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NMR Confirmation That Tryptophan Dehydrogenation Occurs with *Syn* Stereochemistry during the Biosynthesis of CDA in *Streptomyces coelicolor*

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Doubly labeled (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-tryptophan was fed to the Trp-His auxotrophic *Streptomyces coelicolor* strain WH101. Mass spectrometry showed single and double incorporation of the labeled Trp into the calcium-dependent lipopeptide antibiotic (CDA4a). From ^{13}C NMR spectroscopy, it was apparent that the C3′-signal of the (*Z*)-2′,3′-dehydrotryptophan (position 11 in CDA4) was a 1:1:1 triplet indicating that the deuterium atom in the *pro-R* position of the methylene group is retained during Trp-oxidation. This provides definitive proof that Trp dehydrogenation occurs through the loss of the 2′ and *pro*-3′*S* hydrogen atoms with overall *syn* stereochemistry.

Calcium-dependent antibiotics (CDA)[1] are produced by *Streptomyces coelicolor* and belong to the family of nonribosomal lipopeptide antibiotics that include daptomycin,[2] which recently became the first new class of naturally derived antimicrobial agents to be approved for clinical use in more than three decades. In addition to an unusual 2,3-epoxyhexanoyl fatty acid moiety, CDA comprises a number of non-proteinogenic amino acids, including D-4-hydroxyphenylglycine and L-3-methylglutamic acid.[1,3] CDA also contains several oxidized amino acid residues including D-3-hydroxyasparagine or D-3-phosphohydroxyasparagine[4] and a *C*-terminal (*Z*)-2′,3′-dehydrotryptophan (*Z*-ΔTrp).[5] β-Hydroxylation[4,6] and α,β-dehydrogenation[5,7] are common themes in the biosynthesis of nonribosomal peptide natural products. While the enzymes typically implicated in β-hydroxylation of amino acid residues are well characterized,[4,6] there is little known about the enzymes involved in the formation of the α,β-dehydroamino acid residues,[7] within nonribosomal peptides. It is therefore difficult to use protein homology searches to identify the candidate enzyme(s) that may catalyze the formation of the *Z*-ΔTrp residue of CDA. Consequently, we chose to explore the mechanism of Trp dehydrogenation using classical stereochemical studies.[5] In these earlier studies,[5] synthetic *pro*-3′*S*- and *pro*-3′*R*-deuterated Trp precursors were fed separately to the Trp-His auxotrophic *S. coelicolor* strain WH101.[8] ESI-MS analysis of the biosynthetic CDA4a, which was isolated from the individual feeding experiments, led to the preliminary conclusion that hydrogen atoms are abstracted from the 2′- and *pro*-3′*S* positions with overall *syn* stereochemistry during Trp dehydrogenation.[5] Given that the analysis of isotope patterns in MS can be open to misinterpretation, it was necessary to develop an alternative approach to confirm this initial stereochemical assignment.[5]

Described herein is an experiment employing a [^{2}H,^{13}C]-labeled substrate and NMR analysis to definitively assess the stereochemical course of the dehydrogenation reaction during CDA4a biosynthesis. This required a stereocontrolled synthesis of (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-tryptophan (Scheme 1). Accordingly, the indole Grignard salt was generated from indole **1** with methyl-magnesium iodide and selectively methylated at C3 with doubly labeled [$^2H_3$,$^{13}C_1$]-methyl iodide. This involved a modification of the reported procedure,[9] utilizing a 2-fold molar excess, instead of 1 molar equiv, of the indole Grignard salt to give the product **2** in a yield of 50% based on the more valuable [$^2H_3$,$^{13}C_1$]-methyl iodide. Oxidation of **2** with DDQ in aqueous THF[10] afforded the aldehyde **3** in a yield of 53%. In a previous investigation, indole-3-carboxaldehyde was subjected to Erlenmeyer condensation, with *N*-acetylglycine to generate the corresponding *Z*-dehydrotryptophan derivative.[5] However, the modest product yields of this route prompted the use of an alternative method, which involved the condensation of aldehyde **3**, with methyl acetamidomalonate **5**, which was derived by the

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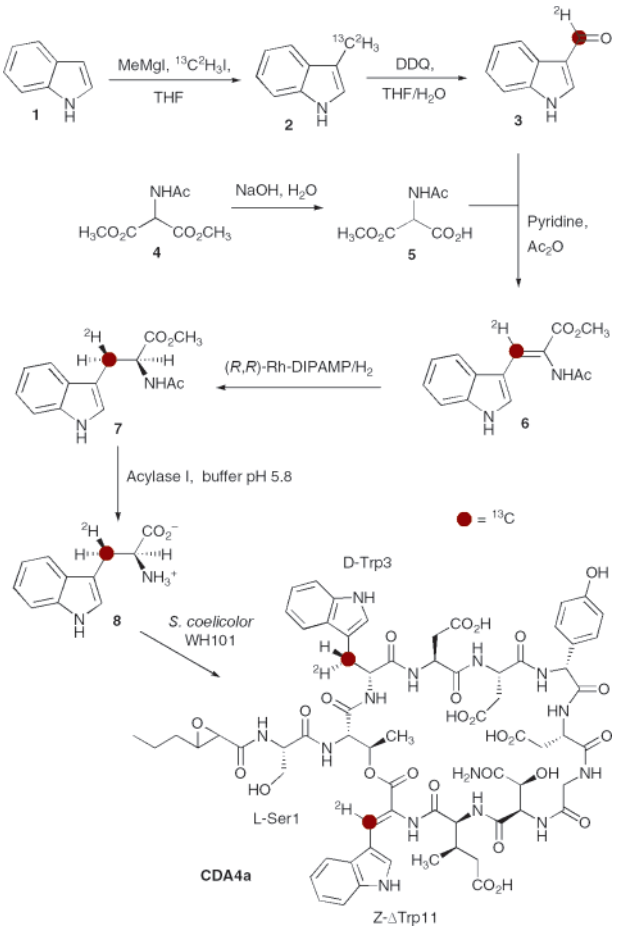
SCHEME 1. Synthesis of (2′S,3′R)-[3′-$^2H_1$,$^{13}C_1$]-Tryptophan (8) and Its Incorporation into CDA4a



saponification of dimethyl acetamidomalonate **4**. Decarboxylative aldol condensation of **3** with **5** in a mixture of acetic anhydride and pyridine[11] gave (*Z*)-[3′-$^2H_1$,$^{13}C_1$]-2′,3′-dehydrotryptophan derivative **6**, in 89% yield with none of the *E*-isomer detected. The *Z*-configuration of **6** was confirmed through NMR comparison with the unlabeled compound, which had been prepared previously and shown to possess the *Z*-configuration by X-ray crystallography.[5] Enantioselective hydrogenation of **6** catalyzed by rhodium(I)-(*R*,*R*)-DIPAMP gave the (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-*N*-acetyltryptophan methyl ester **7** in a yield of 96%; the optical rotation of which corresponds to 95% ee. Finally, hydrolysis of the *N*-acetyl and methyl ester substituents of **7**, by treatment with the aminoacylase from *Aspergillus* sp. (Acylase I),[13] afforded the (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-tryptophan **8** in 96% yield.

The *S. coelicolor* His-Trp auxotrophic strain WH101[8] was then grown in SV2a liquid medium supplemented with L-histidine (50 mg.L^{-1}) and the labeled Trp **8** (37.5 mg.L^{-1}) as previously described.[5] The major CDA product was purified by HPLC and shown to possess the same retention time and

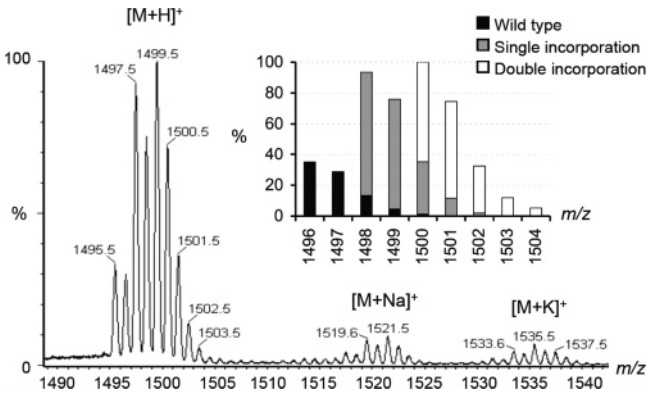


FIGURE 1. ESI-MS of CDA4a isolated from *S. coelicolor* WH101 after incubating with (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-tryptophan **8** for 5 days. Shown are CDA4a *m*/*z* 1495.5 ([M + H]$^+$ $C_{67}H_{79}N_{14}O_{26}$ requires 1495.5) and CDA4a isotopomers in which one and two Trp **8** residues have been incorporated: *m*/*z* 1497.5 ([M + H]$^+$ $^{12}C_{66}$ $^{13}C_1{}^1H_{78}{}^2H_1N_{14}O_{26}$ requires 1497.5) and 1499.5 ([M + H]$^+$ $^{12}C_{65}{}^{13}C_2{}^1H_{77}{}^2H_2N_{14}O_{26}$ requires 1499.5), respectively. The inset depicts the theoretical isotopic distribution for molecular ions ([M + H]$^+$) of a mixture of unlabeled CDA4a (16%) and labeled CDA4a containing one (40%) and two (44%) doubly labeled Trp precursors. The relative peak abundances are established on the assumption that the *pro*-3′*R* deuterium of the substrate appears in both positions 3 and 11 of the product, and the distribution agrees closely with the observed MS calculation. The calculation was performed using Web-based Isotope Patterns Calculator (http://winter.group.shef.ac.uk/chemputer/isotopes.html).

UV spectrum (λ_{max} 274 and 347 nm)[1a] as an authentic sample of CDA4a. ESI-MS analysis of the purified product revealed an ion envelope comprised of three overlapping molecular ions ([M + H]$^+$). The isotopic-mixture contained unlabeled CDA4a (*m*/*z* 1495.5, Figure 1) derived from a small amount of unlabeled Trp present in the media, and isotopically enriched product (*m*/*z* 1497.5 and 1499.5), which was consistent with the incorporation of one and two units of labeled precursor **8** into the product. Note that the double label precursor can be distributed at either position 3 or 11 or together at both places of CDA4a. This again suggests that the *pro*-3′*S* hydrogen atom is abstracted during oxidation of Trp in CDA biosynthesis. By comparison with calculated isotopic patterns for the three molecular ion species it is possible to determine that the approximate levels of single and double Trp incorporation were 40 and 44%, respectively (Figure 1).

The conclusions drawn from the MS data described above were supported by ^{1}H-decoupled ^{13}C NMR analysis of the purified biosynthetically labeled CDA4a. The ^{13}C NMR spectrum revealed ^{13}C enriched triplets δ 28.3 (t, $^1J_{CD}$ = 19.8 Hz) and 129.8 (t, $^1J_{CD}$ = 23.8 Hz) which correspond to the labeled C3′ atoms of Trp and *Z*-ΔTrp at position 3 and 11, respectively (Figure 2). The multiplicity and coupling constants clearly indicate the presence of ^{13}C−^{2}H bonds. The Lorentz−Gauss resolution enhancement[14] was also performed (LB = −3.3 and GF = 0.2), which allowed independent integration of the triplets free from overlap. Consequently, a small but measurable contribution from a CH bond was observed to the central signal of the upfield triplet (Trp-3), whereas no noticeable CH contribution could be assigned in the olefinic C3′-signal of *Z*-ΔTrp-11. This provides definitive proof that *pro*-*R* deuterium of Trp-11, in the penultimate precursor CDA4b, is retained

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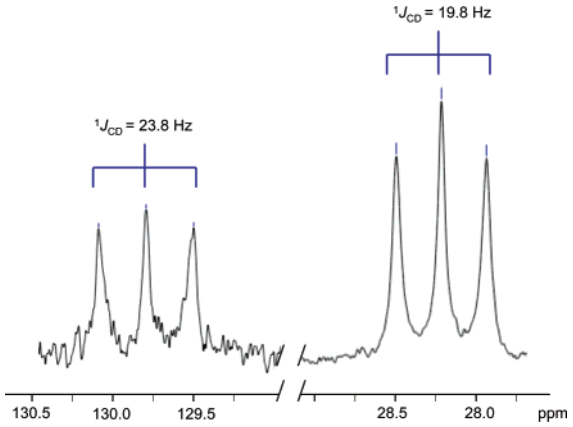

FIGURE 2. ^{13}C NMR of CDA4a in CD_3OD, resulting from feeding of (2′*S*,3′*R*)-[3′-$^2H_1$,$^{13}C_1$]-tryptophan **8** to *S. coelicolor* WH101. Expansion of the C3′ signals of Trp δ 28.3 (t, $^1J_{CD}$ = 19.8 Hz) and *Z*-ΔTrp 129.8 (t, $^1J_{CD}$ = 23.8 Hz) residues of CDA4a is shown.

during dehydrogenation to give the *Z*-ΔTrp-11 residue of CDA4a. Further confirmation was evident from the HMQC spectrum, which shows a strong $^1H-^{13}C$ correlation between C3′and H3′of the Trp at position 3 (δ_C 28.3, δ_H 3.4), while no cross-peak is evident for the C3′ signal of *Z*-ΔTrp (see the Supporting Information). On the other hand, the HMBC spectrum shows clear cross-peaks between C3′ and the indole H2 from both Trp and *Z*-ΔTrp residues.

In summary, the stereochemical course of Trp dehydrogenation is confirmed by NMR spectroscopy to occur through the abstraction of the 2′ and *pro*-3′*S* hydrogen atoms with overall *syn* stereochemistry, which is consistent with earlier MS analysis.[5] The same stereochemical course has also been observed for L-tryptophan 2′,3′-oxidase (LTO) from *Chromobacterium violaceum*,[15] which is proposed[16] to undergo direct dehydrogenation, probably utilizing FAD or a related redox cofactor in a similar fashion to the fatty acyl-CoA dehydrogenase.[17] It is thus most likely that Trp oxidation in CDA biosynthesis occurs by a similar direct dehydrogenation mechanism (Figure 3). In this case, either the cyclic lipopeptide (e.g., CDA4b which possesses a Trp residue at position 11)[1a] or a peptidyl thioester intermediate tethered to the peptidyl carrier protein (PCP) domain of the CDA nonribosomal peptide synthetase (NRPS) are possible substrates. Indeed, a similar mechanism has been proposed to account for the oxidation of proline-*S*-PCP to pyrrole-2-carboxyl-*S*-PCP catalyzed by the FAD dependent dehydrogenase RedW during the biosynthesis of undecylprodigiosin, also in *S. coelicolor*.[18] The stereochemical insight provided by this work will aid the identification and subsequent mechanistic characterization of the putative CDA Trp dehydrogenase/oxidase and related enzymes.

Experimental Section

3-[$^2H_3$,$^{13}C_1$]-Methylindole (2). Methylmagnesium iodide in ether (4.7 mL, 14.1 mmol) was added to indole **1** (1.61 g, 13.7 mmol) in anhydrous THF (20 mL), and the mixture was stirred at room temperature for 30 min under N_2. [$^{13}C_1$,$^2H_3$]-Methyl iodide (1.0 g, 6.85 mmol) in anhydrous THF (2.0 mL) was added over 20 min, and the mixture was stirred and sealed under N_2 for 5 days. A portion of 5% aq NH_4Cl (30.0 mL) was added, and the mixture was extracted with EtOAc (4 × 30 mL), dried over $MgSO_4$, and evaporated under reduced pressure. Purification by column chromatography eluting with by 1% acetone in hexane gave **2** (465 mg, 50%) as a white crystalline solid: R_f 0.6 (80:20 Hex/EtOAc); mp 97−99 °C (lit.[19] mp 98.0−98.5 °C); ^1H NMR (300 MHz, $CDCl_3$) 6.98 (1H, s, H2), 7.10−7.15 (1H, m, H5), 7.17−7.22 (1H, m, H6), 7.35 (1H, d, J = 7.9 Hz, H7), 7.59 (1H, d, J = 7.7 Hz, H4), 7.89 (1H, br s, H1); ^{13}C NMR (75.5 MHz, $CDCl_3$) 8.36 (Sept, J = 19.3 Hz, $^{13}C^2H_3$), 110.9 (C7), 111.8 (d, J = 50.28 Hz, C3), 118.8 (C4), 119.0 (C5), 121.5 (d, ^2J = 5.1 Hz, C2), 121.8 (C6), 128.2 (d, J = 2.7 Hz, C9), 136.2 (d, J = 2.6 Hz, C8); LRMS-ESI$^+$ (*m/z*) 136.0 [M + H]$^+$; HRMS-ESI$^+$ (*m/z*) [M + H]$^+$ calcd for $C_8{}^{13}C_1H_7{}^2H_3N_1$ 136.1030, found 136.1030.

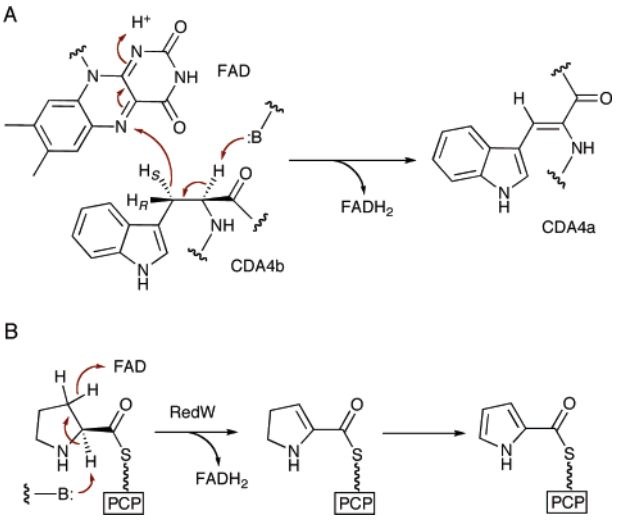

FIGURE 3. (A) Possible mechanism of Trp dehydrogenation during CDA biosynthesis. (B) Proposed mechanism for the oxidation of proline- to pyrrolyl-2-carboxyl-*S*-PCP catalyzed by RedW during biosynthesis of undecylprodigiosin (PCP = peptidyl carrier protein).[18]

Indole-3-[$^2H_1$,$^{13}C_1$]-carboxaldehyde (3). 3-[$^{13}C_1$,$^2H_3$]-Methylindole **2** (225 mg, 1.67 mol) in THF (2.0 mL) was added to DDQ (754 mg, 3.33 mmol) in 90% aq THF (20 mL) at 25 °C and stirred for 3 h under N_2. K_2CO_3 (20 mg, 0.15 mmol) was added, and the mixture was stirred for 15 min and then evaporated to dryness and purified by column chromatography eluting with 3:1 CH_2Cl_2/EtOAc to give **3** (130 mg, 53%) as a white solid: R_f 0.4 (5% MeOH in CH_2Cl_2); mp 195−197 °C (lit. 193−195 °C);[10] ^1H NMR (400 MHz, CD_3OD) δ 7.12−7.17 (2H, m, H6 & H5), 7.36 (1H, d, J = 7.5 Hz, H7), 7.98 (1H, d, J = 1.9 Hz, H2), 8.06 (1H, d, J = 7.9 Hz, H4), 9.77 (1H, brs, H1); ^{13}C NMR (75.5 MHz, CD_3OD) δ 113.3 (C7), 120.1 (d, J = 60.7 Hz, C3), 122.5 (C4), 123.8 (C5), 125.1 (C6), 125.8 (C8), 139.0 (d, J = 3.2 Hz, C9), 139.8 (d, J = 9.4 Hz, C2), 187.3 (t, J = 26.2 Hz, $^{13}C^2HO$); LRMS-EI/CI (*m/z*) 148 [M + H]$^+$; HRMS-ESI (*m/z*) [M + H]$^+$ calcd for $C_8{}^{13}C_1H_7{}^2H_1O_1N_1$ 148.0697, found 148.0703.

[3′-$^2H_1$,$^{13}C_1$]-(*Z*)-*N*2′-Acetyl-2′,3′-dehydrotryptophan Methyl Ester (6). Acetic anhydride (0.5 mL) was added dropwise to a mixture of methyl acetamidomalonic acid **5** (106 mg, 0.61 mmol) and aldehyde **3** (90 mg, 0.61 mmol) in anhydrous pyridine (2.5 mL) at 15 °C. After 3 h of stirring at room temperature, another portion of **5** (106 mg, 0.61 mmol) was added, and the stirring was continued for a further 22 h. Ice (10 g) was added, and the mixture was then extracted with CH_2Cl_2 (3 × 50 mL), dried over $MgSO_4$,

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and evaporated under reduced pressure. $NaOCH_3$ (0.25 mmol) in anhydrous CH_3OH (5 mL) was added to the crude product, and the mixture was stirred overnight and evaporated under reduced pressure. Purification by column chromatography eluting with a gradient of 50% EtOAc in hexane to 100% EtOAC afforded **6** (95 mg, 89% based on unrecovered starting material) as a solid: R_f 0.4 (EtOAc); mp 168−169 °C (lit.[20] mp 167−171 °C); ^{1}H NMR (400 MHz, CD_3OD) δ 2.18 (3H, s, CH_3CO), 3.82 (3H, s, CH_3O), 7.15−7.22 (2H, m, H5 & H6), 7.43 (1H, d, J = 7.8 Hz, H7), 7.73−7.75 (1H, d, J = 7.7 Hz, H4), 7.77 (1H, s, H2), 9.4 (1H, s, NHCO), 11.26 (1H, brs, H1); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 22.7 (CH_3CO), 51.8 (CH_3O), 108.7 (d, J = 64.6 Hz, C3), 112.1 (C7), 118.1 (C4), 120.4 (C5), 120.7 (d, J = 80.8 Hz, C2′), 122.3 (C6), 125.5 (t, J = 20.1 Hz, C3′), 126.9 (d, J = 3.2 Hz, C9) 128.4 (d, J = 3.0 Hz, C2), 135.6 (d, J = 3.5 Hz, C8), 165.7 (d, J = 6.4 Hz, $COOCH_3$), 168.9 ($NHCOCH_3$); LRMS-ESI (m/z) 261.1 $[M + H]^+$, 283.1 $[M + Na]^+$, 299.1 $[M + K]^+$; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{13}{}^{13}C_1H_{14}{}^2H_1N_2O_3$ 261.1174, found 261.1176.

(2′*S*,3′*R*)-[3′-^{2}H$_1$,^{13}C$_1$]-*N*2′-Acetyltryptophan Methyl Ester (7). The alkene **6** (120 mg, 0.46 mmol) was reduced as described previously[5] using rhodium(I)-(*R*,*R*)-DIPAMP (ca. 30 mg) and H_2 (2 atm) to give **7** (116 mg, 96% yield) as a white solid: R_f 0.3 (5% MeOH in DCM); mp 154−155 °C (lit.[21] mp 155−156 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.94 (3H, s, CH_3CO), 3.14−3.48 (1H, dd, $^1J_{HH}$ = 4.8 Hz, $^1J_{CH}$ = 131.6 Hz, H3′), 3.68 (3H, s, CH_3O), 4.91−4.96 (1H, m, H2′), 5.98 (1H, d, J = 6.8 Hz, CONHCH), 6.95 (1H, s, H2), 7.10 (1H, m, H5), 7.18 (1H, m, H6), 7.33 (1H, d, J = 8.1 Hz, H7), 7.51 (1H, d, J = 7.9 Hz, H4), 8.16 (1H, br s, H1); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 23.6 ($NHCOCH_3$), 27.5 (t, J = 19.8 Hz, C3′), 52.6 ($COOCH_3$), 53.2 (d, J = 34.0 Hz, C2′), 110.2 (d, J = 49.7 Hz, C3), 111.3 (C7), 118.5 (C4), 119.7 (C5), 122.2 (C6), 122.6 (d, J = 4.8 Hz, C2), 127.7 (d, J = 5.1 Hz, C9), 136.0 (C8), 169.7 ($COOCH_3$), 172.4 ($NHCOCH_3$); LRMS-ESI (m/z) 263.2 $[M + H]^+$, 285.2 $[M + Na]^+$; $[\alpha]^{26}_D$ +12.2 (c = 2.0, MeOH) (lit.[22] $[\alpha]^{20}_D$ + 12.4 (c = 2.0, MeOH)).

(2′*S*,3′*R*)-[3′-^{2}H$_1$,^{13}C$_1$]-Tryptophan (8). Deprotection of **7** (95 mg, 0.36 mmol) using Acylase I was acheived as described previously[5] resulting in **8** (72 mg, 96%) as a white powder: R_f 0.3 (1:4 H_2O/CH_3CN); mp 282−285 °C (lit.[23] mp 280−285 °C); ^{1}H NMR (400 MHz, D_2O) δ 3.24−3.58 (1H, dd, $^1J_{HH}$ = 4.4 Hz, $^1J_{CH}$ = 130.8 Hz, H3′), 3.99 (1H, t, J = 4.3 Hz, H2′), 7.14 (1H, m, H5), 7.23 (1H, m, H6), 7.25 (1H, s, H2), 7.48 (1H, d, J = 8.1 Hz, H7), 7.68 (1H, d, J = 7.9 Hz, H4); ^{13}C NMR (100.6 MHz, D_2O) δ 26.40 (t, $^1J_{DC}$ = 19.6 Hz, C3′), 55.3 (d, J = 33.9 Hz, C2′), 107.7 (d, J = 48.9 Hz, C3), 112.3 (C7), 118.8 (C4), 119.8 (C5), 122.4 (C6), 125.3 (d, J = 5.1 Hz, C2), 126.9 (d, J = 2.4 Hz, C9), 136.6 (d, J = 1.4 Hz, C8), 174.9 (CO_2); LRMS-ESI (m/z) 207.1 $[M + H]^+$, 229.1 $[M + Na]^+$, 435.2 $[2M + Na]^+$, 451.1 $[M + K]^+$; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{10}{}^{13}C_1H_{12}{}^2H_1N_2O_2$ 207.1068, found 207.1071; $[\alpha]^{26}_D$ −31.3 (lit.[23] $[\alpha]^{25}_D$ −31.8 (c = 1.0, H_2O)).

Feeding and Isolation of Labeled CDA4a from *S. coelicolor* WH101. SV2a[5] liquid medium (1.0 L) was supplemented with filter-sterilized solutions of L-histidine (50 mg) and labeled tryptophan **8** (37.5 mg) divided into four 250 mL portions in 1000 mL conical flasks equipped with a stainless steel spring, inoculated with *S. coelicolor* WH101 spore suspension (10 μL) and incubated at 28 °C in a shaker incubator for 5 days. The cultures supernatants were worked up as described previously[5] and crude CDA extracts were purified by semi-preprative HPLC on a Varian Pro-Star system using a Phenomenex C-18 5 μm, 250 × 10 mm column with a flow rate of 5 mL·min^{-1}. Solvent A was 0.1% aq HCO_2H and solvent B was CH_3CN with 0.1% HCO_2H. The gradient used was 30% B and 70% A for 35 min, increasing to 100% B over 5 min, returning to 30% B and 70% A over 1 min. Fractions eluted over 27.00 to 28.30 min were collected, evaporated under reduced pressure, and subjected to NMR analysis.

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Supporting Information Available: Additional data and copies of spectra and chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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