Chemoenzymatic Formation of Novel Aminocoumarin Antibiotics by the Enzymes CouN1 and CouN7†

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ABSTRACT: The aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A_1 are highly potent inhibitors of the bacterial type II topoisomerase DNA gyrase. The key pharmacophore of both clorobiocin and coumermycin A_1, the 5-methyl-2-pyrrolylcarbonyl moiety, targets the ATP-binding site of GyrB. The 5-methyl-2-pyrrolylcarbonyl group is transferred by the acyltransferases Clo/CouN7 from the carrier proteins Clo/CouN1 to the 3′-hydroxyl of the L-noviosyl scaffold during the late steps of clorobiocin and coumermycin A_1 biosynthesis. We first examined the substrate specificity of the purified thiolation domain protein CouN1 in becoming primed by the phosphopantetheinyltransferase Sfp using a variety of synthetic CoA analogues of the 5-methyl-2-pyrrolylcarbonyl moiety. The acyl-*S*-CouN1 thioesters were then assayed as donors to the 3′-OH group of descarbamoylnovobiocin by the acyltransferase CouN7, resulting in 21 novel variants with heterocyclic acyl groups installed on the noviosyl moiety of the aminocoumarin scaffold. Scaleup of a 5-methylthiophene derivative yielded a compound with activity against both Gram-negative and Gram-positive bacteria. The minimal inhibitory concentration found for the Gram-positive bacteria was comparable to that of novobiocin.

The three members of the aminocoumarin family of antibiotics, novobiocin (**1**), clorobiocin (**2**), and coumermycin A_1 (**3**), competitively target the ATP-binding site of the GyrB subunit of the bacterial type II topoisomerase DNA gyrase (*1*−*3*). DNA gyrase, a heterotetramer (A_2B_2) composed of two subunits, GyrA (97 kDa) and GyrB (90 kDa), is required for DNA replication and for catalysis of biochemical reactions that control the topology of DNA during transcription (*4*). The fluoroquinolone class of antibiotics (e.g., ciproflaxin) that target the A subunits (*5*) of this enzyme has been widely used in the clinic. However, due to their low solubility, poor cell wall penetration, and poor pharmacokinetics, the clinical use of aminocoumarin antibiotics has been restricted (*6*). In the past 10 years, interest in novobiocin has been renewed as a result of its potent activity against bacterial strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (*7*). With the gene sequence of the novobiocin (*8*), clorobiocin (*9*), and coumermycin A_1 (*10*) clusters determined, it is now possible to optimize the aminocoumarin antibiotics by structural variations introduced enzymatically.

The aminocoumarins (Figure 1) contain three structural elements: a central 3-amino-7-hydroxycoumarin that is linked at its 3-amino group to a prenylated 4-hydroxybenzoic acid moiety (novobiocin and clorobiocin) or a 3-methylpyrrole-2,4-dicarboxylic acid moiety (coumermycin) and at its 7-position to a L-noviosyl sugar. In novobiocin, the 3′-hydroxyl position of the 4-methoxy-L-noviosyl ring is carbamoylated, whereas in clorobiocin and coumermycin A_1, it is acylated by a 5-methyl-2-pyrrolylcarbonyl moiety. Studies have shown that this structural modification results in an enhanced gyrase inhibitory activity of clorobiocin over novobiocin (*11*). This suggests that the acyl group attached to the 3′-hydroxyl position of the L-noviosyl sugar plays a crucial role in recognition and interaction with the gyrase enzyme and is therefore a key pharmacophore for antibacterial potency of these aminocoumarin inhibitors. For this reason, we have been interested in introducing structural diversity at the 3′ position of the noviosyl ring to generate novel, potentially more potent, coumarin antibiotics.

We have previously reported the characterization of the three enzymes (Clo/CouN3−5) involved in the formation of the pyrrolyl-2-carboxyl moiety from L-proline during clorobiocin and coumermycin A_1 biosynthesis (Scheme 1A) (*12*). We established that after activation by the adenylation domain Clo/CouN4, L-proline becomes covalently attached to the phosphopantetheinylated thiolation domain Clo/CouN5 where it is oxidized to pyrrolyl-*S*-Clo/CouN5 by the flavoenzyme Clo/CouN3. We recently assigned the functions of the CouN1 and CouN7 proteins involved in transfer of the pyrrolyl moiety during the late stage of coumermycin

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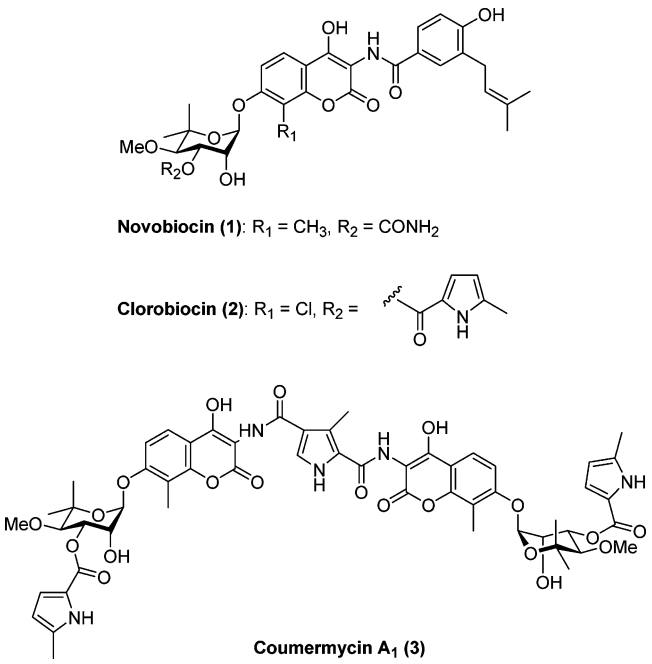


FIGURE 1: Members of the aminocoumarin family of antibiotics.

A_1 biosynthesis (Scheme 1A) (*13*). In that study, we showed that transfer of the pyrrolyl group from the carrier protein CouN5 to the thiolation domain CouN1 is required for its transfer to the L-noviosyl ring by the acyltransferase CouN7. The results of that work also suggest that the methylation of the pyrrolyl moiety by Cou/CloN6 during clorobiocin and coumermycin A_1 formation could occur prior to its transfer to the sugar by action of the N7 enzyme.

With the initial validation that acylated CouN1 serves as a substrate for the acyl transfer catalyst CouN7, we are now ready to examine potential promiscuity of the N1/N7 protein pair for the generation of new aminocoumarin antibiotics (Scheme 1B). In this study, we focus on the biosynthesis of novobiocin analogues. We chose the novobiocin scaffold due to its potency against MRSA bacterial strains and the ease of access to descarbamoylnovobiocin (**4**). We report herein the chemical synthesis of a number of coenzyme A (CoA) substrates and their enzymatic phosphopantetheinylation of apo-CouN1. Using these donor substrates to probe the specificity of CouN7, 21 novel novobiocin analogues were generated by the action of the N1/N7 pair on descarbamoylnovobiocin (**4**) (Scheme 1B). As a model compound, one of the derivatives was scaled up to milligram quantities for the assessment of antibiotic effects. The 5-methylthiophene derivative exhibited activity toward both Gram-negative and Gram-positive bacteria, the latter of which was comparable to that of novobiocin.

MATERIALS AND METHODS

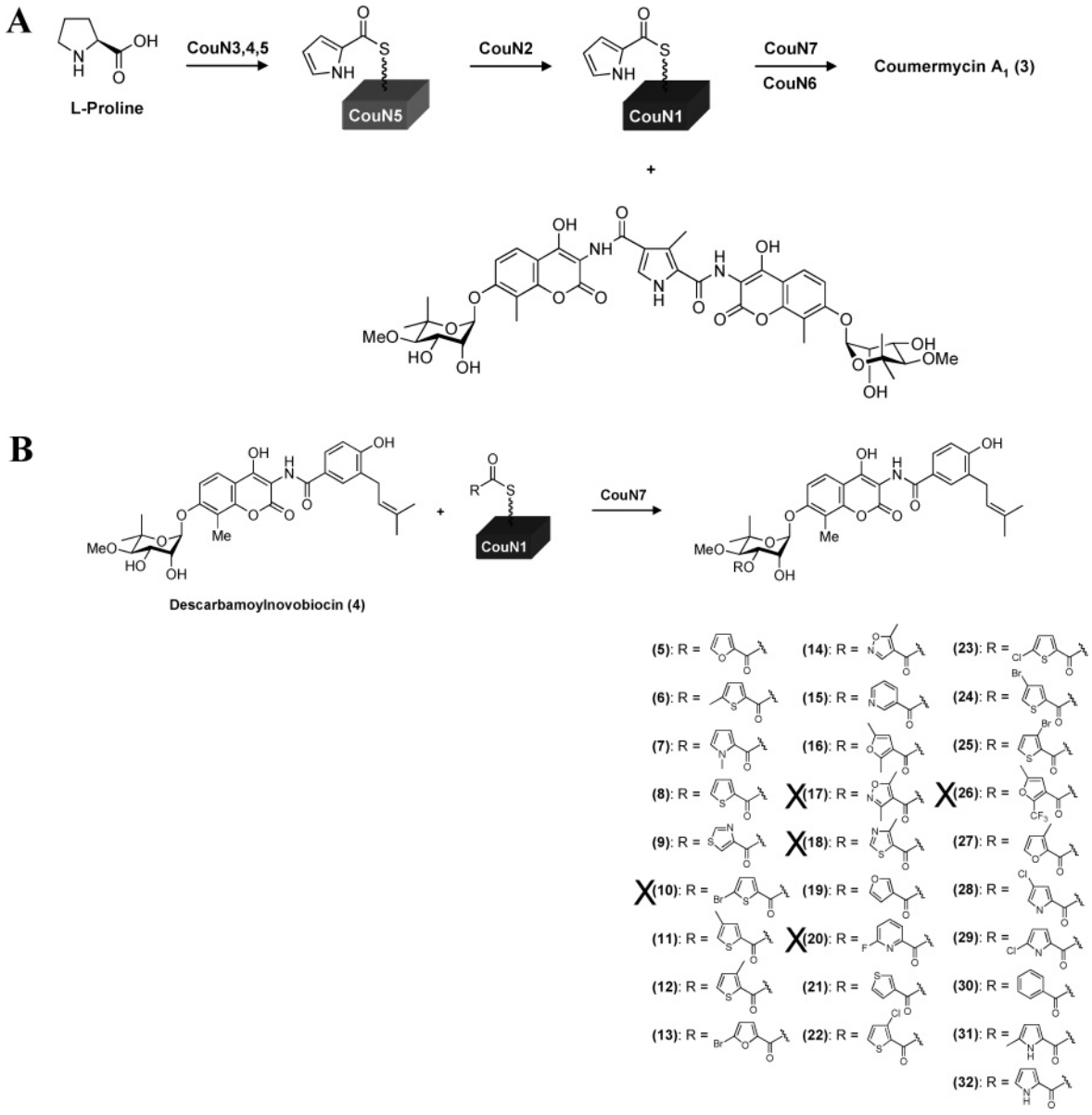
Bacterial Strains, Plasmids, Materials, and Instrumentation. The preparation, overexpression, and purification of the pCouN1-pET28a and pCouN7-pET28a constructs utilized in this study were recently reported (*13*). The PPTase Sfp was expressed and purified as previously described (*14*). The 4-chloropyrrolyl-2-carbonyl-CoA and 5-chloropyrrolyl-2-carbonyl-CoA were generously provided by A. Stapon (D. Kahne's laboratory, Harvard Medical School). They were synthesized from 4-chloropyrrolyl-2-carboxylic acid and 5-chloropyrrolyl-2-carboxylic acid (*15*) using the method described in our previous report on CouN1/CouN7 (*13*). 4-Chloropyrrolyl-2-carbonyl-CoA: ^{1}H NMR (400 MHz, D_2O) δ 8.66 (s, 1H, H-1), 8.39 (s, 1H, H-2), 7.07 (d, J = 1.6 Hz, 1H, H-16), 6.89 (d, J = 1.6 Hz, 1H, H-15), 6.18 (d, J = 5.6 Hz, 1H, H-3), 4.88 (m, 2H, H-4, H-5), 4.62 (m, 1H, H-6), 4.30 (m, 2H, H-8), 4.05 (s, 1H, H-10), 3.89 (m, 1H, H-7), 3.63 (dd, J_1 = 4.4 Hz, J_2 = 9.4 Hz, 1H, H-7), 3.45 (m, 4H, H-11, H-13), 3.14 (m, 2H, H-14), 2.46 (dd, $J_1 = J_2$ = 6.4 Hz, 2H, H-12), 0.97 (s, 3H, H-9), 0.84 (s, 3H, H-9); positive HRMS (ESI) for $C_{26}H_{38}ClN_8O_{18}P_3S$ 895.1046 [M + H]$^+$, calcd 895.1055. 5-Chloropyrrolyl-2-carbonyl-CoA: ^{1}H NMR (400 MHz, D_2O) δ 8.69 (s, 1H, H-1), 8.40 (s, 1H, H-2), 6.96 (d, J = 4 Hz, 1H, H-16), 6.20 (d, J = 5.6 Hz, 1H, H-15), 6.16 (d, J = 4 Hz, 1H, H-3), 4.89 (m, 2H, H-4, H-5), 4.62 (m, 1H, H-6), 4.28 (m, 2H, H-8), 4.06 (s, 1H, H-10), 3.89 (dd, J_1 = 4.4 Hz, J_2 = 8.8 Hz, 1H, H-7), 3.61 (dd, J_1 = 4.4 Hz, J_2 = 9.4 Hz, 1H, H-7), 3.45 (m, 4H, H-11, H-13), 3.13 (m, 2H, H-14), 2.46 (dd, $J_1 = J_2$ = 6.4 Hz, 2H, H-12), 0.97 (s, 3H, H-9), 0.84 (s, 3H, H-9); positive HRMS (ESI) for $C_{26}H_{38}ClN_8O_{18}P_3S$ 895.1034 [M + H]$^+$, calcd 895.1055. Benzoyl-CoA was purchased from Sigma-Aldrich. MALDI-TOF[1] mass spectrometry analyses were carried out at the Dana Farber Cancer Institute. HPLC analyses of enzymatic reaction mixtures were conducted using a Beckman Gold Nouveau System Gold with a Vydac protein and peptide C_{18} column (250 mm × 4.6 mm) (for analysis of acylated CouN1) and a Vydac small pore C_{18} column (250 mm × 4.6 mm) (for analysis of new novobiocin analogues). Preparative HPLC was performed on a Hitachi L6200 instrument using a Phenomenex Luna 5 μm C18 column (250 mm × 21.2 mm). Characterization of the CoA substrates was accomplished as follows. Low-resolution mass spectra (LRESIMS) were obtained on an Agilent Technolgies LC/MSD instrument (model G1956B) using electrospray ionization (ESI), while high-resolution mass spectra (HRESIMS) were obtained at the Harvard University Mass Spectrometry Facilities. NMR spectra were recorded on a Varian Inova 400 (400 MHz for ^{1}H, 162 MHz for ^{31}P, and 375 MHz for ^{19}F) or a Varian Inova 500 (500 MHz for ^{1}H) instrument. Chemical shifts are reported in parts per million. ^{1}H NMR spectra data are reported as follows: D_2O (4.63 ppm). ^{31}P NMR spectra data are reported as follows: phosphoric acid (0 ppm) was used as an external standard. Multiplicities are reported by using the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet; br, broad; J, coupling constant in hertz.

Synthesis of CoA Substrates. Synthesis, characterization, and structures of all novel CoA analogues are reported in the Supporting Information.

HPLC and MALDI-TOF Mass Spectrometry Analysis of Purified Apo-, Holo-, and Acyl-S-CouN1. A Beckman System Gold reversed-phase HPLC system equipped with a Vydac protein and peptide C18 column (250 mm × 4.6 mm) at a flow rate of 1 mL/min was used to separate the apo-, holo-, and acyl-*S*-CouN1 proteins (Figure 2). The HPLC solvents

[1] Abbreviations: DTT, dithiothreitol; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography and mass spectrometry; 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.

Scheme 1: (A) Proposed Coumermycin A_1 Biosynthesis and (B) Preparation of Novel Novobiocin Analogues Using CouN1 and CouN7



were as follows: A, H_2O (0.1% TFA); and B, MeCN. The elution gradient was from 20 to 60% B over 20 min followed by a gradient from 60 to 100% B over 10 min. The conversions from apo-CouN1 to holo-CouN1 and to acyl-*S*-CouN1 were performed over 1 h at room temperature using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), CouN1 (15 μM), Sfp (1 μM), and CoA (100 μM) or acyl-CoA (100 μM). Product elution was monitored at 220 nm. Confirmation of the masses of the apo-, holo-, and acyl-*S*-CouN1 was achieved by linear matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using HPLC-purified proteins (Zip Tip C4, Millipore). For mass analysis, the samples were prepared by using α-sinapinic acid (10 mg/mL in a 60% acetonitrile/H_2O mixture) 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. To verify the ability of CouN7 to catalyze the transfer of the various substrates **5′**−**32′** to the novobiocin scaffold, the corresponding acyl-*S*-CouN1 species were formed by posttranslational priming of apo-CouN1 by the phosphopantetheinyltransferase Sfp. The transformations were carried out in 1 h at room temperature (total volume of 52 μL) using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), CouN1 (300 μM), Sfp (5 μM), and acyl-CoA **5′**−**32′** (500 μM). The initial reconstitution of CouN7 activity was then achieved at room temperature in reaction mixtures (total volume of 75 μL) containing acyl-*S*-CouN1 (208 μM), DMSO (5%), descarbamoylnovobiocin (**4**) (25 μM), BSA (1 mg/mL), $MgCl_2$ (10 mM), Tris-HCl (pH 7.5) (75 mM), and CouN7 (25 μM). Each 75 μL aliquot was quenched with cold methanol (150 μL) at specific time points and kept a −20 °C for at least 30 min before centrifugation (13 000 rpm for 10 min at 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 mm × 4.6 mm) at a flow rate of 1.5 mL/min. The HPLC solvents were as follows: A, H_2O (0.1% TFA); and B, MeCN. The elution

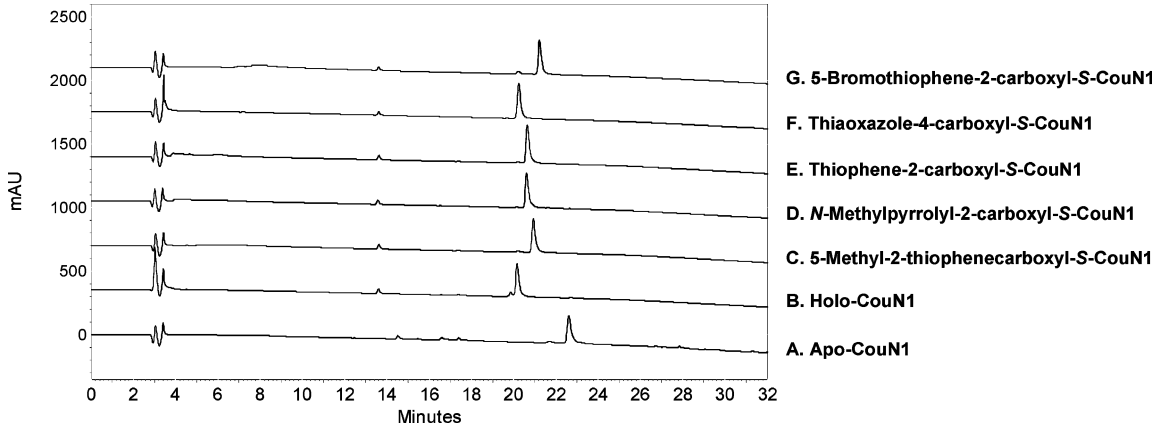


FIGURE 2: HPLC of apo, holo, and acylated forms of *S*-CouN1 from Sfp priming reactions: (A) apo-CouN1 as purified from *E. coli*, (B) holo-CouN1 resulting from Sfp modification of the apo-CouN1 with free CoA, (C) 5-methyl-2-thiophenecarbonyl-*S*-CouN1 prepared by Sfp modification of the apo-CouN1 with 5-methyl-2-thiophenecarbonyl-*S*-CoA (**6′**), (D) *N*-methylpyrrolyl-2-carbonyl-*S*-CouN1 obtained by Sfp modification of the apo-CouN1 with *N*-methylpyrrolyl-2-carbonyl-*S*-CoA (**7′**), (E) thiophene-2-carbonyl-*S*-CouN1 prepared by Sfp modification of the apo-CouN1 with thiophene-2-carbonyl-*S*-CoA (**8′**), (F) thiaoxazole-4-carbonyl-*S*-CouN1 obtained by Sfp modification of the apo-CouN1 with thiaoxazole-4-carbonyl-*S*-CoA (**9′**), and (G) 5-bromothiophene-2-carbonyl-*S*-CouN1 prepared by Sfp modification of the apo-CouN1 with 5-bromo-2-carbonyl-*S*-CoA (**10′**).

Table 1: Rate of Formation of an Acyl-*O*-coumarin Product from **4** with a 200 μM Acyl-*S*-CouN1, 3 μM CouN7 Reaction

product	rate at 30 μM (min^{-1})	highest rate (min^{-1})	concentration (μM) of the acyl donor at the highest rate
21	6.1 ± 0.1	6.3 ± 0.3	60
23	5.46 ± 0.05	5.5 ± 0.05	30
24	4 ± 2	6.1 ± 0.5	120
11	3.95 ± 0.05	4.0 ± 0.05	30
8	3.399 ± 0.004	3.40 ± 0.004	30
22	3.34 ± 0.09	3.6 ± 0.1	60
25	2.73 ± 0.04	2.7 ± 0.04	30
29	2.6 ± 0.2	2.6 ± 0.2	30
27	2.0 ± 0.1	2.2 ± 0.08	60
15	1.39 ± 0.03	1.4 ± 0.03	30
6	0.93 ± 0.02	0.93 ± 0.02	30
19	0.69 ± 0.03	0.78 ± 0.009	60
13	0.64 ± 0.01	0.64 ± 0.01	30
28	0.53 ± 0.02	0.53 ± 0.02	30
5	0.52 ± 0.01	0.63 ± 0.02	90
9	0.49 ± 0.06	0.49 ± 0.06	30
12	0.35 ± 0.07	0.35 ± 0.07	30
14	0.328 ± 0.001	0.45 ± 0.04	60
31	0.22 ± 0.04	0.22 ± 0.04	30
32	0.08 ± 0.01	0.08 ± 0.01	30
7	0.033 ± 0.002	0.033 ± 0.002	30
16	0.023 ± 0.003	0.023 ± 0.003	30

gradient was from 40 to 60% B over 20 min followed by a gradient from 60 to 100% B over 5 min. Product elution was monitored at 340 nm.

Although reactions for determining the efficiency at which the substrates **5′**−**32′** are transferred to the 3′-hydroxyl of the L-noviosyl ring of **4** were performed at 30, 60, 90, 120, 180, 250, 500, and 750 μM acyl-*S*-CouN1 with descarbamoylnovobiocin (**4**) (200 μM) using CouN7 (3 μM), because of substrate inhibition, only the 30 μM reaction was used for comparison (Table 1). Each experiment was carried out in duplicate.

*LCMS Characterization of Analogues **5**−**32***. The formation of all novel novobiocin analogues described above was confirmed by ESI-LCMS using a Shimadzu LCMS-QP8000α instrument equipped with two LC-10AD*VP* liquid chromatography pump modules, a SPD-10AV*VP* UV−vis detector, a SIL-10AD*VP* autosampler module, and a Vydac C18 mass spectrometry column (5 μM, 2.1 mm × 250 mm) (Table 2).

Table 2: LCMS Analysis of Novobiocin Analogues from **4**, CouN1, CouN7 Reactions

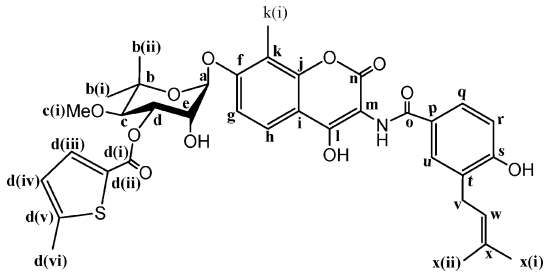
product	calculated $[M + H]^+$	observed $[M + H]^+$
5 ($C_{35}H_{37}NO_{12}$)	664.2	664.4
6 ($C_{36}H_{39}NO_{11}S$)	694.2	694.5
7 ($C_{36}H_{40}N_2O_{11}$)	677.3	677.2
8 ($C_{35}H_{37}NO_{11}S$)	680.2	680.3
9 ($C_{34}H_{36}N_2O_{11}S$)	681.2	681.5
11 ($C_{36}H_{39}NO_{11}S$)	694.2	694.4
12 ($C_{36}H_{39}NO_{11}S$)	694.2	694.1
13 ($C_{35}H_{36}BrNO_{12}$)	743.6	743.4
14 ($C_{35}H_{38}N_2O_{12}$)	679.2	679.3
15 ($C_{36}H_{38}N_2O_{11}$)	692.3	692.0
16 ($C_{37}H_{41}NO_{12}$)	664.2	664.4
19 ($C_{35}H_{37}NO_{12}$)	664.2	664.3
21 ($C_{35}H_{37}NO_{11}S$)	680.2	680.2
22 ($C_{35}H_{36}ClNO_{11}S$)	714.2	714.2
23 ($C_{35}H_{36}ClNO_{11}S$)	714.2	714.3
24 ($C_{35}H_{36}BrNO_{11}S$)	759.6	759.4
25 ($C_{35}H_{36}BrNO_{11}S$)	759.6	759.4
27 ($C_{36}H_{39}NO_{12}$)	678.2	678.4
28 ($C_{35}H_{37}ClN_2O_{11}$)	697.2	697.4
29 ($C_{35}H_{37}ClN_2O_{11}$)	697.2	697.6
30 ($C_{37}H_{39}NO_{11}$)	674.3	674.4

*Deacylation of Analogue **6***. The 75 μL reaction mixture contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP, 100 or 200 μM 5-methylthiophene novobiocin (*6*), 100 or 3 μM CouN7, and 1 mg/mL BSA. Reaction mixtures were incubated at room temperature for various amounts of time and then quenched and analyzed as described previously.

*Scaleup and Characterization of Analogue **6***. The preparative reaction to the make the 5-methylthiophene novobiocin derivative was carried out at room temperature in a total reaction volume of 88 mL. The priming reaction of CouN1 was conducted in a total of 70 mL, and the mixture contained $MgCl_2$ (10 mM), Tris (pH 7.5) (75 mM), 5-methylthiophene CoA (2 mM), CouN1 (1 mM), and Sfp (20 μM). After being incubated for 1 h, this reaction mixture was added to a mixture of descarbamoylnovobiocin in DMSO and the reaction initiated with CouN7 such that the final mixture

contained DMSO (5%), descarbamoylnovobiocin (250 μM), and CouN7 (100 μM). After 1 h, the reaction was quenched with 1.5 volumes of MeOH and the mixture centrifuged at 13 000 rpm to pellet the proteins. Afterward, the pellet was washed with 120 mL of a 50/50 MeOH/H_2O mixture, and the wash combined with the original supernatant was evaporated to a volume of 25 mL. Semipreparative HPLC to purify the novobiocin derivative was performed on a Phenomenex 10 μm C_{18} Luna (250 mm × 21.20 mm) column. The HPLC solvents were as follows: A, H_2O (0.1% TFA); and B, MeCN. The elution gradient was from 10 to 70% B over 10 min, 70 to 85% B over 10 min, 85 to 100% B over 5 min, and then 100% B for 17 min. Product elution was monitored at 340 nm. The overall yield of the reaction was 42% with respect to descarbamoylnovobiocin (6.1 mg of 5-methylthiophene novobiocin from 12 mg of descarbamoylnovobiocin).

The structure of the product was confirmed using LRESIMS and ^{1}H and ^{13}C NMR: ^{1}H NMR (500 MHz, DMSO) δ 11.99 (s, 1H, OH), 10.05 (s, 1H, OH), 9.24 (s, 1H, NH), 7.76−7.72 (m, 4H, H-g, H-h, H-q, H-u), 7.20 [d, J = 9.0 Hz, 1H, H-d(iii)], 6.99 [d, J = 8.0 Hz, 1H, H-d(iv)], 5.81 (bs, 1H, OH), 5.61 (d, J = 2.0 Hz, 1H, H-a), 5.46 (dd, J = 3.5, 10 Hz, 1H, H-d), 5.32 (m, 1H, H-w), 4.24 (s, 1H, H-e), 3.68 (d, J = 10.5 Hz, 1H, H-c), 3.50 [s, 3H, H-c(i)], 3.28 (d, J = 7.5 Hz, 1H, H-v), 2.54 [s, 3H, H-d(vi)], 2.26 [s, 3H, H-k(i)], 1.78 [s, 6H, H-x(i), Hx(ii)], 1.31 [s, 3H, H-b(i)], 1.09 [s, 3H, H-b(ii)]; ^{13}C NMR (125.7 MHz, DMSO) δ 166.52, 160.96, 160.57, 159.18, 158.28, 156.78, 150.53, 148.54, 134.48, 131.44, 130.20, 129.8, 127.39, 127.15, 124.11, 122.52, 121.82, 114.18, 112.78, 110.13, 110.03, 101.51, 98.24, 80.42, 78.16, 71.95, 68.37, 61.15, 28.51, 28.03, 25.54, 22.60, 17.68, 15.36, 8.23; positive LRMS (ESI) found for $C_{36}H_{39}NO_{11}S$ 694.2 $[M + H]^+$, calcd 694.2.



Minimal Inhibitory Concentration Tests. The 5-methylthiophene-2-carboxylate derivative **6** was tested for its antibacterial activity. For the MIC measurements, aminocoumarin stock solutions of known concentration were prepared in DMSO. For each measurement, cells were grown at 37 °C until the OD_{600} reached 0.6 and were then diluted with LB broth to a final concentration of 10^6 cfu/mL. The diluted cultures were poured into wells of microtiter plates (Becton Dickinson, 96-well flat-bottom polystyrene plates). Compounds were added to the first column of wells and then underwent a series of double dilutions across the plate. Cells were incubated for 22 h at 37 °C, after which time a 0.33 mg/mL solution of thiazolyl blue tetrazolium bromide in sterilized water was added to each well. The MICs were determined as the lowest concentration of antibacterial agent that completely inhibited bacterial respiration, as indicated by staining.

RESULTS

Preparation of CoA Substrates. A general coupling procedure for the preparation of unnatural acyl CoA derivatives was developed. Briefly, the acyl chloride of the desired carboxylic acid was added in small portions to a H_2O/THF solution containing CoA. Pyridine was used as the acid scavenger (the detailed procedures can be found in the Supporting Information). All of the derivatives were characterized by ^{1}H NMR, ^{31}P NMR, and high-resolution ESIMS. Derivatives containing fluoride were characterized by ^{19}F NMR (see the Supporting Information).

Enzymatic Loading of Apo-CouN1 with Various CoA Substrates. Recently, we reported the overproduction and purification of the CouN1/CouN7 carrier protein/enzyme pair involved in the transfer of the key pharmacophore, the pyrrolyl moiety, to the noviosylcoumarin scaffold during clorobiocin and coumermycin A_1 biosynthesis (*13*). CouN1 was proven to be a thiolation domain capable of accepting both the pyrrolyl **31′** and 5-methylpyrrolyl-2-carboxylate **32′** substrates. To introduce structural diversity into the key position of the aminocoumarin antibiotics, we have now performed initial loading experiments of apo-CouN1 using Sfp, a well-established phosphopantetheinyltransferase, and a variety of synthetic acyl-CoA substrates **5′**−**30′**. MALDI-TOF mass spectrometry and HPLC analyses (as exemplified in Figure 2) confirmed that the acyl-*S*-pantetheinyl-phosphate moieties of all these acyl-CoAs can be covalently attached to apo-CouN1 by the action of Sfp.

Characterization of CouN7 Activity. Previous in vivo studies by Heide and co-workers suggested that Clo/CouN7 are the acyltransferases involved in attachment of the pyrrolyl moiety to the deoxysugar of the clorobiocin and coumermycin A_1 scaffolds (*16*). We have recently validated that CouN7 is the transferase responsible for the transfer of the acyl group from the carrier protein CouN1 to the 4-methoxy-L-noviosyl of descarbamoylnovobiocin (**4**). In this study, we sought to characterize CouN7 for acyl donor promiscuity and determine whether it would transfer other acyl moieties loaded onto holo-CouN1. Of the 26 synthetic acyl-CoA substrates that were generated, **5′**−**30′**, and installed as thioesters on CouN1, Figure 3A shows the time course (up to 19 h) for CouN7-mediated conversion of the 4-chloropyrrole-2-carbonyl moiety from 4-chloropyrrolyl-*S*-CouN1 to descarbamoylnovobiocin, yielding compound **28**. Similarly, Figure 3B shows the transfer (up to 3 h) of the 5-methylthiophene-2-carbonyl moiety and Figure 3C the transfer (up to 1 h) of the 3-bromothiophene-2-carbonyl moiety. In this study, CouN7 successfully transferred the acyl groups of 21 of the 26 acylated forms of CouN1 to the descarbamoylnovobiocin scaffold (**4**). We observe in these initial studies that compounds **10**, **17**, **18**, **20**, and **26** cannot be prepared under these conditions by action of CouN7, as denoted by the X's in Scheme 1B. This was primarily caused by the instability of the final novobiocin derivative that appeared to decompose to several peaks on the HPLC spectrum (data not shown).

Next, a preliminary kinetic comparison of the 21 active CouN1-loaded substrates was conducted. The optimal reaction rate for each of the substrates at a single substrate concentration (concentrations vary between 30 and 120 μM) was used for the purpose of comparison because an as yet uncharacterized substrate inhibition, which was observed for

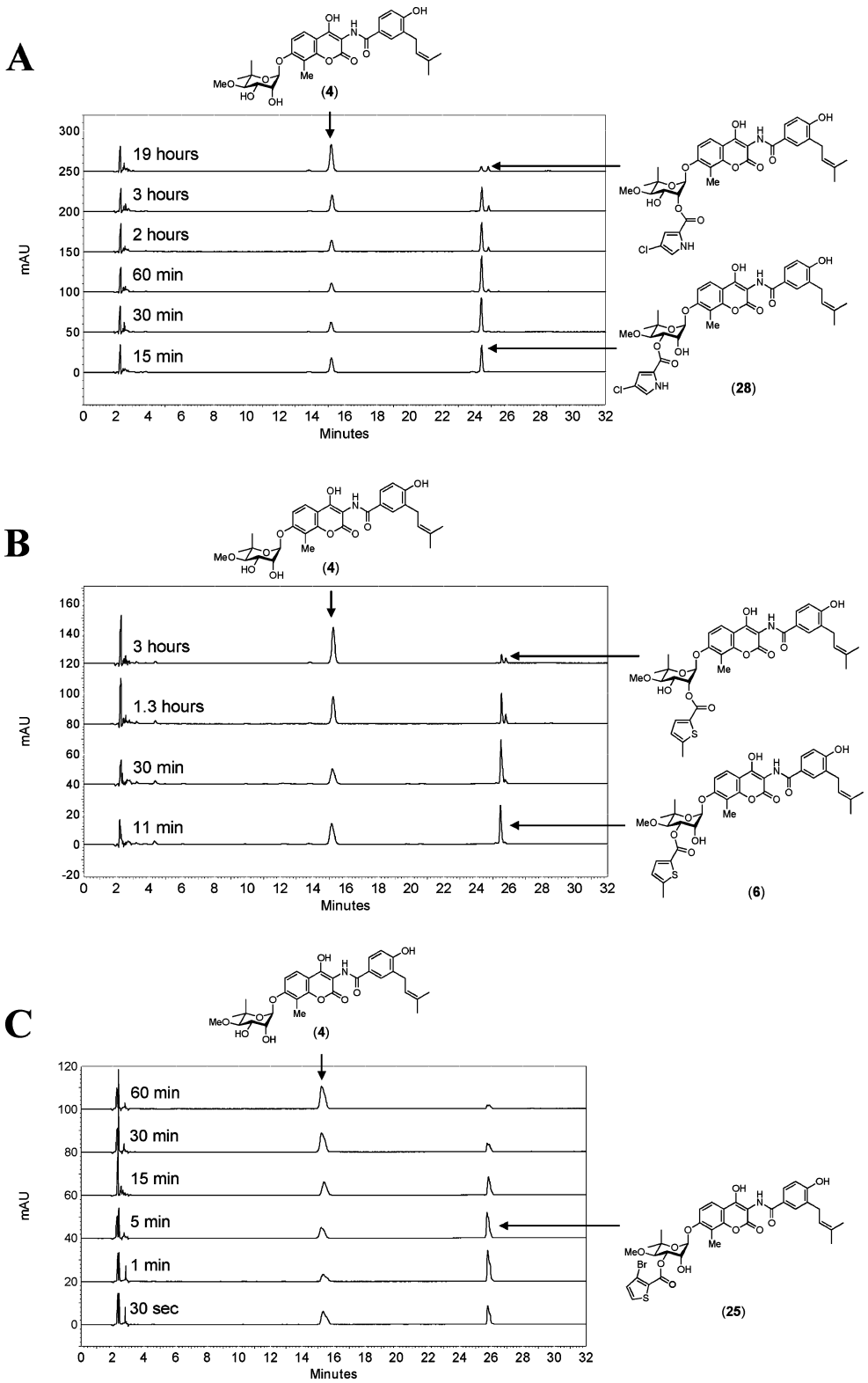


FIGURE 3: (A) Time course for the conversion of 200 μM descarbamoylnovobiocin (**4**) into 4-chloropyrrolylnovobiocin (**28**) at room temperature by the action of 25 μM CouN7 with 300 μM 4-chloropyrrolyl-*S*-CouN1 at pH 7.5. (B) Time course for the conversion of 200 μM descarbamoylnovobiocin (**4**) into 5-methyl-2-thiophenecarbonylnovobiocin (**6**) at room temperature by the action of 25 μM CouN7 with 300 μM 5-methyl-2-thiophenecarbonyl-*S*-CouN1 at pH 7.5. (C) Time course for the conversion of 200 μM descarbamoylnovobiocin (**4**) into 3-bromo-2-thiophenecarbonylnovobiocin (**6**) at room temperature by the action of 25 μM CouN7 with 300 μM 3-bromo-2-thiophenecarbonyl-*S*-CouN1 at pH 7.5.

all of the CouN1-loaded substrates, precluded determination of Michaelis−Menten parameters. The results listed in Table 1 show that CouN7 tolerates a variety of single heteroatoms within the aromatic ring and a number of single substitutions around the ring. However, it seems to disfavor more than one heteroatom in the aromatic ring, as in the case of the thiazole and isoxazole substrates, and multiple substitutions around the ring.

Other Transformations of the Novobiocin Derivatives. During formation of the 21 novel novobiocin derivatives,

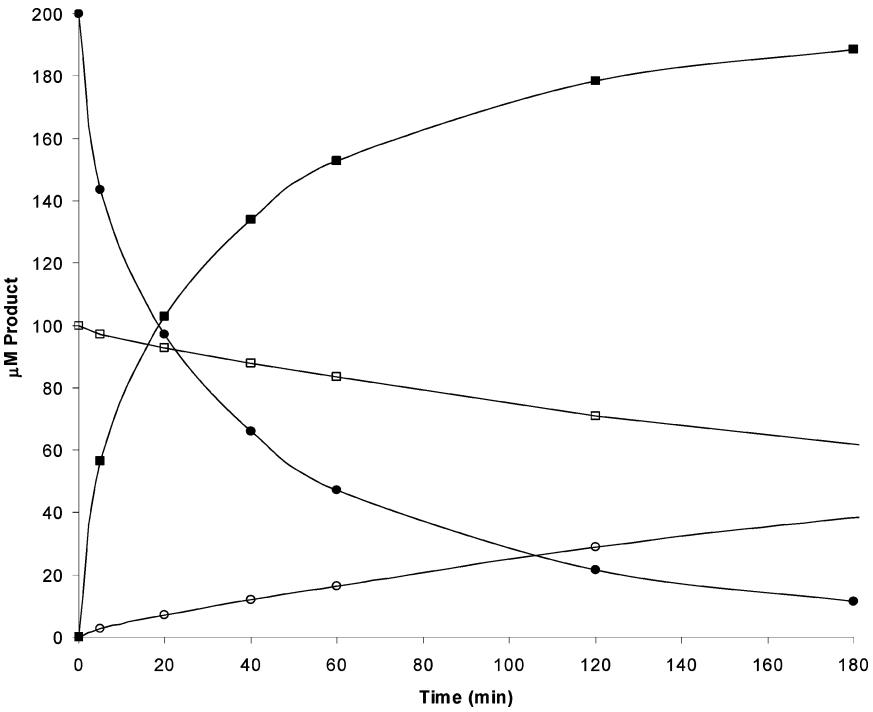


FIGURE 4: Time course for CouN7-mediated deacylation of the 5-methylthiophene novobiocin derivative. Filled circles and squares represent the time course at 100 μM CouN7, and empty circles and squares represent the time course at 3 μM CouN7. Squares depict data for the 5-methylthiophene novobiocin derivative and circles data for the descarbamoylnovobiocin scaffold.

we noticed that for some of the compounds a second peak directly adjacent to the product peak would appear after some time, as shown in both panels A and B of Figure 3. These peaks had UV−vis spectra identical to that of the major product peak and were identical in mass. We propose that this peak doublet represents a nonenzymatic migration of the acyl substituent from the 3′-hydroxyl to the 2′-hydroxyl of the noviose sugar. Such 3′−2′ migrations had been observed during the initial isolation 50 years ago and are well-known for the aminocoumarin class of antibiotics (*9*, *17*−*19*). They are presumably a CouN7 enzyme-independent chemical process. To test this, we enzymatically generated the 5-methylthiophene novobiocin derivative (**6**) and isolated only the major product peak. Following redissolution of the compound in buffer at room temperature overnight, the HPLC trace once again contained a doublet peak (data not shown) with the second peak being identical in mass and UV−vis spectrum to the major product peak. We subsequently confirmed that this second peak indeed represented migration of the appended acyl substituent to the 2′-hydroxyl of the noviose by NMR during our scaleup efforts, which will be described shortly. We have not characterized the rates of the rearrangement.

During the course of determining rates of product formation, we also observed for many of the derivatives that the amount of product would initially build up as expected; then, after a longer period of time, the level of acylated aminocoumarin would start to decrease, with a reciprocal increase in the level of descarbamoylnovobiocin starting material (Figure 3A−C). We postulated that this was in fact due to CouN7-catalyzed deacylation of the final novobiocin derivative because control experiments showed that the products were stable at room temperature in the absence of enzyme for at least several days. To test this hypothesis, we again used the 5-methylthiophene novobiocin product and incubated it with CouN7 at either 100 or 3 μM, the level of CouN7 present in our kinetic assays. As one can see in Figure 4, CouN7 catalyzes the net hydrolytic deacylation of 100 μM 5-methylthiophene novobiocin (**6**) to the descarbamoylnovobiocin scaffold (**4**) at a rate of 8.2 min^{-1}. It should be noted that this rate drops off precipitously with lower concentrations of **6**, and at levels that are <10% of the starting concentration, the reversal via deacylation is almost negligible.

*Scaleup Reaction of Compound **6** for the Formation of a Novel Novobiocin Analogue.* We chose to scale up the 5-methylthiophene derivative **6**, as it showed an intermediate rate of formation among all the derivatives (Table 1) and would serve as a good model compound. Using the procedure outlined in Materials and Methods, we were able to obtain several milligrams of 5-methylthiophene novobiocin. The scaleup reaction afforded a mixture of both the 3′- and 2′-acylated scaffolds with the desired 3′-acylated derivative as the predominant product. Prior evidence showed that the 2′-*O*-acyl rearrangement products are not active with GyrB (*17*, *19*), and therefore, since the ratio of the 2′-acyl product was determined by both HPLC and NMR, we could proceed with the MIC experiments (Table 3).

We chose two representatives of Gram-positive bacteria: *Bacillus subtilis* PY79 and *Bacillus cereus* ATCC 14893. Knowing that in the case of the Gram-negatives, membrane permeability might affect the antibacterial activity, we chose two bacterial strains, wild-type *Escherichia coli* MC4100 and the mutated *E. coli* NR698 imp4213, with differential membrane permeability. We tested compound **6** along with novobiocin and descarbamoylnovobiocin scaffold as controls. As compound **6** contained a mixture of the 3′- and 2′-acylated derivatives, we used HPLC purification to afford two mixtures. The first contained 40% of the 2′-acylated compound, while the second contained 10% of the 2′-acylated

Table 3: Minimal Inhibitory Concentrations (MICs)

	MIC (μg/mL)			
bacterial strain	novobiocin scaffold	novobiocin	90% 5-methyl-thiophene novobiocin	60% 5-methyl-thiophene novobiocin
E. coli MC4100	>600	>600	>600	>600
E. coli imp4213[a]	>200	1	55	59
B. subtilis PY79	>200	1	5	10
B. cereus ATCC 14893	>200	1	4	7

[a] From ref *27*.

and 90% of the desired 3′-acylated compound. The novobiocin scaffold minus the 3′-carbamoyl group loses all antibacterial activity for all of the tested strains. Interestingly, compound **6** exhibits activity comparable to that of novobiocin against the Gram-positives. In the case of the Gram-negative strains, lower activity was observed. Both novobiocin and compound **6** were found to have poor antibacterial activity against *E. coli* MC4100 with a MIC of >200 μg/mL. However, for *E. coli* NR698 imp4213, novobiocin exhibited marked activity similar to that found for the Gram-positives while **6** exhibited moderate activity. These outcomes might be explained by the lower membrane permeability of **6** in comparison to that of novobiocin. The mixture of **6** containing 40% of the 2′-acylated derivative was consistently less active than the mixture containing 10% of the 2′-acylated compound. This is in agreement with the observation that the 2′-acylated scaffolds exhibit no antibacterial activity.

DISCUSSION

The members of the aminocoumarin class of natural products are intriguing because of their potent inhibition of bacterial DNA gyrase by competitive binding to the ATP site of the GyrB subunit (*1*). X-ray analyses of novobioicin and clorobiocin with an *N*-terminal 24 kDa fragment of GyrB have established the relative orientation of the antibiotics compared to ATP (*20*). The aminocoumarin portion forms a planar scaffold that presents the unusual L-noviosyl sugar, in glycoside linkage to O_7 of the bicyclic coumarin. In turn, the acyl substituent esterified to the 3′-oxygen of the noviosyl ring contacts the key subsite of GyrB. In novobiocin, this 3′-*O*-acyl moiety is a carbamoyl group, while in clorobiocin and the dimeric coumermycin, it is the 5-methylpyrrolyl unit. The ca. 10-fold increase in potency of clorobiocin over novobiocin (*11*) has been attributed to the displacement of bound water molecules by the methylpyrrole compared to the small carbamoyl moiety.

Despite the fact that GyrA subunit inhibition by quinolone antibacterial agents has led to wide clinical use of such molecules as ciprofloxacin and levaquin, the GyrB-targeting aminocoumarins have not been utilized clinically due to a variety of physical, chemical, and pharmacodynamic issues. This has raised the question of whether replacements on the 5-methylpyrrolyl pharmacophore of clorobiocin and coumermycin would lead to optimization of antibacterial properties. In turn, this has focused our attention on the late stages of aminocoumarin antibiotic biosynthesis where the regioselective acylation of the noviosylcoumarin scaffold occurs at the end of the pathway.

Overall, aminocoumarins such as clorobiocin are biosynthesized from four parts: (1) tyrosine to generate the bicyclic aminocoumarin scaffold and (2) the prenylated hydroxybenzoate that is enzymatically ligated to form clorobiocic acid (3) TDP-L-noviose is fashioned from TDP-D-glucose by a series of dedicated enzymes and then the noviosyl ring is appended to O_7 of the coumarin by the glycosyltransferase CloM (4) the pyrrole-2-carboxyl unit is elaborated from L-proline via a series of aminoacyl-*S*-carrier protein species and then ligated by the CloN7 being discussed here.

In prior studies, we have heterologously expressed and purified the streptomycete NovN, the novobiocin carbamoyltransferase, from *E. coli* (*21*). While we could reconstitute the carbamoylation step, NovN was narrowly specific for the acyl donor, carbamoyl phosphate, and would not utilize other acyl donors for pharmacophore diversification (*22*). Recently, we have turned to the Clo/CouN1, Clo/CouN7 pair involved in the cognate acylation of the noviosyl coumarin scaffolds of clorobiocin and coumermycin (*16*, *23*). The apo form of the carrier protein CouN1 can be loaded with 5-methylpyrrolyl-*S*-pantetheinyl-phosphate via 5-methylpyrrolyl-CoA and the promiscuous phosphopantetheinyltransferase Sfp from *B. subtilis*. CouN7 can then be validated as the pyrrolyl acyltransferase with the desacyl forms of coumermycin and novobiocin as acceptor substrates (*13*).

Now the possibility that CouN7, unlike NovN, might exhibit broad tolerance to the acyl donor could be assayed, providing that a series of alternate acyl-*S*-CouN1 forms could be prepared. To that end, we report the synthesis of 26 acyl-CoA derivatives, predominantly containing five-ring heterocyclic acyl groups as a first set of 5-methylpyrrolyl analogues. Each heterocyclic acyl-CoA preparation was optimized such that multimilligram quantities could be purified and used as substrates for Sfp with apo-CouN1. The physiological role of Sfp is to transfer the unsubstituted HS-pantetheinyl-P (Ppant) arm from CoASH onto apo carrier proteins in *Bacillus*, but from prior experience with Sfp in loading many apo carrier proteins with acyl- and alkyl-CoAs (*16*, *24*, *25*), we anticipated great tolerance. Indeed, all 26 acyl-*S*-pantetheinyl-P moieties were transferred to apo-CouN1, allowing HPLC purification of substrate quantities of the 10 kDa acyl-*S*-pantetheinyl-CouN1 forms as potential donors for CouN7.

The acceptor substrate for CouN7 should be the mono- and bis-desacyl forms of the dimeric coumermycin. However, this is not readily available from either commercial or fermentation sources. Therefore, as a first effort, we turned to the desacyl form of the novobiocin scaffold. Novobiocin is commercially available, and the chemical deacylation step works well to yield the descarbamoylnovobiocin (**4**) as a surrogate acceptor substrate to assay CouN7. Initial studies show CouN7 to be encouragingly promiscuous toward the acyl donor, with 21 of the 26 acyl variants presented on the pantetheinyl CouN1 accepted for clean transfer to the noviosyl 3′-OH. In this first effort, we stayed close to the pyrrole ring framework but varied heteroatoms and the placement of ring substituents. For acyl-*S*-CouN1 donors assayed at one concentration, 30 μM, the k_{obs} for CouN7-mediated acyl transfer varied over a 12-fold range, from 6 to 0.5 min^{-1}, with thiophenes at the high end and furans at the low end. N-Methylation of the pyrrole was not tolerated

well, nor was dimethylation of the furan ring. This promiscuity augurs well for making and testing a larger library of acyl-CoAs to load onto apo-CouN1 by Sfp action to further test the spectrum of CouN7 action.

The use of the novobiocin scaffold for the production of new synthetic 3′-acylated derivatives proves to be challenging. The presence of four alcohols, two on the L-noviose and two on the coumarin ring system, the amide, the lactone, and the presence of a double bond that impairs the use of protecting groups that require catalytic hydrogenation for removal are all a part of the difficulties of using a chemical strategy for the preparation of new novobiocin derivatives. This increases the importance of the chemoenzymatic approach serving as a practical tool for that purpose. With the development of a method for the preparation of CoA derivatives and with the discovered high substrate tolerance of the CouN1/N7 system, we chose to test the utility of the system for scaling up novel derivatives to quantities that would allow for minimal inhibitory concentration tests in an examination of possible antibacterial activity.

Focusing on derivative **6** as a model compound, we were able to obtain several milligrams of 5-methylthiophene novobiocin using the CouN1/CouN7 system. Interestingly, the addition of 5-methylthiophene to the novobiocin scaffold restored the antibacterial activity that was lost with removal of the carbamoyl group from the natural compound. In the case of the tested Gram-positive bacteria, activity was restored to the same order of magnitude as that of novobiocin, whereas in the case of the Gram-negative bacteria, though activity was restored, the effect was smaller. These results demonstrate that the 5-methylthiophene moiety could act as a successful pharmacophore and highlight the antibacterial potential of new novobiocin derivatives produced using the chemoenzymatic method presented in this paper. Furthermore, we were able to unambiguously show that acylation of the 2′-hydroxyl of the noviose would yield an inactive novobiocin analogue. This underscores the importance of the CouN1/CouN7 system in controlling regiospecificity during installation of new acyl substituents.

By using a chemoenzymatic approach, we demonstrated that novel novobiocin analogues can be produced in milligram quantities by the action of the pair of enzymes CouN1 and CouN7. This work also clearly demonstrates the promiscuity of this biosynthetic pair of enzymes in the formation of 21 novel coumarin compounds. In the future, trying other noviosylcoumarin scaffolds as acceptors for CouN7-mediated variant acyl transfers to evaluate the extent to which the underlying coumarin scaffolds condition both CouN7 efficiency and subsequent antibiotic activity will be of interest. These include the clorobiocin and coumarin desacyl scaffolds, but also simocyclinone (*26*) and incomplete fragments of those natural products. These should be useful directions for altering the pharmacophore to affect the potency of these natural product antibiotic scaffolds.

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SUPPORTING INFORMATION AVAILABLE

Synthesis and full characterization of all the CoA derivatives used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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