Installation of the Pyrrolyl-2-carboxyl Pharmacophore by CouN1 and CouN7 in the Late Biosynthetic Steps of the Aminocoumarin Antibiotics Clorobiocin and Coumermycin A_1[†]

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ABSTRACT: The 5-methyl-2-pyrrolylcarbonyl moiety of the aminocoumarin antibiotics clorobiocin and coumermycin A_1 is the key pharmacophore for targeting the ATP-binding site of GyrB for inhibition of the bacterial type-II topoisomerase DNA gyrase. During the late stage of clorobiocin and coumermycin A_1 biosynthesis, the pyrrolyl-2-carboxyl group is transferred from the peptidyl carrier proteins Clo/CouN1 to the 3′-hydroxyl of the 4-methoxy-L-noviosyl scaffold by the action of the acyltransferases Clo/CouN7. CouN1 and CouN7 have now been heterologously expressed and purified from *Escherichia coli*. The apo form of CouN1 is converted to the acyl-holo form by loading with pyrrolyl-2-carboxyl-*S*-pantetheinyl moieties from synthetic pyrrolyl- and 5-methylpyrrolyl-CoAs by the action of the phosphopantetheinyl transferase Sfp. CouN7 acts as an acyltransferase, moving the pyrrole acyl moieties from CouN1 to the noviose sugar of descarbamoylnovobiocin. When the 5-methylpyrrolyl-2-carboxyl-thioester of CouN1 is the cosubstrate, the in vitro product differs from clorobiocin only in a CH_3 for Cl group change on the coumarin ring. Double transfer of this acyl moiety by CouN7 to the penultimate intermediate in coumermycin A_1 assembly completes that antibiotic biosynthetic pathway.

Clorobiocin (**1**), novobiocin (**2**), and coumermycin A_1 (**4**), members of the aminocoumarin family of antibiotics, are potent competitive inhibitors of the bacterial type-II topoisomerase DNA gyrase required for DNA replication and transcription (Figure 1A) (*1*−*3*). DNA gyrase is a heterotetramer (A_2B_2) composed of two subunits, GyrA (97 kDa), responsible for DNA breakage and reunion, and GyrB (90 kDa), involved in adenosine triphosphate (ATP)[1] binding and hydrolysis. The A subunits of this enzyme are also the target of the widely used quinolone antibiotics such as ciprofloxacin.

In addition to the core 3-amino-7-hydroxycoumarin bicyclic scaffold, clorobiocin and coumermycin A_1 are characterized by a 4-methoxy-L-noviosyl ring acylated at the 3′-hydroxyl position by a 5-methyl-2-pyrrolylcarbonyl moiety. In novobiocin, a carbamoyl group is found in place of the 5-methyl-2-pyrrolylcarbonyl of clorobiocin and coumermycin A_1. The high-resolution crystal structure of a 24 kDa GyrB fragment complexed with clorobiocin revealed key interactions between the imino group of the pyrrolyl moiety that sits in a conserved hydrophobic pocket and the ATP-binding site of GyrB (Figure 1C) (*4*). Previous studies have demonstrated that, in vitro, gyrase inhibition is enhanced by substitution of the 3′-noviose carbamoyl of novobiocin by the 5-methyl-2-pyrrolylcarbonyl group of clorobiocin (*5*). This suggests that the 5-methyl-2-pyrrolylcarbonyl moiety is a particularly important recognition unit for the antibacterial potency of these coumarin inhibitors and that introduction of structural diversity at the 3′ position of the L-deoxysugar could generate novel, potentially more potent, aminocoumarin antibiotics. For this reason, we have been interested in the characterization of the biosynthetic formation of the 5-methylpyrrolyl-2-carbonyl ring and its attachment to L-noviose, encoded by the seven genes *cloN1*−*7* and *couN1*−*7* in the clorobiocin and coumermycin A_1 biosynthetic gene clusters (Figure 1B).

We recently assigned the functions of the Clo/CouN3, Clo/CouN4, and Clo/CouN5 proteins that convert L-proline to the acyl-*S*-proteins pyrrolyl-*S*-Clo/CouN5 during clorobiocin and coumermycin A_1 biosynthesis (*6*). In that study, we showed by high-performance liquid chromatography (HPLC), radioactive assays, and electrospray ionization (ESI)−Fourier transform mass spectrometry (FTMS) that the adenylation (A) domains Clo/CouN4 transform L-proline into L-prolyl-

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[1] Abbreviations: A, adenylation; ATP, adenosine triphosphate; DH, dehydrogenase; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; IPTG, isopropryl-*β*-D-thiogalactopyranoside; MALDI−TOF, matrix-assisted laser desorption ionization time-of-flight; PCP, peptidyl carrier protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris-(2-carboxyethylphosphine); TFA, trifluoroacetic acid.

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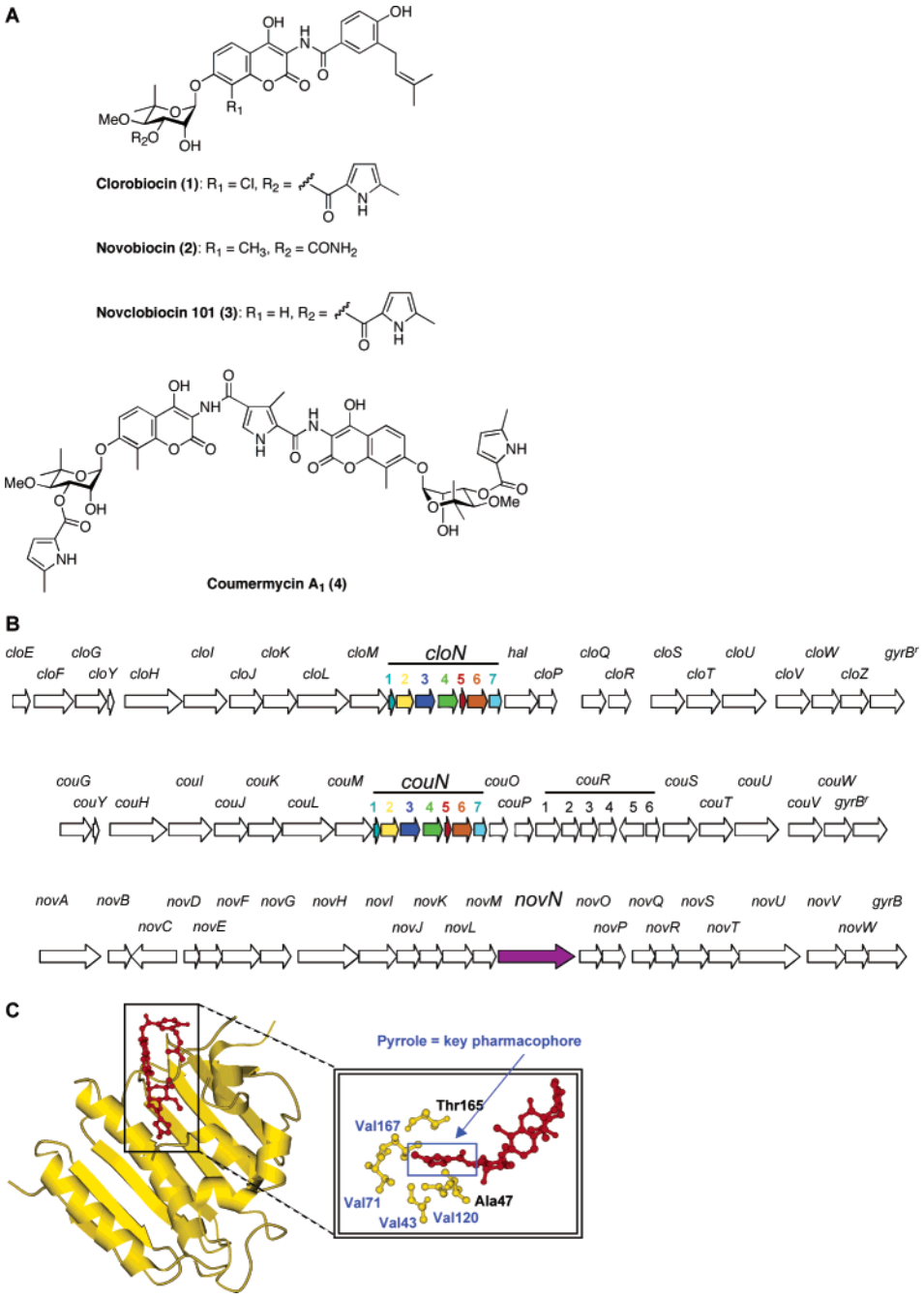
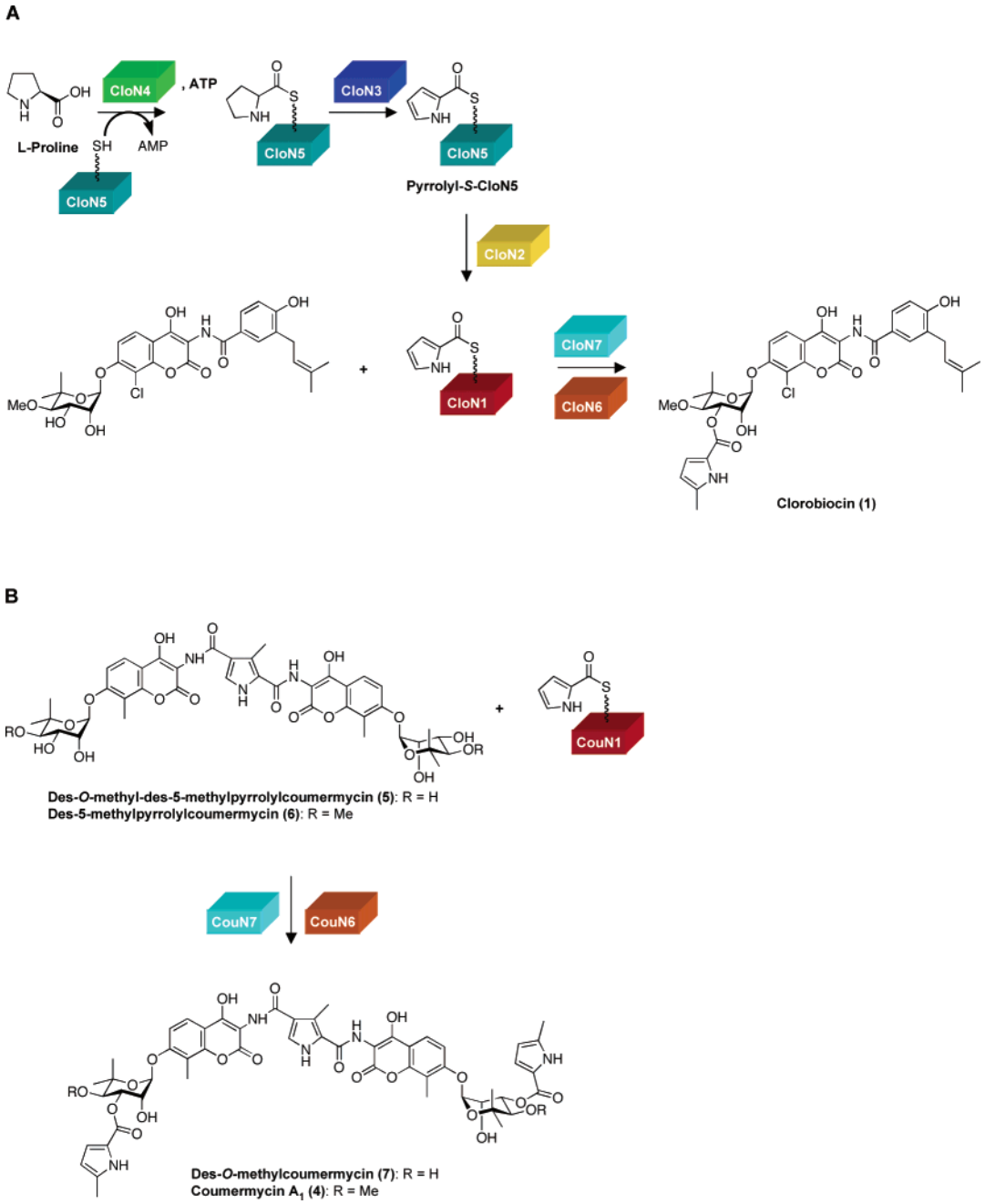


FIGURE 1: (A) Aminocoumarin family of antibiotics: clorobiocin, novobiocin, novclobiocin 101, and coumermycin A_1 structures and (B) gene clusters. (C) Clorobiocin bound to GyrB.

AMP prior to its covalent attachment as a phosphopantetheinyl thioester onto the holo peptidyl carrier proteins (PCPs) Clo/CouN5. The pyrrolyl-*S*-PCP is then formed from L-prolyl-*S*-PCP by two successive two-electron oxidations by the dehydrogenase (DH) flavoenzyme CloN3 (Scheme 1A).

Early work by Xu et al. proposed that the pyrrolyl group is then transferred directly from the N5 PCPs to the L-deoxysugar by the acyltransferases Clo/CouN2 (*7*). However, recent deletion studies of the genes *cloN1* and *cloN7* from the clorobiocin cluster (*8*) by Freitag and co-workers suggest additional intermediate steps of pyrrolyl-2-carboxyl acyl transfer and CloN1 as a second acyl carrier protein. In their scheme, during clorobiocin biosynthesis, the pyrrolyl moiety on CloN5 gets transferred first to the CloN1, acting as a carrier protein, by the action of the acyltransferase CloN2 prior to attachment to the 3′-hydroxyl of L-noviose by the action of the acyltransferase CloN7 and the last step in the pathway would then be C-methylation at position 5 by the methyltransferase CloN6 (Scheme 1A) (*9*). By analogy, the enzymes CouN1 and CouN7 are proposed to transfer the pyrrolyl moiety twice, to each of the 3′-OH of the two noviosyl sugars, during coumermycin A_1 biosynthesis (Scheme 1B) (*10*). The absence of orthologues of *cloN1* and *cloN7* genes in the novobiocin cluster (*11*) (Figure 1B) that contains a carbamoyl in place of the pyrrolyl functionality is consistent with the N1/N7 role in acyl transfer. In novobiocin maturation, NovN would serve the combined role of Clo/CouN2 and Clo/CouN7 in a single rather than sequential double acyl transfer step.

Scheme 1: (A) Proposed Clorobiocin Biosynthesis and (B) Proposed Late Steps of Coumermycin A_1 Biosynthesis Involving CouN1 and CouN7



To address directly the proposal of transfer of the pyrrolyl-2-carboxyl acyl moiety between carrier proteins N1 and N5 prior its to attachment to the L-noviosyl sugar by sequential action of acyltransferases N2 and N7, we determined to prepare the pyrrolyl-*S*-CouN1 protein thioester and evaluate it as a substrate for CouN7. This required the synthesis of pyrrolyl-coenzyme A (CoA), described in this paper, and its enzymatic acylation of CouN1 to serve as a potential substrate for CouN7. We also report the cloning and heterologous overproduction of both CouN1 and CouN7 in *Escherichia coli*, as well as their purification as *N*-His_6-tagged proteins (Figure 2). For initial validation of CouN7 as a pyrrolyl transfer catalyst, we have used as a cosubstrate scaffolds descarbamoylnovobiocin (**8**) (Scheme 2) and the des-*O*-methyl-des-5-methylpyrrolylcoumermycin (**5**) and des-5-methylpyrrolylcoumermycin (**6**) biosynthetic intermediates (Scheme 1B) (*12*).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Materials, and Instrumentation. Chemically competent *E. coli* TOP10 and BL21(DE3) cell strains were purchased from Invitrogen. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs. DNA primers for polymerase chain reaction (PCR) amplification were purchased from Integrated DNA Technologies. *Pfu* Turbo DNA polymerase was purchased from Stratagene. The pET28a overexpression vector was purchased from Novagen. The PPTase Sfp was expressed and purified as previously described (*13*). DNA sequencing and matrix-assisted laser desorption ionization

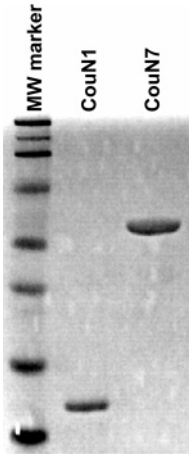


FIGURE 2: Tris (15%)/HCl SDS−PAGE of purified CouN1 and CouN7 detected by Coomassie Blue staining.

(MALDI) mass spectrometry were performed at the Dana Farber Cancer Institute. HPLC analysis of enzymatic reaction mixtures was carried out using a Beckman Gold Nouveau System Gold with a Vydac protein and peptide C18 column (250 × 4.6 mm) (for analysis of acylated PCPs) and a Vydac small-pore C18 column (250 × 4.6 mm) (for analysis of product formation by CouN7).

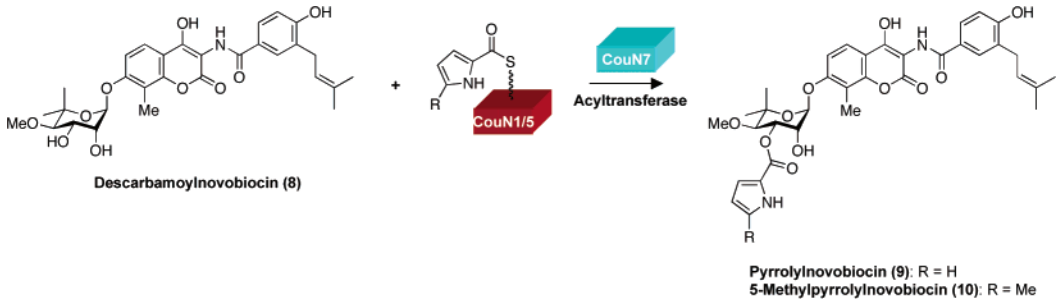
Preparation of pCouN1-pET28a, pCouN5-pET28a, and pCouN7-pET28a Overexpression Constructs. We previously reported the preparation of pCouN5-pET28a (*6*). The genes encoding CouN1 and CouN7 were PCR-amplified from genomic DNA isolated from *Streptomyces rishiriensis* (DSM 40489). The forward (5′-CTACGCCATATGGTCACTGGACGCACCGAGCTCGAG-3′) and reverse (5′-ATGGGCAAGCTTCTATGCCTCGGCAGCGGCCGCGG-3′)primers used for the amplification of the *couN1* gene introduced *Nde*I and *Hin*dIII restriction sites (underlined), respectively. For the amplification of the *couN7* gene, the forward (5′-GCATGACTCATATGGCGAACCACGGACCGG-3′) and reverse (5′-CGTGAATTCTTAGGGACCGTTGTGCGAGCGC-3′) primers used introduced *Nde*I and *Eco*RI restriction sites (underlined), respectively. PCRs were carried out using *Pfu* Turbo DNA polymerase as described by Stratagene. The amplified genes were inserted into the linearized pET28a vector via the corresponding *Nde*I/*Hin*dIII and *Nde*I/*Eco*RI restriction sites for CouN1 and CouN7, respectively. Expression of pCouN1-pET28a and pCouN7-pET28a was done following transformation into *E. coli* TOP10 competent cells. All expression clones were characterized by DNA sequencing (Dana Farber Cancer Institute) and compared to their corresponding gene sequence from *S. rishiriensis* strain DSM 40489 (GenBank entry AF235050).

Overproduction and Purification of CouN1, CouN5, and CouN7. We reported the purification of CouN5 in a previous study (*6*). Purified pCouN1-pET28a and pCouN7-pET28a plasmids were transformed into *E. coli* BL21(DE3) competent cells for production and purification. Transformants harboring the pCouN1-pET28a and pCouN7-pET28a constructs were grown under control of a T7 promoter in Luria−Bertani (LB) medium (6× 2 L batches) supplemented with kanamycin (50 μg mL^{-1}). All cells were grown at 25 °C to an OD_{600} of around 0.5. They were subsequently induced by the addition of isopropryl-β-D-thiogalactopyranoside (IPTG) (final concentration of 100−200 μM) and shaken for an additional 14 h at 25 °C. Cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C, Sorvall RC5B centrifuge, SLA-3000 rotor) and resuspended in buffer A [25 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0), 400 mM NaCl, and 10% (v/v) glycerol]. Resuspended cells were lysed (2 passes at 10 000−15 000 psi, Avestin EmulsiFlex-C5 high-pressure homogenizer), and the cell debris was removed by centrifugation (35 000 rpm, 30 min, 4 °C, Beckman L7 Ultracentrifuge, 45Ti rotor). Imidazole (final concentration of 2 mM) was added to the supernatant, which was then incubated with 4 mL of Ni−NTA agarose resin (Qiagen) at 4 °C for 2 h with gentle rocking. The resin was loaded onto a column and washed with 10 mL of buffer A containing 2 mM imidazole and with 10 mL of buffer A containing 5 mM imidazole. The desired proteins were eluted from the column in a stepwise imidazole gradient [10 mL fractions of 20, 40, 60, 200, and 500 mM (20 mL) imidazole]. Fractions containing the pure target proteins [as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)] were combined and dialyzed overnight at 4 °C against 2 L of buffer B [50 mM Tris-HCl (pH 8.0 adjusted at room temperature), 100 mM NaCl, 1 mM dithiothreitol (DTT), and 10% (v/v) glycerol]. A second dialysis was carried out for 4 h at 4 °C in 2 L of buffer B. Proteins were concentrated using either Amicon Ultra PL-10 or Amicon Ultra PL-5 for either CouN7 or CouN1, respectively. Proteins were flash-frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad).

Synthesis of Pyrrolyl-2-carboxyl-CoA and 5-Methylpyrrolyl-2-carboxyl-CoA: General Protocol. Ethyl 5-methyl-1*H*-pyrrole-2-carboxylate was synthesized by the method of Curran and Keaney (*14*) and subsequently hydrolyzed to the corresponding carboxylic acid (*15*) using 1:1 EtOH/2 N NaOH (aqueous). The coupling of pyrrolyl-2-carboxylic acid and 5-methylpyrrolyl-2-carboxylic acid to coenzyme A trilithium salt was achieved utilizing the following general procedure: To a round-bottom flask equipped with a reflux condenser, dry toluene (5 mL), pyrrolyl-2-carboxylic acid (0.1 g, 0.9 mmol, 1 equiv), and catalytic *N*,*N*-dimethylformamide (DMF, 7 μL, 0.09 mmol, 0.1 equiv) was treated with thionyl chloride (328 μL, 4.5 mmol, 5 equiv). The reaction was then heated at 70 °C for 1 h with stirring. This was then concentrated by rotary evaporation, and then dry toluene (5 mL) was added and concentrated. The residue was then put on a vacuum pump for 30 min. Crude 1*H*-pyrrolyl-2-carbonyl chloride (3.3 mg, 25.6 μmol, 1 equiv) was added to coenzyme A trilithium salt (20 mg, 25.6 μmol, 1 equiv) in 1:1 THF/H_2O (166 μL) containing pyridine (1.9 μL, 25.6 μmol, 1 equiv). The reaction was stirred for 1 h at room temperature. A total of 50 μL of this solution was diluted to 1 mL with H_2O and then injected onto an Agilent Zorbax 5 μm 300SB-C18 semi-prep HPLC column. The various pyrrolyl-2-carboxyl-*S*-coenzyme A's were eluted using a gradient of 0.1% trifluoroacetic acid (TFA) (aqueous) to 50% MeCN in H_2O (0.1% TFA) over 60 min at a flow rate of 5 mL min^{-1} with elution of the thioesters detected at 310 nm.

Pyrrolyl-2-carboxyl-S-CoA. HPLC: 16−17 min elution. ^{1}H NMR (D_2O, 400 MHz) δ: 8.67 (s, 1H), 8.37 (s, 1H), 7.15 (m, 1H), 7.02 (m, 1H), 6.28 (m, 1H), 6.19 (d, J = 5.2 Hz, 1H), 4.89 (m, 2H), 4.62 (m, 1H), 4.29 (m, 2H), 4.04 (s,

Scheme 2: Pyrrolylnovobiocin (**9**) and 5-Methylpyrrolylnovobiocin (**10**) Formation by Pyrrolyl Transfer from CouN1 to Descarbamoylnovobiocin (**8**) by the Action of CouN7



1H), 3.88 (dd, J = 2.4, 8.4 Hz, 1H), 3.62 (dd, J = 1.6, 9.2 Hz, 1H), 3.46 (t, J = 6.0 Hz, 2H), 3.42 (t, J = 6.0 Hz, 2H), 3.13 (t, J = 6.0 Hz, 2H), 2.45 (t, J = 6.0 Hz, 2H), 0.96 (s, 3H), 0.83 (s, 3H). HRMS [M + H]: calcd for $C_{26}H_{40}N_8O_{17}P_3S$, 861.1445; found, 861.1463.

5-Methylpyrrolyl-2-carboxylic Acid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 12.54 (br s, 1H), 8.86 (br s, 1H), 6.95 (t, J = 3.2 Hz, 1H), 6.00 (t, J = 3.2 Hz, 1H), 2.33 (s, 3H). ESI/MS [M − H]: calcd for $C_6H_6NO_2$, 124.1; found, 124.1.

5-Methylpyrrolyl-2-carboxyl-S-CoA. HPLC: 19−20 min elution. ^{1}H NMR (D_2O, 400 MHz) δ: 8.69 (s, 1H), 8.38 (s, 1H), 6.93 (d, J = 4.4 Hz, 1H), 6.20 (d, J = 5.6 Hz, 1H), 6.00 (d, J = 4.4 Hz, 1H), 4.89 (m, 2H), 4.62 (m, 1H), 4.29 (m, 2H), 4.06 (s, 1H), 3.89 (m, 1H), 3.61 (dd, J = 2.8, 9.4 Hz, 1H), 3.46 (t, J = 6.0 Hz, 2H), 3.41 (t, J = 6.4 Hz, 2H), 3.11 (t, J = 6.0 Hz, 2H), 2.46 (t, J = 6.0 Hz, 2H), 2.27 (s, 3H), 0.97 (s, 3H), 0.83 (s, 3H). HRMS [M + H]: calcd for $C_{27}H_{42}N_8O_{17}P_3S$, 875.1601; found, 875.1595.

HPLC Analysis of Purified Apo, Holo, Pyrrolyl-S-PCPs, and 5-Methylpyrrolyl-S-PCPs (CouN1 and CouN5). Apo-, holo-, pyrrolyl-*S*-PCPs, and 5-methylpyrrolyl-*S*-PCPs (CouN1 and CouN5) were separated by reversed-phase HPLC (Beckman System Gold) with a Vydac protein and peptide C18 column (250 × 4.6 mm) at a flow rate of 1 mL min^{-1}. The HPLC solvents were, for A, H_2O (0.1% TFA) and, for B, MeCN. The elution gradient was 20−60% B over 20 min followed by 60−100% B over 10 min. Product elution was monitored at 220 nm. The conversions from apo-PCP to holo-PCP and to pyrrolyl-*S*-PCP and 5-methylpyrrolyl-*S*-PCP were performed over 1 h at room temperature using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), tris-(2-carboxyethylphosphine) (TCEP) (pH 7.0) (1 mM), PCP (CouN1 or CouN5) (15 μM), Sfp (1 μM), and CoA (100 μM), pyrrolyl-CoA (100 μM), or 5-methylpyrrolyl-CoA (100 μM), respectively.

MALDI−Time-of-Flight (TOF) Mass Spectrometry. To determine the mass of apo, holo, pyrrolyl-*S*-PCPs, and 5-methylpyrrolyl-*S*-PCPs (CouN1 and CouN5), mass spectrometry analyses on HPLC-purified proteins (Zip Tip C4, Millipore) were performed with a linear MALDI−TOF mass spectrometer. Samples were prepared by using α-sinapinic acid (10 mg mL^{-1} in 60% acetonitrile/H_2O) as the matrix. Cal3 (MH^+ = 5734.51, 12 361.96, and 16 952.27 Da) was used for calibration of the instrument, which was performed before each experiment.

Characterization of CouN7 Activity. Prior to the confirmation of the ability of CouN7 to catalyze the pyrrolyl and 5-methylpyrrolyl transfer to the novobiocin scaffold, the pyrrolyl-*S*-PCP and 5-methylpyrrolyl-*S*-PCP (CouN1 or CouN5) formations were achieved in 1 h at room temperature (52 μL total volume) using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), PCP (CouN1 or CouN5) (300 μM), Sfp (5 μM), and pyrrolyl-CoA or 5-methylpyrrolyl-CoA (500 μM). Initial reconstitution of CouN7 activity was then performed at room temperature in reactions (75 μL total volume) containing pyrrolyl-*S*-PCP or 5-methylpyrrolyl-*S*-PCP (208 μM), dimethylsulfoxide (DMSO) (5%), descarbamoylnovobiocin (**8**) (25 μM), bovine serum albumin (BSA) (1 mg mL^{-1}), $MgCl_2$ (10 mM), Tris-HCl (pH 7.5) (75 mM), ATP (4 mM), and CouN7 (25 μM). Each 75 μL aliquot was quenched with cold methanol (150 μL) at specific time points (0 min, 5 min, 15 min, 30 min, and 2 h) and kept a −20 °C for at least 30 min before centrifugation (13 000 rpm, 10 min, 4 °C) to remove precipitated proteins. Ammonium acetate (pH 3.5) (180 mM) was added to the supernatant prior to analysis by reversed-phase HPLC (Beckman System Gold) with a Vydac small-pore C18 column (250 × 4.6 mm) at a flow rate of 1.5 mL min^{-1}. The HPLC solvents were, for A, H_2O (0.1% TFA) and, for B, MeCN. The elution gradient was 40−60% B over 20 min followed by 60−100% B over 5 min. Product elution was monitored at 340 nm. The CouN7 products were confirmed by liquid chromatography−mass spectrometry (LCMS) [ESI calculated for pyrrolylnovobiocin (**9**) ($C_{35}H_{38}N_2O_{11}$), m/z 661.2 [M − H]$^-$; found, 661. ESI calculated for 5-methylpyrrolylnovobiocin (**10**) ($C_{36}H_{40}N_2O_{11}$), m/z 675.3 [M − H]$^-$; found, 675].

The pyrrolyl-*S*-CouN1, 5-methylpyrrolyl-*S*-CouN1, and descarbamoylnovobiocin (**8**) were used as variable substrates for the determination of kinetic parameters (K_m and k_{cat}). For the determination of the K_m and k_{cat} values for pyrrolyl-*S*-CouN1 and 5-methylpyrrolyl-*S*-CouN1, the concentration of the descarbamoylnovobiocin (**8**) was kept constant (200 μM) and the concentration of pyrrolyl-*S*-CouN1 and 5-methylpyrrolyl-*S*-CouN1 were varied (30, 30, 90, 120, 180, 250, and 285 μM). Each reaction (75 μL total volume) performed in the above-described reaction buffer was initiated by the addition of CouN7 (3 μM) and terminated after 35 min by quenching the reaction mixture with cold methanol (150 μL). The K_m value for descarbamoylnovobiocin (**8**) was determined at a constant concentration of pyrrolyl-*S*-CouN1 (140 μM) and over a range of descarbamoylnovobiocin concentrations (5, 10, 20, 30, 50, 70, and 90 μM). All reactions were quenched after 30 min. Each experiment was carried out in triplicate.

*Formation of Des-O-methyl-dipyrrolylcoumermycin (**13**) and Des-O-methylcoumermycin (**7**).* With des-*O*-methyl-des-5-methylpyrrolylcoumermycin (**5**) (25 μM), des-*O*-methyl-

Table 1: MALDI−TOF Mass Analysis of Purified Apo, Holo, and Loaded Proteins

protein	calculated $[M + H]^+$	calculated $[M + Na]^+$	observed $[M + H]^+$	observed $[M + Na]^+$
apo-CouN1[a]	12 299 (12 168)	12 321 (12 190)		(12 197)
holo-CouN1[a]	12 639 (12 508)	12 661 (12 530)	12 636	
CouN7[a]	32 864 (32 733)	32 886 (32 755)		(32 755)
apo-CouN5[a]	11 885 (11 754)	11 907 (11 776)	11 936 (11 726)	
holo-CouN5[a]	12 225 (12 094)	12 247 (12 116)	12 227 (12 070)	

[a] Missing the first methionine.

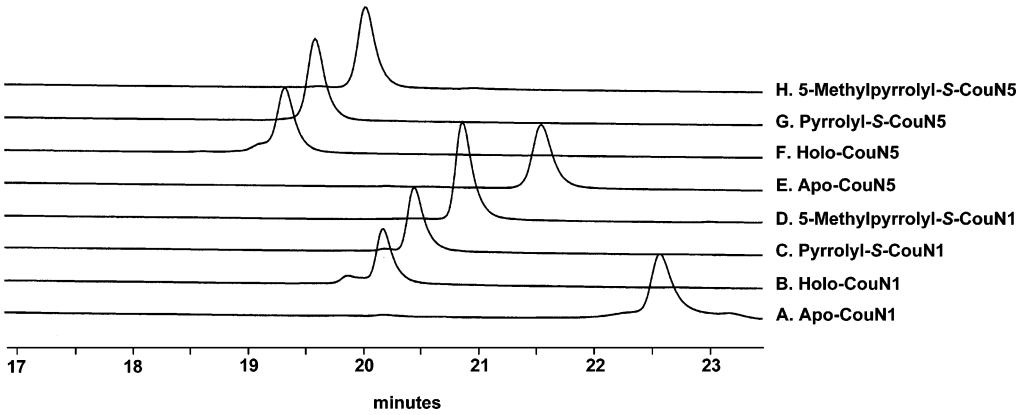


FIGURE 3: HPLC analysis of PCP domains. (A) Apo-CouN1 as purified from *E. coli*. (B) Holo-CouN1 resulting from Sfp modification of the apo-CouN1 with free CoA. (C) Pyrrolyl-*S*-CouN1 obtained by Sfp modification of the apo-CouN1 with pyrrolyl-CoA. (D) 5-Methylpyrrolyl-*S*-CouN1 obtained by Sfp modification of the apo-CouN1 with 5-methylpyrrolyl-CoA. (E) Apo-CouN5 as purified from *E. coli*. (F) Holo-CouN5 resulting from Sfp modification of the apo-CouN5 with free CoA. (G) Pyrrolyl-*S*-CouN5 obtained by Sfp modification of the apo-CouN5 with pyrrolyl-CoA. (H) 5-Methylpyrrolyl-*S*-CouN5 obtained by Sfp modification of the apo-CouN5 with 5-methylpyrrolyl-CoA.

dipyrrolylcoumermycin (**13**) and des-*O*-methylcoumermycin (**7**) were prepared using the procedure described for the preparation of pyrrolylnovobiocin (**9**) and 5-methylpyrrolylnovobiocin (**10**). The products were separated by reversed-phase HPLC (Beckman System Gold) with a Vydac small-pore C18 column (250 × 4.6 mm) at a flow rate of 1.0 mL min^{-1}. The HPLC solvents were, for A, H_2O (0.1% TFA) and, for B, MeCN. The elution gradient was 15−100% B over 30 min. Product elution was monitored at 340 nm. The identity of peaks c, d, f, and g in Figure 6A and peaks j, k, m, and o in Figure 6B remains to be determined.

RESULTS

Heterologous Expression, Overproduction, and Purification of the CouN1 and CouN7 Enzymes. To confirm the proposed roles for CouN1 and CouN7 proteins during coumermycin A_1 biosynthesis, we began with heterologous expression of the proteins in *E. coli* as *N*-His_6-tagged constructs. As shown in Figure 2, the 10.1 kDa CouN1 PCP and the 32.9 kDa acyltransferase CouN7 were purified to homogeneity after metal (Ni)-affinity chromatography. Yields varied between 8 and 12 mg L^{-1} of protein for both CouN1 and CouN7. Analysis of purified proteins by MALDI−TOF mass spectrometry (Table 1) provided molecular weights corresponding to the predicted molecular weights.

Characterization of the PCP Domains CouN1 and CouN5. The CouN1 protein is predicted to behave as a free-standing PCP domain because it bears high overall similarity with other PCPs around its proposed 4′-phosphopantetheine (4′-Ppant) attachment site ((**L**/I)**G**X**D**<u>**S**</u>(**L**/I)) (in bold are the amino acid residues found in CouN1, and underlined is the active binding site of 4′-Ppant) (*16*). Because of a lack of PCP-specific phosphopantetheinyl transferases in *E. coli* (*17*), CouN1 is overproduced in its expected apo (non-pantetheinylated inactive) form. However, it can be converted to its holo (active) form by attachment of a phosphopantetheinyl arm on a specific serine residue by the action of the well-established phosphopantetheinyl transferase Sfp (*18*) and CoASH as observed in Table 1, by an increase of 340 Da by MALDI−TOF mass spectrometry analysis and by a significant shift in HPLC mobility (lines A and B in Figure 3).

As noted in parts A and B of Scheme 1, it is proposed that during clorobiocin and coumermycin A_1 biosynthesis the pyrrolyl-2-carboxyl group gets transferred to Clo/CouN1 from Clo/CouN5 by the action of the acyltransferases Clo/CouN2. In the producing bacterial cell, the tandem action of five enzymes (Clo/CouN1, Clo/CouN2, Clo/CouN3, Clo/CouN4, and Clo/CouN5) is required to attain pyrrolyl-*S*-Clo/CouN1. This is a circuitous, low-yield path to the proposed substrate for Clo/CouN7, and further, we have been unable to produce either CouN2 or CloN2 in soluble, active form in *E. coli*. Therefore, we circumvented the five enzymes by using apo CouN1, Sfp, and synthetic pyrrolyl-*S*-CoA. The pyrrolyl-*S*-CouN1 species can easily be distinguished from apo-CouN1 by MALDI−TOF analysis and HPLC (Table 1 and line C in Figure 3). Because the proposed transfer of the pyrrolyl group from CouN5 to CouN1 by the acyltransferase CouN2 has not been yet characterized in vitro, there is a possibility that the pyrrolyl covalently tethered to CouN5 could get attached to the L-deoxysugar by the action of CouN7. For this reason, we also loaded the pyrrolyl moiety onto CouN5 by the action of Sfp and pyrrolyl-CoA. As shown in Table 1 and lines E−G in Figure 3, the conversion from apo-CouN5 to holo- and pyrrolyl-*S*-CouN5 is complete in 1 h.

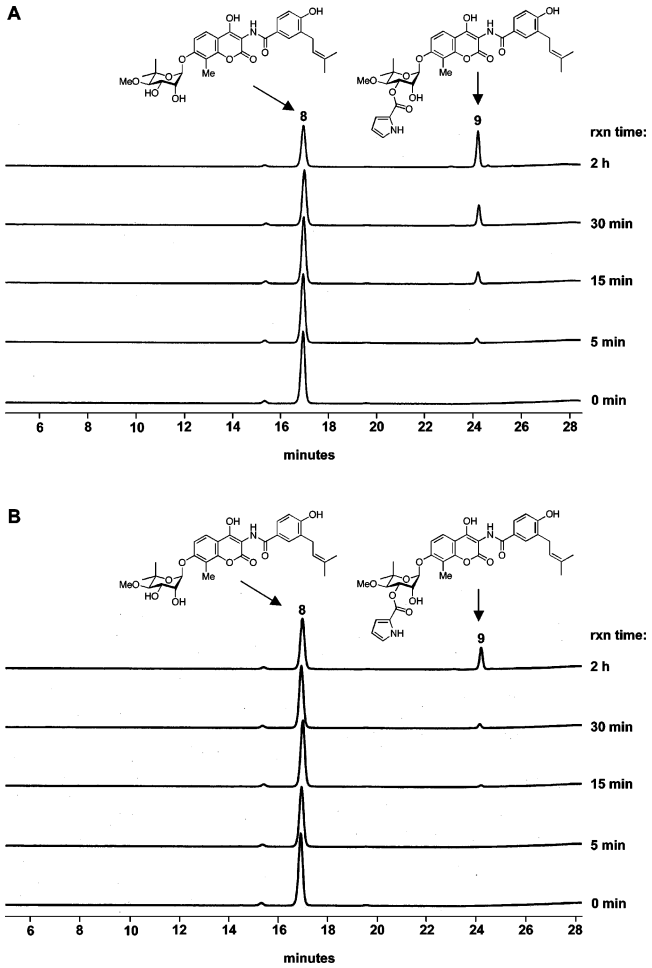


FIGURE 4: (A) Conversion of descarbamoylnovobiocin (**8**) into pyrrolylnovobiocin (**9**) at room temperature by the action of CouN7 in the presence of pyrrolyl-*S*-CouN1 and ATP at pH 7.5. (B) Conversion of descarbamoylnovobiocin (**8**) into pyrrolylnovobiocin (**9**) at room temperature by the action of CouN7 in the presence of pyrrolyl-*S*-CouN5 and ATP at pH 7.5.

Characterization of CouN7 Activity. In novobiocin biosynthesis, the carbamoyltransferase NovN is functionally homologous to CouN7. In NovN assays, in contrast to what would be believed, ATP is required for the reaction to proceed. In a similar manner, we originally used ATP in our CouN1/CouN7 reactions even though it has now been proven that ATP is not required for the reaction to work (data not shown). Previous in vitro investigations by our group (*12*, *19*) and the mutational approach by Xu and coworkers (*20*) into tailoring the noviosyl ring of the aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A_1 demonstrated that NovN transfers a carbamoyl group to the deoxysugar of a variety of coumarin substrates. While our in vitro attempts to incorporate other acyl groups by the action of NovN proved that the enzyme was narrowly specific for carbamoyl transfer, efforts with NovM and NovP, the two prior enzymes in the novobiocin pathway, gave us access (*19*) to sufficient descarbamoylnovobiocin (**8**) to test as an acceptor cosubstrate with CouN7. Initial experiments with pyrrolyl-*S*-CouN1 showed that CouN7 transfers the pyrrolyl-2-carbonyl moiety from CouN1 to descarbamoylnovobiocin to yield a new compound [pyrrolylnovobiocin (**9**)] (Figure 4A), whose formation was dependent upon both CouN1 and CouN7. Further confirmation of product formation was obtained by LCMS analysis (ESI for $C_{35}H_{38}N_2O_{11}$: calculated, *m*/*z* 661.2 $[M - H]^-$; found, 661) of the new species purified by HPLC.

To analyze if CouN7 interacts exclusively with CouN1, a similar reaction using pyrrolyl-*S*-CouN5 instead of its CouN1 counterpart was performed. Using pyrrolyl-*S*-CouN5 in conjunction with CouN7, the pyrrolylnovobiocin (**9**) product could also be detected by HPLC (Figure 4B). However, the reaction proceeded 3 times slower than with the CouN1 carrier protein.

Kinetic Characterization of CouN7. The kinetic parameters for CouN7 were determined by keeping the concentration of descarbamoylnovobiocin (**8**) constant at 200 μM. The K_m for descarbamoylnovobiocin was determined to be 60 μM by using pyrrolyl-*S*-CouN1 (data not shown). With the proposed natural substrate, pyrrolyl-*S*-CouN1, a typical hyperbolic curve was obtained by addition of CouN7 over varying pyrrolyl-*S*-CouN1 concentrations, providing a K_m of 510 μM for pyrrolyl-*S*-CouN1. The maximal rate for this reaction was observed to be 0.18 min^{-1} (data not shown). The kinetic parameters for CouN7 were also determined for 5-methylpyrrolyl-*S*-CouN1 (Figure 5). A K_m of 1.1 mM and k_{cat} of 0.73 min^{-1} were obtained for 5-methylpyrrolyl-*S*-CouN1. The identity of the product formed was confirmed by LCMS analysis (ESI calculated for $C_{36}H_{40}N_2O_{11}$, *m*/*z* 675.3 $[M - H]^-$; found, 675). The 5-methylpyrrolyl-2-carbonyl is a 2-fold better substrate by catalytic efficiency criterion (k_{cat}/K_m) than the pyrrolyl-2-carbonyl moiety. To what extent the CouN6-mediated C-methylation of the pyrrole ring occurs before CouN7 action (*9*) or afterward will require in vitro characterization of CouN6.

The descarbamoylnovobiocin scaffold is an artificial cosubstrate for CouN7 or CloN7. In our previous studies on in vitro reconstitution of the coumermycin A_1 pathway, tandem incubations of the ligase CouL, the glycosyltransferase CouM, and the noviosyl-*O*-methyltransferase CouP (*12*) with the appropriate intermediates generated the des-5-methylpyrrolyl-scaffold of coumermycin A_1 (**6**), where both 3′-*O*-5-methylpyrrolyl-2-carbonyl groups were absent. As noted in parts A and B of Figure 6, product **6** was used with pyrroyl-*S*-CouN1 (or 5-methylpyrrolyl-*S*-CouN1) to generate two new peaks in the HPLC traces corresponding to the des-*O*-methyl-monopyrrolylcoumermycin (**11** or **12**) and des-*O*-methyl-dipyrrolylcoumermycin (**13**) [or des-*O*-methyl-mono-5-methylpyrrolylcoumermycin (**14** or **15**) and des-*O*-methylcoumermycin (**7**)]. Incubations of the CouM product with CouP gave partial 3′-O-methylation to a mix of mono- and dimethyl products (line A in Figure 6C). Addition of 5-methylpyrrolyl-*S*-CouN1 and CouN7 generated new peaks in the HPLC trace (line B in Figure 6C). The product from the double acyl transfer shown in Scheme 1B is coumermycin A_1 as indicated by cochromatography with the authentic antibiotic (line C in Figure 6C).

DISCUSSION

A variety of biologically active natural products incorporate as key pharmacophore one or more pyrrolyl-2-carboxylate moieties into their scaffold. In contrast to the macrocycles in the heme, chlorophyll, and vitamin B_{12} families (*21*−*23*), where aminolevulinate is the precursor to the pyrrole units, the proteinogenic amino acid L-proline is the

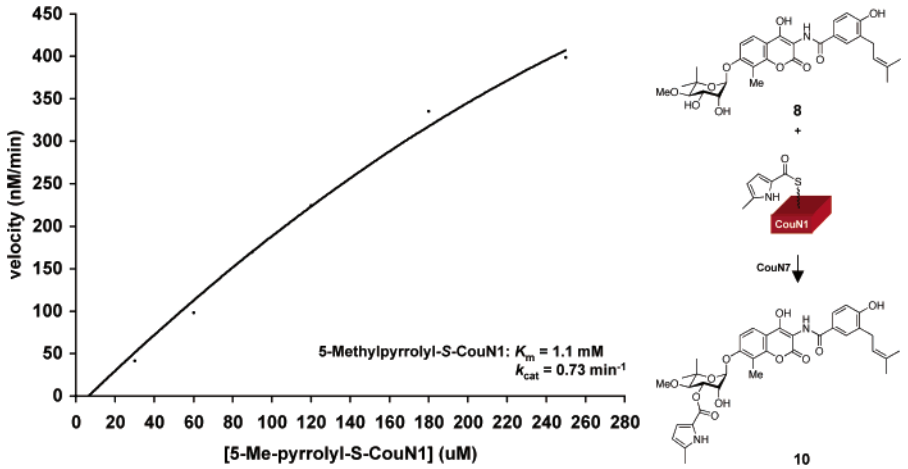


FIGURE 5: Michaelis−Menton kinetics for 5-methylpyrrolyl-*S*-CouN1.

source of the pyrrolyl-2-carboxylate moiety incorporated into a variety of secondary metabolites. The antifungal agents pyrrolnitrin (*24*, *25*) and pyoluteorin (*26*, *27*) contain mono- and dichlorinated pyrrole moieties, respectively. Both play important roles in the biocontrol activity of their producing *Pseudomonas* strains. The clinically relevant (antimicrobial, anticancer, antifungal, and immunosuppressive activity) red streptomycete metabolites undecylprodigiosin and prodigiosin have tripyrrole structures biosynthesized from a dipyrrole derived from L-proline (*28*, *29*). In the aminocoumarin antibiotics clorobiocin and coumermycin A_1, the 5-methylpyrrolyl-2-carbonyl moieties that sit in the ATP-binding site of the biological target GyrB are the most important units for potent inhibition. Understanding the enzymatic route by which the pyrrolyl-2-carboxyl functionality becomes attached to the L-deoxysugar would provide a promising entry point for late-stage diversification at this key position for these coumarin antibiotics.

In earlier studies (*6*, *26*, *29*, *30*), we have deciphered the logic that bacteria use to shunt a fraction of the intracellular pool of L-proline and other proteinogenic amino acids down conditional metabolic pathways to pyoluteorin and prodigiosin, as well as the pyrrolic acyl group that is a key pharmacophore in the GyrB subunit active site for the clorobiocin and coumermycin A_1 antibiotics. A three-step sequence using nonribosomal peptide synthetase-type enzymatic machinery is in play. L-Proline is activated by an adenylating enzyme to create L-prolyl-AMP held in the active site. The activated carboxyl group is captured by the holo form of a PCP via the terminal thiolate of the phosphopantetheinyl prosthetic group. The covalent tethering of the L-prolyl moiety as a pantetheinyl thioester to a 10 kDa PCP domain/subunit achieves two objectives: sequestering a fraction of the L-proline pool to be diverted into secondary metabolites and activating the carboxyl group for transfer to cosubstrate nucleophiles. The third protein is a flavoprotein dehydrogenase that carries out sequential two-electron oxidations of the L-prolyl-*S*-PCP to the pyrrolyl-*S*-PCP and does not act on free L-proline.

The determination of the gene clusters for all three aminocoumarins (novobiocin, clorobiocin, and coumermycin A_1) from their producing streptomycetes was achieved by Heide and co-workers (*8*, *10*, *11*) (Figure 1B). Inspection of these gene clusters revealed highly homologous open-reading frames (ORFs) for construction of the aminocoumarin scaffold, for the prenylated hydroxybenzoate piece, for noviosyl sugar construction and its transferase, and the regiospecific methyltransferase that decorates the 4′-OH of the noviosyl moiety. Novobiocin differs from clorobiocin and coumermycin in the identity of the pharmacophoric acyl group added to the 3′-OH of the noviosyl ring at the end of the pathway. A carbamoyl group is added by the single enzyme NovN as the last step in novobiocin assembly, with a gain of 200-fold in antibacterial potency. Instead, in clorobiocin and coumermycin A_1, a 5-methylpyrrolyl-2-carboxyl moiety is transferred to the noviosyl 3′-OH group, which leads to a 10-fold increase in antibiotic activity over novobiocin (*31*) (parts A and B of Scheme 1).

Heide and colleagues further noted that in place of the single gene *novN* there were seven adjacent genes in the *clo* and *cou* clusters that they designated *cloN1*−*7* and *couN1*−*7* (Figure 1B). It was reasonable to assume the seven encoded Clo and Cou proteins would be involved in pyrrolyl-2-carboxylate formation and would transfer to the noviosyl-aminocoumarin scaffolds. A series of careful genetic and in vivo studies by Heide and colleagues validated these expectations (*7*, *9*, *32*). Meanwhile, as noted above, we purified three of the ORFs N3, N4, and N5 from the Clo/Cou systems after expression in *E. coli* and established that they act to generate pyrrolyl-*S*-Clo/CouN5 (*6*). The remaining four ORFs are predicted to be two acyltransferases, N2 and N7, another PCP, N1, and a radical self-assembled monolayer (SAM) type of *C*-methyltransferase, N6.

In subsequent efforts, although our group can readily overproduce CloN2 or CouN2 heterologously, we have been unable to obtain them in soluble form in *E. coli* nor have we been able to refold the insoluble proteins to active catalysts despite many efforts and conditions. Thus, we were stymied in the last stages of reconstitution and enzymatic attachment of the activity-conferring pyrrolyl pharmacophore to the rest of the scaffold. The recent in vivo efforts by Heide and co-workers on knockouts in the producer organism (*9*) led them to propose that N7 was the acyltransferase carrying out the actual pyrrolyl-2-carboxyl attachment to the noviosyl ring and that the donor might be pyrrolyl-*S*-Cou/CloN1. Therefore, during clorobiocin and coumermycin A_1 biosynthesis, it is proposed that the second to last step consists of the introduction of the pyrrolyl-2-carboxyl moiety from

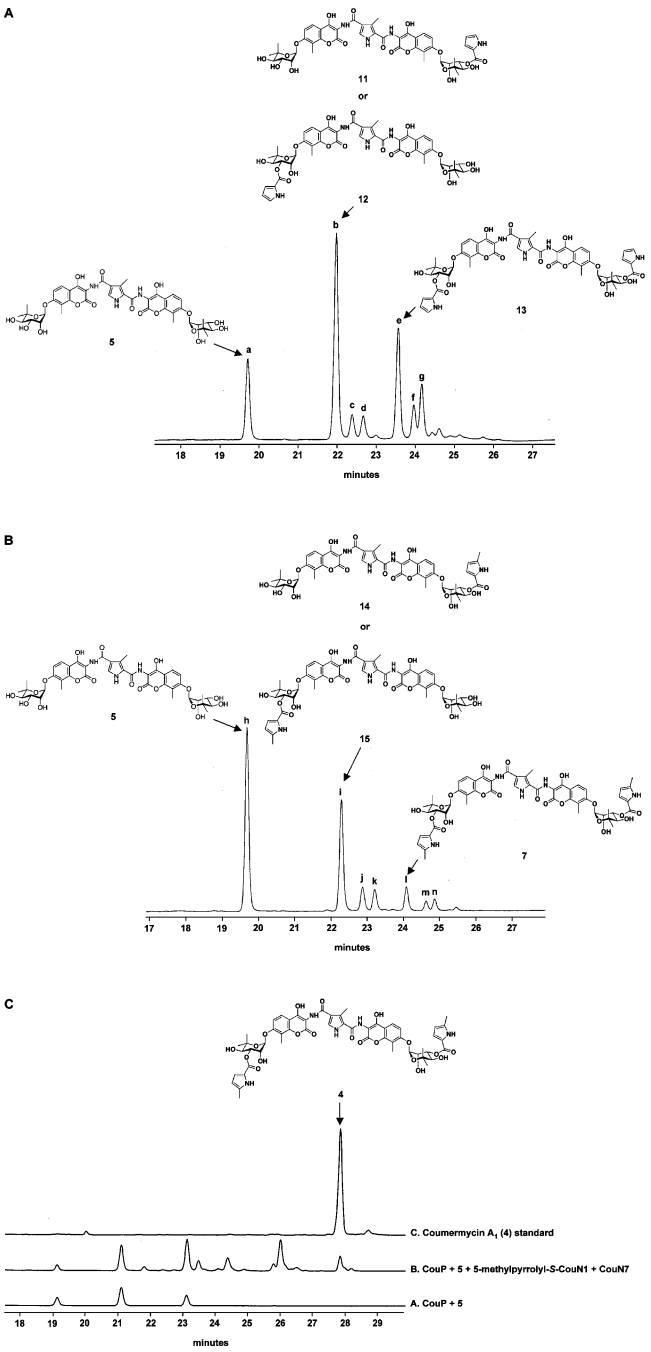


FIGURE 6: (A) Conversion of des-*O*-methyl-des-5-methylpyrrolylcoumermycin (**5**) into des-*O*-methyl-monopyrrolylcoumermycin (**11** or **12**) and des-*O*-methyl-dipyrrolylcoumermycin (**13**) at room temperature by the action of CouN7 in the presence of pyrrolyl-*S*-CouN1. (B) Conversion of des-*O*-methyl-des-5-methylpyrrolylcoumermycin (**5**) into des-*O*-methyl-mono-5-methylpyrrolylcoumermycin (**14** or **15**) and des-*O*-methylcoumermycin (**7**) at room temperature by the action of CouN7 in the presence of 5-methylpyrrolyl-*S*-CouN1. (C) Coumermycin A_1 (**4**) formation by the action of CouP and CouN7 on des-*O*-methyl-des-5-methylpyrrolylcoumermycin (**5**) and 5-methylpyrrolyl-*S*-CouN1.

pyrrolyl-*S*-CouN1 by the action of the acyltransferases (Clo/CouN7), which occurs immediately after noviosylation by the glycosyltransferases (CloM and CouM).

The studies that we report here validate this proposal by direct assays with the purified proteins CouN1 and CouN7, bypassing the recalcitrant CouN2 enzyme. The approach relies on our past development of methodologies to prime PCPs [and acyl carrier proteins (ACPs)] with a promiscuous phosphopantetheinyl transferase, Sfp from *B. subtilis* (*18*, *33*). Although Sfp is a dedicated PPTase for the apo forms of carrier protein domains in the surfactin nonribosomal peptide synthetase (NRPS) assembly line (*33*), it has shown high tolerance both for apo carrier proteins and transferring acyl moieties and has become a central reagent in NRPS and polyketide synthase (PKS) specificity studies.

The approach also relies on the ability to synthesize pyrrolyl-CoA and 5-methylpyrrolyl-CoA. While one might think that this a straightforward exercise in acyl activation chemistry, our earlier efforts to make pyrrolyl-2-carboxyl thioesters were thwarted by the electronic deactivating effect of the pyrrole ring on carboxylate reactivity (*34*). In the end, conditions involving nucleophilic catalysis promoted by pyridine, inspired by the Schotten−Baumann reaction, sufficed for reliable preparation of CoA thioesters of 2-carboxylpyrroles as noted in the Materials and Methods. Sfp, in turn, obliged at transferring the 5-methylpyrrolyl-2-carboxyl-*S*-pantetheinyl-*P* moiety to the side-chain CH_2OH of the active-site serine of the apo PCP in both CouN5 and CouN1 (lines D and H in Figure 3). This was the first demonstration that indeed CouN1 is a free-standing PCP, the second (along with CloN5/CouN5) in this seven-protein pathway for generating and transferring the five-membered ring heterocyclic acyl group. Additionally, it provides the donor substrate for assay of CouN7. Absent from this shortcut to pyrrolyl-*S*-CouN1, provided by pyrrolyl-CoA and Sfp, this substrate is only accessible by the prior action of CouN4 and CouN3 on primed CouN5 and transfer by the unavailable CouN2, noted above.

To assay CouN7 required not only the pyrrolyl-*S*-CouN1 donor but also a noviosyl-containing acceptor substrate. Although it is clorobiocin and coumermycin that bear this acyl group in the natural antibiotics, Heide and colleagues have shown by in vivo gene replacement that a novclobiocin 101 (**3**) can be obtained by fermentations, indicating that the CloN1−7 machinery is compatible with a novobiocin-like scaffold (*9*). Therefore, we first utilized the descarbamoylnovobocin (**8**), which we have obtained in prior studies (*19*), as a potential acceptor with the 4-methoxy-noviosyl group presented on the coumarin scaffold. Indeed, purified CouN7 shows robust pyrrolyl-2-carboxyl acyl transfer activity, validating its catalytic role and the pathway predictions made by the Heide group from their in vivo efforts (*9*), even though Clo/CouN2 has yet to be assayed in an active form. We have also carried out initial studies with the less accessible des-*O*-methyldesacyl coumermycin scaffold (**5**) to show initial mono- and bisacylation with both the pyrrolyl-2-carboxyl and 5-methylpyrrolyl-2-carboxyl forms of CouN1.

The last of the seven ORFs in the L-proline to pyrrole arm of the coumarin antibiotic biosynthetic pathway yet uncharacterized is Clo/CouN6, the proposed *C*-methyltransferase for the pyrrolyl-2-carboxyl unit. It has been unclear if CouN6 carries out its putative radical C−C coupling before or after CouN7 has attached the pyrrolyl-2-carboxyl unit to the 3′-hydroxyl of the novoisyl ring. In this regard, the pyrrolyl-CoA/Sfp technology allows for the synthesis of 5-methylpyrrolyl-CoA and its covalent tethering to CouN1. As shown in this paper, CouN7 is quite efficient at accepting the 5-methylpyrrolyl unit and thereby completes the antibiotic pathway. This alone does not resolve when Clo/CouN6 operates in the producing streptomycetes, but it does open

the door for enzymatic diversification of both the pharmacophoric heterocycle and the aminocoumarin scaffold at the last step of antibiotic formation. We note that the two products of CouN7 action described here are methylated analogues of novclobiocin 101 and clorobiocin in which the 8 position of the coumarin nucleus is CH_3 (as found in novobiocin and coumermycin) rather than the H and Cl substituents of novclobiocin 101 and clorobiocin, respectively. Similar variation in the hetrocycle of both arms of coumermycin A_1 should be accessible by the action of CouN7 with different acyl-*S*-CouN1 donors.

The logic of the seven *Clo/CouN1*−*7* genes seems now to be revealed, for recruitment of L-proline and shunting it through a series of carrier protein thioester intermediates for eventual regioselective transfer of the aromatized five-ring unit to the aminocoumarin scaffold. This arm of the pathway completes assembly of the four-component parts in clorobiocin and the five parts of the coumermycin scaffold. Most notably, it installs the key heterocyclic specificity determinant for high-affinity interaction with the B subunit of bacterial DNA gyrase. It is not fully clear why there are two free-standing PCPs (N5 and N1) and two acyltransferases (N2 and N7) when one of each might have sufficed. This may reflect evolving differential recognition of the free-standing carrier proteins by their cognate acyltransferases. We have noted a comparable logic for sequential use of two PCPs in the coronamic acid biosynthetic pathway with a dedicated acyltransferase (CmaE) for shuttling the aminoacyl group to the second PCP, for subsequent recognition by a nonheme chlorinase CmaB (*35*).

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REFERENCES

1. Maxwell, A., and Lawson, D. M. (2003) The ATP-binding site of type II topoisomerases as a target for antibacterial drugs, *Curr. Top. Med. Chem. 3*, 283−303.
2. Maxwell, A. (1999) DNA gyrase as a drug target, *Biochem. Soc. Trans. 27*, 48−53.
3. Levine, C., Hiasa, H., and Marians, K. J. (1998) DNA gyrase and topoisomerase IV: Biochemical activities, physiological roles during chromosome replication, and drug sensitivities, *Biochim. Biophys. Acta 1400*, 29−43.
4. Tsai, F. T. F., Singh, O. M. P., Skarzynski, T., Wonacott, A. J., Weston, S., Tucker, A., Pauptit, R. A., Breeze, A. L., Poyser, J. P., O'Brien, R., Ladbury, J. E., and Wigley, D. B. (1997) The high-resolution crystal structure of a 24-kDa gyrase B fragment from *E. coli* complexed with one of the most potent coumarin inhibitors, clorobiocin, *Proteins: Struct., Funct., Genet. 28*, 41−52.
5. Hooper, D. C., Wolfson, J. S., McHugh, G. L., Winters, M. B., and Swartz, M. N. (1982) Effects of novobiocin, coumermycin A1, clorobiocin, and their analogs on *Escherichia coli* DNA gyrase and bacterial growth, *Antimicrob. Agents Chemother. 22*, 662−671.
6. Garneau, S., Dorrestein, P. C., Kelleher, N. L., and Walsh, C. T. (2005) Characterization of the formation of the pyrrole moiety during clorobiocin and coumermycin A_1 biosynthesis, *Biochemistry 44*, 2770−2780.
7. Xu, H., Kahlich, R., Kammerer, B., Heide, L., and Li, S.-M. (2003) CloN2, a novel acyltransferase involved in attachment of the pyrrole-2-carboxyl moiety to the deoxysugar of clorobiocin, *Microbiology 149*, 2183−2191.
8. Pojer, F., Li, S.-M., and Heide, L. (2002) Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: New insights into the biosynthesis of aminocoumarin antibiotics, *Microbiology 148*, 3901−3911.
9. Freitag, A., Wemakor, E., Li, S.-M., and Heide, L. (2005) Acyl transfer in clorobiocin biosynthesis: Involvement of several proteins in the transfer of the pyrrole-2-carboxyl moiety to the deoxysugar, *ChemBioChem 6*, 2316−2325.
10. Wang, Z.-X., Li, S.-M., and Heide, L. (2000) Identification of the coumermycin A_1 biosynthetic gene cluster of *Streptomyces rishiriensis* DSM 40489, *Antimicrob. Agents Chemother. 44*, 3040−3048.
11. Steffensky, M., Mhlenweg, A., Wang, Z.-X., Li, S.-M., and Heide, L. (2000) Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891, *Antimicrob. Agents Chemother. 275*, 1214−1222.
12. Freel Meyers, C. L., Oberthr, M., Heide, L., Kahne, D., and Walsh, C. T. (2004) Assembly of dimeric variants of coumermycins by tandem action of the four biosynthetic enzymes CouL, CouM, CouP, and NovN, *BIochemistry 43*, 15022−15036.
13. Quadri, L. E. N., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases, *Biochemistry 37*, 1585−1595.
14. Curran, T. P., and Keaney, M. T. (1996) A novel pyrrole synthesis: One-pot preparation of ethyl 5-methylpyrrole-2-carboxylate, *J. Org. Chem. 61*, 9068−9069.
15. Olson, S. H., and Slossberg, L. H. (2003) Synthesis of coumermycin A_1, *Tetrahedron Lett. 44*, 61−63.
16. Stachelhaus, T., Hser, A., and Marahiel, M. A. (1996) Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases, *Chem. Biol. 3*, 913−921.
17. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) A new enzyme superfamily—The phosphopantetheinyl transferases, *Chem. Biol. 3*, 923−936.
18. Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases, *Biochemistry 37*, 1585−1595.
19. Freel Meyers, C. L., Oberthr, M., Xu, H., Heide, L., Kahne, D., and Walsh, C. T. (2004) Characterization of NovP and NovN: Completion of novobiocin biosynthesis by sequential tailoring of the noviosyl ring, *Angew. Chem., Int. Ed. 43*, 67−70.
20. Xu, H., Heide, L., and Li, S.-M. (2004) New aminocoumarin antibiotics formed by a combined mutational and chemoenzymatic approch utilizing the carbamoyltransferase NovN, *Chem. Biol. 11*, 655−662.
21. Jordan, P. M. (1991) In *Biosynthesis of Tetrapyrroles* (Jordan, P. M., Ed.) Vol. 19, pp 1−66, Elsevier, Amsterdam, The Netherlands.
22. Beale, S. I., and Weinstein, J. D. (1991) In *Biosynthesis of Tetrapyrroles* (Jordan, P. M., Ed.) Vol. 19, pp 155−236, Elsevier, Amsterdam, The Netherlands.
23. Friedmann, H. C., Klein, A., and Thauer, R. K. (1991) In *Biosynthesis of Tetrapyrroles* (Jordan, P. M., Ed.) Vol. 19, pp 139−154, Elsevier, Amsterdam, The Netherlands.
24. Dong, C., Flecks, S., Unversucht, S., Haupt, C., van Pee, K. H., and Naismith, J. H. (2005) Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination, *Science 309*, 2216−2219.
25. van Pee, K. H., and Ligon, J. M. (2000) Biosynthesis of pyrrolnitrin and other phenylpyrrole derivatives by bacteria, *Nat. Prod. Rep. 17*, 157−164.
26. Dorrestein, P. C., Yeh, E., Garneau-Tsodikova, S., Kelleher, N. L., and Walsh, C. T. (2005) Dichlorination of a pyrrolyl-*S*-carrier protein by $FADH_2$-dependent halogenase PltA during pyoluteorin biosynthesis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 13843−13848.
27. Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J., and Loper, J. E. (1999) Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5, *J. Bacteriol. 181*, 2166−2174.
28. Williamson, N. R., Simonsen, H. T., Ahmed, R. A. A., Goldet, G., Slater, H., Woodley, L., Leeper, F. J., and Salmond, G. P. C. (2005) Biosynthesis of the red antibiotic, prodigiosin, in *Serratia*: Identification of a novel 2-methyl-3-amyl pyrrole (MAP) assembly pathway, definition of the terminal condensing enzyme, and implications for undecylprodigiosin biosynthesis in *Streptomyces*, *Mol. Microbiol. 56*, 971−989.

29. Garneau-Tsodikova, S., Dorrestein, P. C., Thomas, P., Kelleher, N. L., and Walsh, C. T. (2006) Characterizaton of the formation of the dipyrrole moiety during prodigiosin biosynthesis, manuscript submitted for publication.
30. Dorrestein, P. C., Blackhall, J., Straight, P. D., Fischbach, M. A., Garneau-Tsodikova, S., Edwards, D. J., McLaughlin, S., Lin, M., Gerwick, W. H., Kolter, R., Walsh, C. T., and Kelleher, N. L. (2006) Activity screening of carrier domains within nonribosomal peptide synthetases using complex substrate mixtures and large molecule mass spectrometry, *Biochemistry 45*, 1537−1546.
31. Gellert, M., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1976) Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase, *Proc. Natl. Acad. Sci. U.S.A. 73*, 4474−4478.
32. Xu, H., Wang, Z.-X., Schmidt, J., Heide, L., and Li, S.-M. (2002) Genetic analysis of the biosynthesis of the pyrrole and carbamoyl moieties of coumermycin A_1 and novobiocin, *Mol. Gen. Genet. 268*, 387−396.
33. Weinreb, P. H., Quadri, L. E., Walsh, C. T., and Zuber, P. (1998) Stoichiometry and specificity of *in vitro* phosphopantethinylation and aminoacylation of the valine-activating module of surfactin synthetase, *Biochemistry 37*, 1575−1584.
34. Thomas, M. G., Burkart, M. D., and Walsh, C. T. (2002) Conversion of L-proline to pyrrolyl-2-*S*-PCP during undecylprodigiosin and pyoluteorin biosynthesis, *Chem. Biol. 9*, 171−184.
35. Vaillancourt, F. H., Yeh, E., Vosburg, D. A., O'Connor, S. E., and Walsh, C. T. (2005) Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis, *Nature 436*, 1191−1194.

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