direct dehydrogenation or hydroxylation and elimination. In order to study the biosynthesis of capreomycidine more closely, we have heterologously expressed *vioC* in *E. coli* and report here the characterization of VioC as a non-heme iron, α-ketoglutarate-dependent oxygenase that utilizes free L-Arg as substrate and generates 3*S*-hydroxy-L-Arg as product.

The *vioC* gene was amplified by PCR from cosmid pTOV101 harboring a 30 kb fragment of *S. vinaceus* chromosomal DNA.[7] The primers introduced a *Nde*I site overlapping the start codon and created *Bam*HI/*Eco*RI restriction sites downstream of the stop codon. The amplified DNA fragment was cloned and checked by DNA-sequence analysis and then transferred into the *E. coli* expression vector pET28a encoding a *N*-terminal His_6-tag. The resulting plasmid, pET28vioC, was used to transform *E. coli* (DE3) Rosetta cells. Production cultures were grown at 20 °C, and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were cultured for an additional 5 h at 20 °C, harvested, washed, and stored at −80 °C. Frozen cells were thawed on ice, resuspended, and lysed by sonication. The cleared supernatant was subjected to Ni^{2+}-chelate chromatography, and fractions containing His_6-VioC were pooled, dialyzed to remove imidazole, and stored at 4 °C. Figure 1 shows the SDS-PAGE analysis of the overexpressed and purified VioC. Size-exclusion chromatography indicated that native VioC is a monomer.

Our initial analysis of VioC evaluated free L-Arg as the substrate. Assays were conducted in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, and included 1 mM L-Arg, 1 mM α-ketoglutarate, 25 μM $FeSO_4$, 0.5 mM dithiothreitol

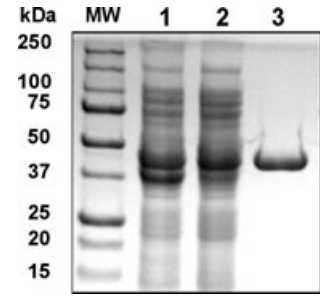


Figure 1. *SDS-PAGE analysis of the expression in* E. coli *and purification of VioC. Lane 1, total protein; lane 2, soluble protein; lane 3, purified VioC. The calculated subunit MW of VioC is 41.6 kDa.*

(DTT), 0.1 mM ascorbate, and appropriately diluted VioC in a total volume of 200 μL. Reactions were initiated by the addition of VioC and incubated at 30 °C for 0.5 to 3 h. Protein was precipitated with cold ethanol, and the supernatant was decanted and stored at −20 °C. Aliquots were removed and derivatized with dansyl chloride (DNS-Cl) to permit HPLC analysis with UV detection.[12] Figure 2 illustrates results from representative assays, showing the separation of DNS-L-Arg and the derivatized VioC reaction product. The product was not seen in boiled controls or when α-ketoglutarate was omitted from the assay. Leaving $FeSO_4$ out of the assay decreased the amount of product formed tenfold, and addition of 1 mM EDTA completely halted product formation. Additionally, the reaction was completely stereospecific for L-Arg, and a turnover product was not detected when N^G-methyl-L-Arg was evaluated as an alternate substrate (Table 1). Studies on arginine metabolism and the intermediacy of **3** in the streptothricin F pathway had

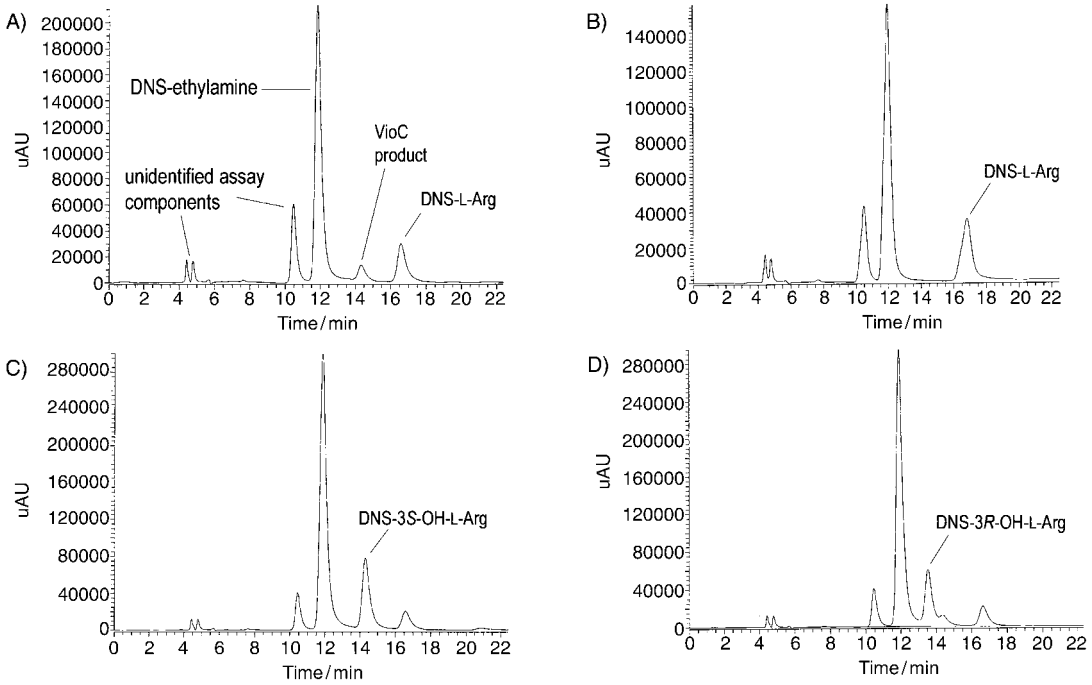


Figure 2. *HPLC chromatograms identifying the VioC reaction product. A) Complete assay, B) boiled control, C) coinjection of A and DNS-3S-OH-L-Arg, D) coinjection of A and DNS-3R-OH-L-Arg.*

Table 1. Effect of assay composition on VioC product formation.

Assay[a]	% conversion
complete	60
boiled VioC	0
(−) α-ketoglutarate	0
(−) $FeSO_4$	6
(+) 1 mM EDTA	0
(−) ascorbate	15
(−) DTT	51
D-arginine	0
N^G-methyl-L-arginine	0

[a] 3 h incubation, 5 nM VioC.

established that DNS-capreomycidine has a longer retention time than DNS-Arg under these conditions.[13] Therefore, the best candidate for the product was β-hydroxy-L-Arg, a compound previously proposed by Gould et al. to be a precursor to **3**[14] and speculated to be the product of VioC.[10]

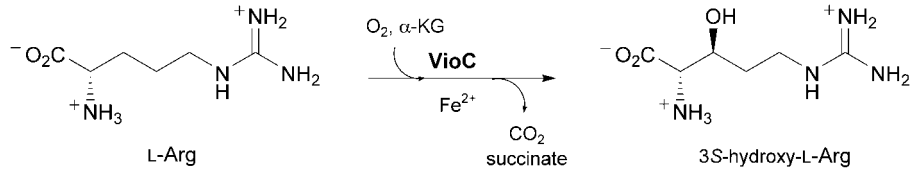
ESI-MS analysis of the dansylated VioC reaction product showed a molecular ion at $m/z = 430.5$ corresponding to the Li^+ adduct of DNS-hydroxy-L-Arg. To prove the oxidation occurred at C-3 and to establish the stereochemistry of the newly introduced hydroxyl, samples of 3*R*-hydroxy-L-Arg and 3*S*-hydroxy-L-Arg were derivatized and analyzed under the same conditions as the VioC assays. The chromatograms in Figure 2 clearly show that the product of the VioC-catalyzed reaction is 3*S*-hydroxy-L-Arg. The overall reaction is summarized in Scheme 1.

The finding that VioC acts on free L-Arg to form 3*S*-hydroxy-L-Arg was somewhat unexpected. We predicted in our initial report on cloning the viomycin pathway that VioC might act on an arginyl-*S*-protein species. This was based in part on work by Walsh's group demonstrating that β-hydroxylation or α,β-dehydrogenation of several proteinogenic amino acids destined for secondary metabolite biosynthesis occurred on amino acyl derivatives tethered as thioesters to the phosphopantetheine cofactor of peptidyl carrier proteins (PCPs).[15–18] Furthermore, the gene product in the public databases with the greatest similarity to VioC is SttL from the streptothricin F cluster (43% identity, 58% similarity). The streptolidine moiety of streptothricin F (**4**) originates from L-Arg, and isotope-labeling experiments implicate capreomycidine as an intermediate in the rearrangement (Scheme 2).[19,20] However, 3*S*- and 3*R*-hydroxy-L-[5,5-$^2H_2$]Arg and [^{13}C]capreomycidine did not label **4** in vivo, whereas the more advanced precursor [^{13}C]streptolidine was efficiently incorporated into **4**.[14,21] Even radioisotope-trapping[14] and derivatization[13] experiments with cultures of *S. lavendulae* containing [^{14}C]arginine failed to provide evidence for the formation of free β-hydroxy-L-Arg. Together these data indirectly supported an enzyme-bound intermediate(s) between L-Arg and streptolidine. However, it may be possible that there are two routes leading to **3** inasmuch as the α-H and *both* β-H are lost in the incorporation of arginine into **4**; this suggests the intermediacy of β-ketoarginine.[20]

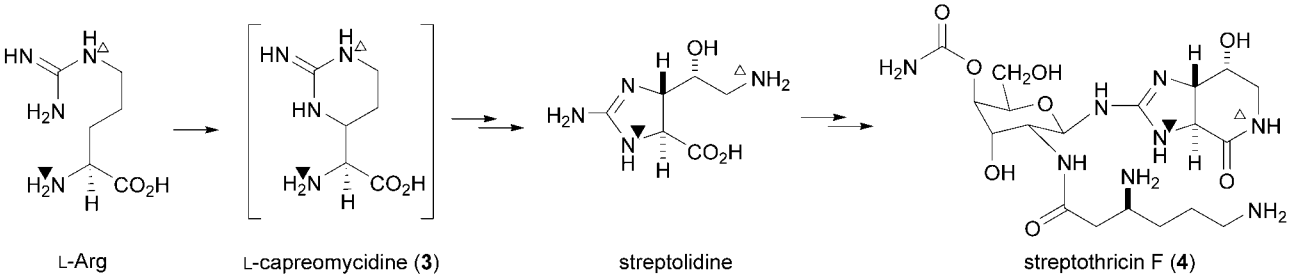
In summary, this report describes the first characterization of an arginine β-hydroxylase. Whereas the isomeric γ-hydroxyarginine is found in several plants[22] and is a component of a mollusk adhesion protein,[23] and *N*-hydroxyarginine is formed as an intermediate in the generation of nitric oxide by nitric oxide synthase,[24] this study provides the first evidence for the natural occurrence of β-hydroxyarginine. An in-depth characterization of VioC will be reported shortly, and associated studies on the transformation of 3*S*-hydroxy-L-Arg to capreomycidine by VioD are reported in the next article.[25]

Experimental Section

Cloning of vioC: Further DNA sequencing of a cosmid harboring the *vioC* oxygenase gene (pTOV101) revealed a prior sequencing error, introducing an extra T at nucleotide 886, that led us to revise the start site of the open reading frame to nucleotide 786 (GenBank Accession No. AY225601). The resulting start codon for *vioC* is the same as that reported by Thomas et al.[10] To construct the expression plasmid pET28vioC, the primers VioCf (5′-ACGCATATGACTGAGAGCCCCACGACGCA-3′, *Nde*I site underlined) and VioCr (5′-CCAGAATTCGGATCCTCATCGCTGCCCCAGGACC-3′, *Bam*HI/*Eco*RI sites



Scheme 1. Overall reaction catalyzed by VioC.



Scheme 2. Pattern of L-Arg incorporation and proposed involvement of capreomycidine in streptothricin F biosynthesis.

underlined) were used to amplify *vioC* with cosmid pTOV101 serving as DNA template. The gel-purified PCR product was sequenced and then cloned into the *Nde*I and *Eco*RI sites of pET28a (Novagen). The resulting plasmid was designated pET28vioC.

Expression and purification of recombinant VioC: Plasmid pET28-vioC was used to transform *E. coli* (DE3) Rosetta cells (Novagen) for production of VioC as a *N*-terminal His_6-tagged protein. Single colonies isolated from fresh transformation plates were grown overnight at 30 °C in LB medium (5 mL) containing kanamycin (50 μg mL^{-1}) and subsequently used to inoculate of LB/kanamycin medium (500 mL). Cells were grown at 20 °C until the A_{600} = 0.6–0.9, at which point expression was induced with IPTG (0.5 mM). Cells were cultured for an additional 5 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, 300 mM NaCl) containing phenylmethylsulfonyl fluoride (0.1 mM), 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 35 300 *g* for 30 min at 4 °C to give a cleared lysate from which His_6-VioC was purified by using a Ni^{2+}-NTA spin column (Qiagen) according to the manufacturer's directions. The VioC was eluted with buffer A containing imidazole (200 mM). Efficiency of purification was verified by SDS-PAGE. Fractions containing VioC were pooled and dialyzed against sodium phosphate (50 mM), pH 7.6, and NaCl (300 mM) to remove imidazole and then stored at 4 °C.

Oxygenase assay: Typical assays with arginine or related compounds as substrate were conducted in MOPS (50 mM), pH 7.0, and included substrate (1 mM), α-ketoglutarate (1 mM), $FeSO_4$ (25 μM), DTT (0.5 mM), ascorbate (0.1 mM) and appropriately diluted VioC in a total volume of 200 μL. Reactions were initiated by the addition of VioC and incubated at 30 °C for 0.5 to 3 h. Protein was precipitated with cold ethanol, and the supernatant was decanted and stored at −20 °C prior to derivatization with dansyl chloride (DNS-Cl) to permit HPLC analysis with UV detection.[12] 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 reaction mixtures were vortexed briefly and kept at room temperature for 1 h, at which point excess DNS-Cl was consumed with a 2% solution of ethylamine (20 μL). Reverse-phase HPLC analysis (Beckman Ultrasphere C_{18} column, 5 μm; 4.6×250 mm) was performed on a ThermoFinnigan Surveyor system with photodiode array detection under isocratic conditions of 80% potassium phosphate (0.1 mM), pH 7.5, 20% MeCN.

Characterization of the VioC reaction product: The purified product of the VioC reaction was analyzed by ESI-MS on a ThermoFinnigan LCQ Advantage instrument by direct injection. A molecular ion was seen at *m/z* = 430.5, which corresponded to $[M+Li]^+$. Analysis of DNS-Arg showed molecular ions at *m/z* = 408.5 $[M+H]^+$ and 414.5 $[M+Li]^+$. The authentic samples of 3*R*- and 3*S*-hydroxy-L-Arg used as standards were the same materials used in the labeled precursor-incorporation studies described in ref. [14] and are deuterated at C-5. Both isomers of DNS-3-hydroxy-L-[5,5-$^2H_2$]Arg produced an ion at *m/z* = 432.6 $[M+Li]^+$. DNS-3*R* and 3*S*-hydroxy-L-[5,5-$^2H_2$]Arg were readily separated by RP-HPLC. Each isomer was separately coinjected with aliquots of the same VioC reaction mixture.

Acknowledgements

We are indebted to Dr. Steven Gould for the generous gift of labeled β-hydroxyarginine standards. Quan Zhang and Morgan Parker are acknowledged for excellent technical and analytical support. This work was supported by NIH grant GM 69320.

Keywords: antibiotics · arginine · biosynthesis · iron · proteins · viomycin

[1] A. Finlay, G. L. Hobby, F. A. Hochstein, T. M. Lees, T. F. Lenert, J. A. Meens, S. Y. P'An, P. P. Regna, J. B. Routin, B. A. Sobin, K. B. Tate, J. H. Kane, *Am. Rev. Tuberc.* **1951**, *63*, 1–3.
[2] E. B. Herr, M. E. Honey, G. E. Pittenger, C. E. Higgins, *Proc. Indiana Acad. Sci.* **1960**, *69*, 134.
[3] A. Nagata, T. Ando, R. Izumi, H. Sakakibara, T. Take, K. Hayano, J. Abe, *J. Antibiot.* **1968**, *21*, 681–687.
[4] Y. F. Liou, N. Tanaka, *Biochem. Biophys. Res. Commun.* **1976**, *71*, 477–483.
[5] P. Marrero, M. J. Cabanas, J. Modolell, *Biochem. Biophys. Res. Commun.* **1980**, *97*, 1047–1052.
[6] J. Modolell, D. Vazquez, *Eur. J. Biochem.* **1977**, *81*, 491–497.
[7] X. Yin, T. O'Hare, S. J. Gould, T. M. Zabriskie, *Gene* **2003**, *312*, 215–224.
[8] R. W. Busby, M. D.-T. Chang, R. C. Busby, J. Wimp, C. A. Townsend, *J. Biol. Chem.* **1995**, *270*, 4262–4269.
[9] Z. Zhang, J. Ren, D. K. Stammers, J. E. Baldwin, K. Harlos, C. J. Schofield, *Nat. Struct. Biol.* **2000**, *7*, 127–133.
[10] M. G. Thomas, Y. A. Chan, S. G. Ozanick, *Antimicrob. Agents Chemother.* **2003**, *47*, 2823–2830.
[11] S. J. Gould, D. A. Minott, *J. Org. Chem.* **1992**, *57*, 5214–5217.
[12] Y. Tapuhi, D. E. Schmidt, W. Lindner, B. L. Karger, *Anal. Biochem.* **1981**, *115*, 123–129.
[13] M. D. Jackson, Ph.D. Dissertation, Oregon State University (USA), **2000**.
[14] S. J. Gould, J. Lee, J. Wityak, *Bioorg. Chem.* **1991**, *19*, 333–350.
[15] H. Chen, M. G. Thomas, S. E. O'Connor, B. K. Hubbard, M. D. Burkart, C. T. Walsh, *Biochemistry* **2001**, *40*, 11 651–11 659.
[16] H. Chen, C. T. Walsh, *Chem. Biol.* **2001**, *8*, 301–312.
[17] H. Chen, B. K. Hubbard, S. E. O'Connor, C. T. Walsh, *Chem. Biol.* **2002**, *9*, 103–112.
[18] M. G. Thomas, M. D. Burkart, C. T. Walsh, *Chem. Biol.* **2002**, *9*, 171–184.
[19] S. J. Gould, K. J. Martinkus, C.-H. Tann, *J. Am. Chem. Soc.* **1981**, *103*, 4639–4640.
[20] K. J. Martinkus, C.-H. Tann, S. J. Gould, *Tetrahedron* **1983**, *39*, 3493–3505.
[21] M. D. Jackson, S. J. Gould, T. M. Zabriskie, *J. Org. Chem.* **2002**, 2934–2941.
[22] P. Rozan, Y. H. Kuo, F. Lambein, *Phytochemistry* **2001**, *58*, 281–289.
[23] V. V. Papov, T. V. Diamond, K. Biemann, J. H. Waite, *J. Biol. Chem.* **1995**, *270*, 20 183–20 192.
[24] M. A. Marletta, A. R. Hurshman, K. M. Rusche, *Curr. Opin. Chem. Biol.* **1998**, *2*, 656–663.
[25] X. Yin, K. L. McPhail, K.-j. Kim, T. M. Zabriskie, *ChemBioChem* **2004**, *5*, 1278–1281.

Received: March 29, 2004

