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Stereochemical Course of Tryptophan Dehydrogenation during Biosynthesis of the Calcium-Dependent Lipopeptide Antibiotics

Bagher Amir-Heidari,[†] Jenny Thirlway, and Jason Micklefield*

School of Chemistry and Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester M1 7ND, U.K.

jason.micklefield@manchester.ac.uk

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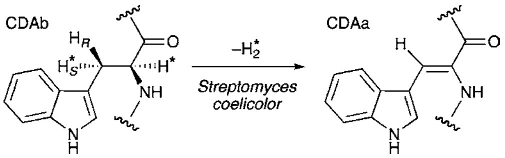
ABSTRACT



Hydrogen atoms are abstracted from the C2′ and C3′-*pro-S* positions of an (*S*)-tryptophanyl precursor, with overall *syn* stereochemistry, during the biosynthesis of the C-terminal *Z*-2′,3′-dehydrotryptophan residue of the calcium-dependent lipopeptide antibiotics (CDAs) in *Streptomyces coelicolor*. The absence of β-hydroxytryptophanyl, or other possible intermediates, further suggests a direct dehydrogenation mechanism similar to that proposed for the L-tryptophan 2′,3′-oxidase from *Chromobacterium violaceum*.

In addition to proteinogenic amino acids, nonribosomal peptides typically comprise a wide range of unusual amino acid residues, which in part accounts for their structural diversity and broad spectrum of biological activities.[1] A common theme in the biosynthetic diversification process is the hydroxylation of amino acid precursors or residues within nascent or mature nonribosomal peptides.[2] The resulting β-hydroxylated amino acid residues can be further modified through oxidation, glycosylation, methylation, macrolactonization, and other transformations.[3] For example, we recently identified an asparaginyl oxygenase (AsnO) that β-hydroxylates an asparagine precursor prior to phosphorylation by a phosphotransferase enzyme (HasP) leading to the 3-phosphohydroxyasparagine residue found within the decapeptide lactone core of the calcium-dependent lipopeptide antibiotics (CDAs) (Figure 1).[4] Also present within the a-series CDAs is a C-terminal *Z*-2′,3′-dehydrotryptophan (*Z*-ΔTrp) residue.[5] Many other nonribosomal peptides and related natural products also contain dehydroamino acids including telomycin, Keramamide F, and the microsclerodermins G/I which all possess *Z*-ΔTrp residues.[6]

[†] Current address: School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

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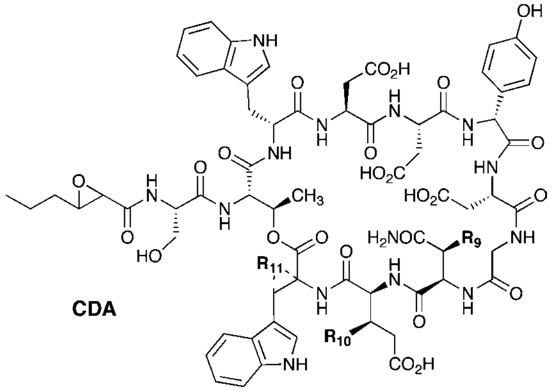
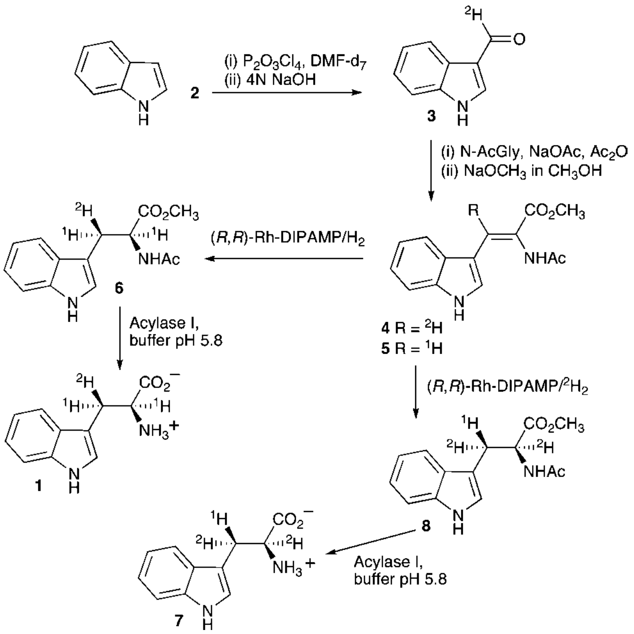


Figure 1. Calcium-dependent antibiotics (CDAs) produced by the wild-type *S. coelicolor*:[5] CDA1, $R_9 = OPO_3H_2$ and $R_{10} = H$; CDA2, $R_9 = OPO_3H_2$ and $R_{10} = CH_3$; CDA3, $R_9 = OH$ and $R_{10} = H$; CDA4, $R_9 = OH$ and $R_{10} = CH_3$. The a-series contains *Z*-ΔTrp ($R_{11} = \pi$-bond) and the b-series contains L-Trp (R_{11} = H,H) at position 11.

Little is known about the nature of the enzymes involved in the dehydrogenation of amino acid residues during nonribosomal peptide biosynthesis. However, related enzyme activities have been characterized. Notably, the tryptophan side chain oxidase (TSO) from *Pseudomonas*[7] and the L-tryptophan 2′,3′-oxidase (LTO) from *Chromobacterium violaceum*[8] both catalyze the oxidation of tryptophan residues to their corresponding α,*β*-dehydro derivatives by different mechanisms. Despite this, protein sequence alignment of the LTO, TSO, and other enzymes involved in the formation of α,*β*-dehydroamino acids[9] reveals little sequence similarity. Moreover, none of these enzymes[7−9] display any obvious sequence similarities with putative proteins encoded by genes from within or outside the CDA biosynthetic cluster. In light of this, we set out to probe the mechanism of tryptophan dehydrogenation during CDA biosynthesis, using classical stereochemical studies. To do this, we proposed to feed synthetic stereospecifically C3′-deuterated tryptophans to a *Streptomyces coelicolor* strain WH101.[10] The WH101 strain is derived from the *S. coelicolor* M145 parental strain but possesses a deletion in the *priA* gene which encodes a bifunctional enzyme that is essential for both histidine and tryptophan biosynthesis. Using the Trp auxotrophy, it should be possible to follow the fate of the Trp-C3′-deuterium atoms during CDA biosynthesis using electrospray ionization (ESI) mass spectrometry.

Scheme 1. Synthesis of (2′*S*,3′*R*)-[3′-$^2H_1$]-Tryptophan **1** and (2′*S*,3′*S*)-[2′,3′-$^2H_2$]-Tryptophan **7**



The synthesis of (2′*S*,3′*R*)-[3′-$^2H_1$]-tryptophan **1** was achieved via the Vilsmeier formylation of indole **2** with deuterated dimethylformamide (DMF-d_7) (Scheme 1). Literature procedures for this formylation typically utilize DMF as the solvent.[11] However, to conserve the labeled precursor, the reaction was optimized using toluene as a solvent, such that a 1.2:1 molar ratio of indole to DMF-d_7 was required to generate indole-3-deuterocarboxaldehyde **3** in 64% yield (based on DMF-d_7). The deuteroaldehyde **3** was then subjected to an Erlenmeyer condensation with *N*-acetyl glycine.[12] This resulted in an intermediate azalactone, which was subjected to methanolysis to give *Z*-[3′-$^2H_1$]-2′,3′-dehydrotryptophan derivative **4** in 44% overall yield, with none of the *E*-isomer. The *Z*-configuration of **4**, and the similarly prepared unlabeled isotopomer **5**, was confirmed by NMR and X-ray crystallography (see Supporting Information). Enantioselective hydrogenation of **4**, using the rhodium(I)-(*R*,*R*)-DIPAMP catalyst,[13] gave (2′*S*,3′*R*)-[3′-$^2H_1$]-*N*-acetyltryptophan methyl ester **6** in a yield of 85%. ^1H NMR clearly shows that **6** is diastereomerically pure, consistent with the fact that the Wilkinson-type catalysts are all known to hydrogenate exclusively with *syn* stereochemistry.[13e] Also, optical rotation indicated that **6** is ≥95% ee,

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which is in line with the enantioselectivity observed in many related hydrogenation reactions using Rh(I)-(*R*,*R*)-DIPAMP.[13] Attempts to hydrolyze the acetyl and methyl ester protecting groups of **6**, under basic or acidic conditions, resulted in either decomposition or epimerization at C2′. To overcome this, **6** was incubated with the aminoacylase from *Aspergillus* sp. (Acylase I)[14] in phosphate buffer at pH 5.8 for an extended period of 3.5 h, which results in the hydrolysis of both the *N*-acetyl and methyl ester groups. Acylase I is highly enantioselective for L-amino acid derivatives,[14] allowing for a very effective double kinetic resolution, resulting in enantiomerically pure (2′*S*,3′*R*)-[3′-$^2H_1$]-tryptophan **1** in a yield of 86%.

The doubly labeled (2′*S*,3′*S*)-[2′,3′-$^2H_2$]-tryptophan **7**, with the opposite configuration at C3′, was similarly prepared by asymmetric reduction of *Z*-2′,3′-dehydrotryptophan derivative **5** using deuterium gas and Rh(I)-(*R*,*R*)-DIPAMP.[13] The dideuterated intermediate **8** (≥95% ee) was then hydrolyzed with the acylase to give the product **7**, which again proved to be enantiomerically pure. With both labeled tryptophans in hand, it remained to establish conditions that would allow for maximum incorporation of the synthetic precursors into a *Z*-ΔTrp-containing (a-series) CDA. To do this, the Trp-His auxotrophic *S. coelicolor* strain, WH101, was grown on a series of defined minimal liquid media supplemented with His and Trp and the culture supernatants were extracted onto C18 (bond-elute) cartridges. However, electrospray ionization (ESI) LC-MS analysis of the resulting extracts showed no production of CDAs. Despite this, WH101 was shown to produce CDAs when grown on a modified SV2 liquid medium[5] with reduced soy peptone supplemented with His (50 μg mL^{-1}) and Trp (37.5 μg mL^{-1}) (SV2a). Given that the soy peptone of SV2a provides ca. 6 μg mL^{-1} of Trp, it is thus possible to supply ca. 85% of required Trp, in the stereospecifically deuterated forms. Accordingly, WH101 was grown in SV2a with either the (2′*S*,3′*S*)- or (2′*S*,3′*R*)-deuterated tryptophans **7** or **1** and the culture supernatants were analyzed by ESI-LC-MS. In each case, the major product was CDA4a with a minor amount of CDA3a which have retention times in agreement with authentic standards (see Supporting Information). However, the protonated, sodiated, and potassiated molecular ions of CDA4a and CDA3a produced from media enriched in the (2′*S*,3′*S*)-deuterated tryptophan **7** are clearly 1 mass unit higher than molecular ions of unlabeled CDA4a and CDA3a (Figure 2 and Supporting Information). This is consistent with retention of the C3′-*pro-S* deuterium atom in the D-Trp residue at position 3 of CDA and loss of the C3′-*pro-S* deuterium atom during oxidation to *Z*-ΔTrp at position 11. Clearly, the C2′ deuterium atom of **7** is necessarily lost during the epimerization of L-Trp to D-Trp3 and the dehydrogenation to *Z*-ΔTrp11.[15]

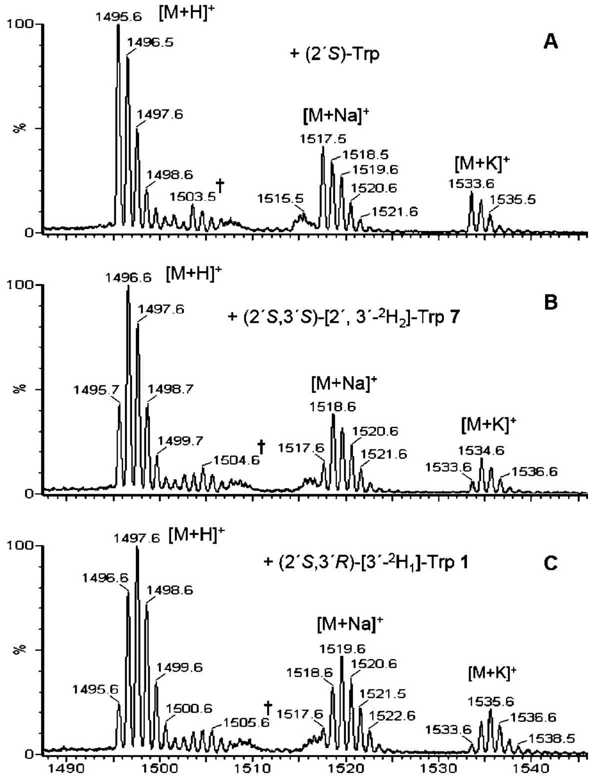

Figure 2. (A) Mass spectrum of CDA4a from *S. coelicolor* WH101 grown on SV2a supplemented with (2*S*)-tryptophan: *m*/*z* 1495.6 ([M + H]$^+$ $C_{67}H_{79}N_{14}O_{26}$ requires 1495.5); 1517.5 ([M + Na]$^+$ $C_{67}H_{78}N_{14}O_{26}Na$ requires 1517.5); 1533.6 ([M + K]$^+$ $C_{67}H_{78}N_{14}O_{26}K$ requires 1533.5). (B) CDA4a from WH101 supplemented with (2′*S*,3′*S*)-[2′,3′-$^2H_2$]-tryptophan **7** shows that the major isotopomer of the protonated, sodiated, and potassiated molecular ions is 1 mass unit higher than the wild-type CDA4a as a result of incorporation of a single deuterium atom. (C) CDA4a from WH101 supplemented with (2′*S*,3′*R*)-[3′-$^2H_1$]-tryptophan **1** shows molecular ions that are 2 mass units higher than the wild-type, consistent with the incorporation of two deuterium atoms. An identical pattern was observed in the MS of CDA3a, obtained from feeding labeled and unlabeled Trp (see Supporting Information). †Signals due to a residual trace of CDA3a [M + Na]$^+$.

In agreement with this, the monodeuterated (2′*S*,3′*R*)-tryptophan **1** with opposite configuration at C3′ gives CDA4a and CDA3a products which are isotopically enriched by two deuterium atoms (+2 amu). The C3′-*pro-R* deuterium atom of **1** is thus retained in the D-Trp3 and *Z*-ΔTrp11 residues of CDA. This confirms that the C3′-*pro-S* hydrogen atom is abstracted during the *syn* dehydrogenation of the C-terminal L-Trp residue of CDA. Interestingly, analysis of the isotopic patterns of CDA4a and CDA3a produced from **1** and **7** indicates ca. 60% level of isotopic enrichment, which is lower than the 85% which is predicted on the basis of the isotopic

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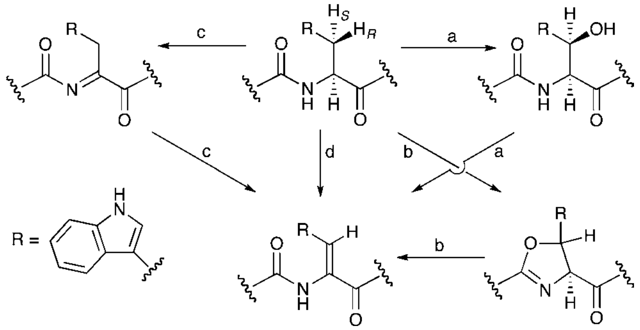
[15] Epimerization during nonribosomal peptide biosynthesis has been shown to result in the complete exchange of the α-proton of amino acyl thioester intermediates with the solvent: Stachelhaus, T.; Walsh, C. T. *Biochemistry* **2000**, *39*, 5775−5787.

composition of the Trp in the medium. This could be due to the fact that WH101 in the absence of PriA is deficient in indole-3-glycerol phosphate.[10] As a result, the α-subunit of the tryptophan synthase α2β2 complex is unable to generate indole. This would allow the β-subunit to catalyze the reversible PLP-dependent cleavage of labeled L-Trp to form indole and L-Ser, which could exchange with unlabeled L-Ser.[16] In addition *S. coelicolor* is known to excrete indole-3-acetic acid when fed with L-Trp.[17] This requires a Trp transaminase to generate indole pyruvate prior to decarboxylation and oxidation.[17] The facile enolization of indole pyruvate followed by the reverse transamination could also account for the loss of the Trp-C3′ deuterium label.

There are several possible pathways by which the *Z*-ΔTrp residue of CDA might be biosynthesized. First, β-hydroxylation followed by dehydration (Scheme 2, path a) could take place as in the biosynthesis of a dehydroproline residue of pristinamycin II_A.[9c] However, no β-hydroxylated Trp containing CDA intermediates have been isolated to date. Moreover, both heme-dependent and non-heme hydroxylases typically affect hydroxylation of unactivated methylene groups, with retention of stereochemistry.[18] In addition, most related dehydratases, including those predicted to be involved in the biosynthesis of α,β-dehydroamino acid residues in the nonribosomal peptide thiostrepton, operate with anti stereochemistry.[19] This pathway would thus be expected to result in the abstraction of the C3′-*pro-R* hydrogen atom of an L-tryptophanyl precursor. Alternatively, Trp dehydrogenation catalyzed by the *Pseudomonas* TSO[7] is suggested to occur via an indolyloxazoline intermediate (Scheme 2, path b), followed by isomerization. However, the TSO reaction results in a mixture of *E*- and *Z*-ΔTrp isomers.[7] Moreover, in CDA biosynthesis, the formation of such an oxazoline intermediate in a cyclic peptide precursor could be prohibited due to geometrical constraints. Similarly, the mechanism proposed for the cyclic peptide oxidases[9a] involves tautomerization of imine intermediates resulting in both *E*- and *Z*-dehydroamino acids (Scheme 2, path c), which is not in accordance with the stereochemical course of the CDA Trp dehydrogenation. On the other hand, L-tryptophan 2′,3′-oxidase (LTO) from *Chromobacterium violaceum*[8] catalyzes the dehydrogenation of *N*-Boc-L-Trp to the *Z*-ΔTrp derivative abstracting the C3′-*pro-S* proton with identical *syn* stereochemistry.[20] It is therefore most likely that a direct dehydrogenation, similar to the mechanism proposed for LTO[8] and the fatty acyl-CoA dehydrogenase,[21] occurs during the biosynthesis of the *Z*-ΔTrp residue in CDA (Scheme 2, path d). This knowledge will assist with the future identification and characterization of the enzymes involved in the biosynthesis of α,β-dehydroamino acid residues, which will be valuable in efforts aimed at engineering new non-ribosomal peptides of therapeutic importance.

Scheme 2. Proposed Mechanisms for the Dehydrogenation of Amino Acid Residues[a]



[a] Pathway: (a) β-hydroxylation followed by dehydration;[4,9c,18,19] (b) oxidation to an oxazoline intermediate followed by isomerization;[7] (c) oxidation to an imine followed by tautomerization;[9a] (d) direct dehydrogenation.[8]

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Supporting Information Available: Crystal structure of compound **5**, additional HPLC and MS data, and full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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