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Covalent CouN7 Enzyme Intermediate for Acyl Group Shuttling in Aminocoumarin Biosynthesis

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SUMMARY

The last stages of assembly of the aminocoumarin antibiotics, clorobiocin and coumermycin A_1, which target the GyrB subunits of bacterial DNA gyrase, involve enzymatic transfer of the pyrrolyl-2-carbonyl acyl group from a carrier protein (CloN1/CouN1) to the 3′-OH of the noviosyl moiety of the antibiotic scaffold. The enzyme, CouN7, will catalyze both the forward and back reaction on both arms of the coumermycin scaffold. This occurs via an *O*-acyl-Ser_{101}-CouN7 intermediate, as shown by transient labeling of the enzyme with [^{14}C]acetyl-*S*-CouN1 as donor and by inactivating mutation of the active site, Ser_{101}, to Ala. The intermediacy of the pyrrolyl-2-carbonyl-*O*-CouN7 allows net pyrrole transfer between distinct aminocoumarin scaffolds, for example, between the descarbamoylnovobiocin scaffold and coumermycin A_1 and vice versa. CouN7 also allows shuttling of surrogate acyl groups between noviosyl-aminocoumarin scaffolds to generate new antibiotic variants.

INTRODUCTION

The aminocoumarin antibiotics are natural products that kill bacteria by blockade of topoisomerase II activity by acting as competitive inhibitors of ATP hydrolysis in the GyrB subunits of DNA gyrase [1–3], shutting down DNA synthesis [4]. DNA gyrase is also the target of the synthetic quinolone antibacterial agents, such as ciprofloxacin, although they target the GyrA subunits [4, 5]. The three most prevalent members of the aminocoumarin family, all produced by streptomycetes, are novobiocin **1**, clorobiocin **2**, and coumermycin A_1 **3** (Figure 1). Novobiocin and clorobiocin each have three parts: a prenyl-hydroxybenzoate in amide linkage to the aminocoumarin, in turn glycosylated on a phenolic oxygen by an unusual L-deoxyhexose, termed noviose. They differ at two places: a methyl versus chlorine at position 8 of the coumarin scaffold, and, more importantly, in the identity of the acyl group on the 3′-OH of the noviosyl ring. Novobiocin has a carbamoyl group, while clorobiocin has a 5-methylpyrrolyl-2-carbonyl acyl moiety. The X-ray structures of novobiocin and clorobiocin bound to a 24 kDa ATPase fragment of GyrB compared to ATP binding [6–8] show not only that the antibiotics overlap the ATP binding site, but also that the carbamoyl and pyrrolyl-2-carbonyl groups are key pharmacophoric units interacting with the GyrB active site. The third member of the aminocoumarin family is the pseudodimeric coumermycin with a 4-methyl-2,5-dicarboxypyrrole linker containing the noviosyl-aminocoumarin in amide linkage to both carboxyls of the linker [9]. The key acyl substituent at the 3-position of each of the noviosyl units of coumermycin is, as in clorobiocin, the 5-methylpyrrolyl-2-carbonyl unit.

Heide and his colleagues have carried out pioneering work to sequence all three biosynthetic gene clusters [10–12], perform genetic deletions to validate gene functions [13–20], and have done in vivo manipulations and replacements to make a small set of hybrid aminocoumarins and characterize them for antibiotic activity [13, 15, 21–27]. Notably, they identified seven ORFs, CloN1–7 and CouN1–7, in the Clo and Cou clusters that are involved in generating and transferring the 5-methylpyrrolyl-2-carbonyl unit in the producer organisms. In complementary studies, we have shown that the activities of the purified Clo/CouN3,4,5 are to activate L-proline (N4), tether it as prolyl-N5, and then desaturate (N3) it to the pyrrolyl-2-carbonyl thioester [28]. Most recently, we have validated the in vivo-based experiments of Heide and colleagues [20] that CouN1 is a free-standing peptidyl carrier protein or thiolation domain and CouN7 is the enzyme that shuttles the pyrrolyl-2-carbonyl or 5-methylpyrrolyl-2-carbonyl to the desacyl form of clorobiocin/novobiocin to create the pharmacophore for antibiotic activity [29, 30] (M. Fridman, C.J.B., T. Lupoli, D. Kahne, C.T.W., and S.G.-T., data not shown).

In this work, we have studied the mechanism of CouN7, an α,β-hydrolase family member, and establish that it generates a covalent acyl-*O*-Ser_{101} enzyme intermediate during catalysis. The active site residue accepts the acyl group from acyl-*S*-CouN1 donor and then delivers it to the oxygen nucleophile of the desacylated noviosyl

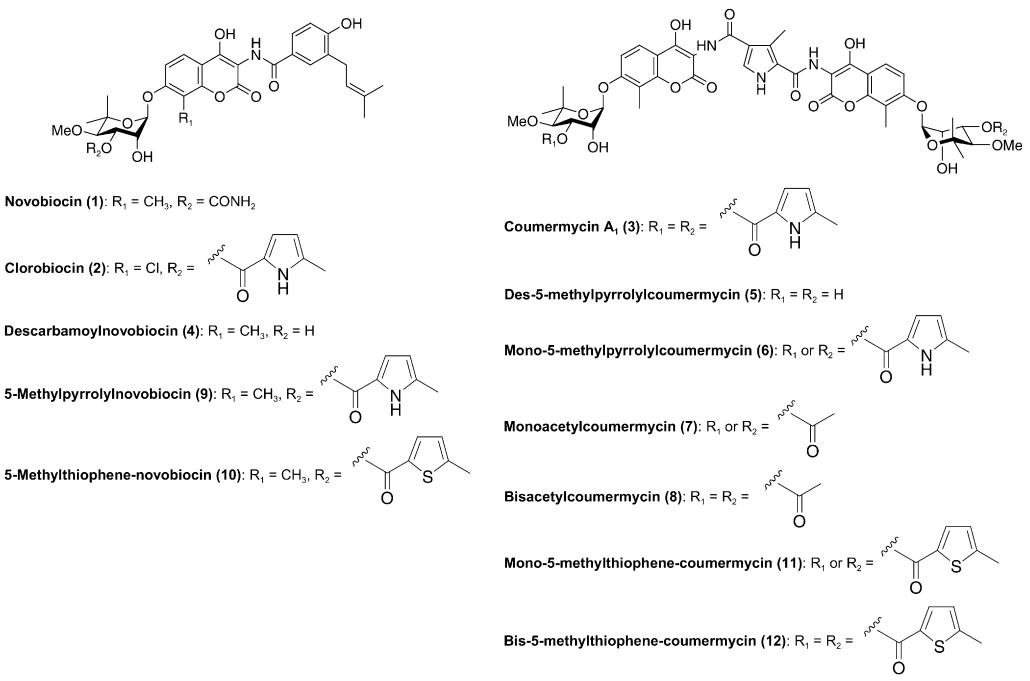


Figure 1. Aminocoumarin Molecules Discussed in This Study

ring of the aminocoumarin scaffold. In accord with this mechanism, we show CouN7 will catalyze a net deacylation of coumermycin A_1 **3**, removing either one or both of the 5-methylpyrrolyl-2-carbonyl units in a net hydrolytic reaction as the 5-methylpyrrolyl-*O*-CouN7 is intercepted by water. In the presence of descarbamoylnovobiocin **4**, the acyl-*O*-CouN7 is instead captured by the cosubstrate, leading to a net 5-methylpyrrolyl-2-carbonyl transfer. In principle, this acyl group shuttle mechanism of CouN7 should enable regioselective acyl transfer to the des-5-methylpyrrolylcoumermycin **5** scaffold to generate antibiotic variants with one or two novel acyl group pharmacophores. We demonstrate this by transfer of the 5-methylthiophene-2-carbonyl group from novobiocin to the des-5-methylpyrrolylcoumermycin scaffold, generating mono- and bisacylated variants.

RESULTS

CouN7 Catalyzes Mono- and Bis-5-Methylpyrrolyl-2-Carbonyl Transfer to the Des-5-Methylpyrrolylcoumermycin Scaffold

CouN7 has been identified genetically as the last/next-to-last enzyme in coumermycin A_1 assembly, transferring the pyrrolyl/5-methylpyrrolyl-2-carbonyl acyl moiety to the noviosyl rings of both arms of the pseudodimeric antibiotic scaffold [20]. Because of availability of the monomeric descarbamoylnovobiocin **4** [31], in initial studies with CouN7 purified after heterologous expression in *Escherichia coli*, we tested the enzyme for regioselective acyl transfer to the 3′-OH of the noviosyl ring of descarbamoylnovobiocin to make pyrrolylnovobiocins [29]. The presumed in vivo substrate for CouN7, the des-5-methylpyrrolylcoumermycin scaffold **5**, is available from the sequential action of three enzymes: CouL, CouM, and CouP [32]. CouL does a double ligation of the aminocoumarin nucleus to 2,5-dicarboxy-4-methylpyrrole to make the bis amide on which CouM then transfers two noviosyl groups from TDP-L-noviose. CouP makes the 4′-*O*-methylnoviose on both arms of the maturing scaffold to yield **5** as putative acceptor substrate for CouN7.

With **5** as acceptor substrate and 5-methylpyrrolyl-2-carbonyl-*S*-pantetheinyl CouN1 [29] as donor substrate, the activity of CouN7 toward des-5-methylpyrrolylcoumermycin was assayed. As illustrated in Figure 2A, CouN7 catalyzes a time-dependent utilization of **5** with formation of two new compounds, as observed by HPLC analysis. The initial peak (seen at 2 min incubation time) has the mass spectrum of the mono-5-methylpyrrolylcoumermycin **6** (1003.3 $[M + H]^+$ calculated, 1003.6 observed), while, at later times, the second peak that grows in has the mass spectrum of **3** (1110.4 $[M + H]^+$ calculated, 1110.7 observed), and coelutes with authentic coumermycin A_1. Under the incubation conditions of Figure 2A, about 90% of substrate **5** is converted equally to the mono-5-methylpyrrolyl intermediate **6** and the doubly acylated final antibiotic product **3** (Figure 2B).

Steady-state kinetic analysis (Figure 2C) shows a catalytic rate constant (k_{cat}) of 5.9 ± 0.2 min^{-1} and a Michaelis constant (K_M) for the acceptor substrate **5** of 2.9 ± 0.3 μM. This k_{cat} is about an order of magnitude faster than that of 0.73 min^{-1}, and the K_M about 20-fold lower (60 μM) [29], than that recently reported by us for transfer to the surrogate acceptor descarbamoylnovobiocin **4** [29], for an

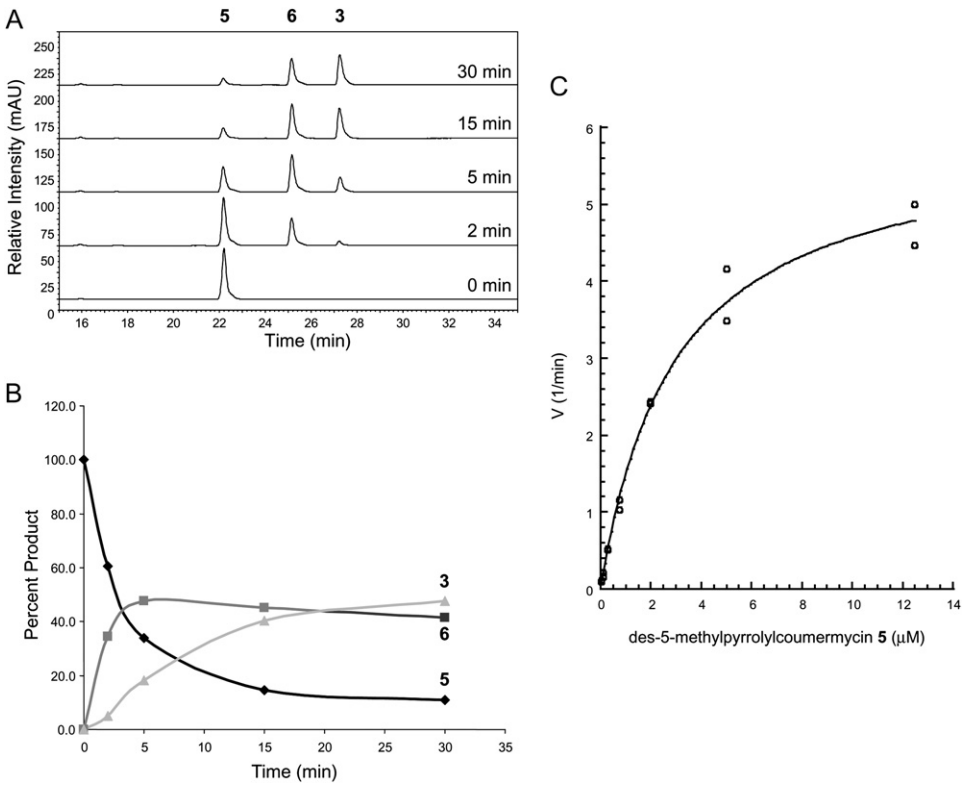


Figure 2. CouN7 Generates Coumermycin A_1 3 from 5-Methylpyrrolyl-*S*-CouN1 and Des-5-Methylpyrrolylcoumermycin 5

(A) HPLC time course of reaction containing 5.7 μM des-5-methylpyrrolylcoumermycin **5**, 85.7 μM 5-methylpyrrolyl-*S*-CouN1, and 20 μM CouN7. Products were monitored at 340 nm.

(B) Quantification of products from the reaction in (A). Triangles represent coumermycin A_1 **3**, squares represent mono-5-methylpyrrolylcoumermycin **6**, and diamonds represent des-5-methylpyrrolylcoumermycin **5**.

(C) Michaelis-Menten kinetic parameters for utilization of des-5-methylpyrrolylcoumermycin **5** in the forward acylation reaction with 100 μM 5-methylpyrrolyl-*S*-CouN1, 0.1 μM CouN7, and various concentrations of des-5-methylpyrrolylcoumermycin **5**, ranging from 0.05 to 12 μM.

improvement in catalytic efficiency of about 170-fold with the native substrate of CouN7.

Catalysis Proceeds via an Acyl-Enzyme Intermediate Involving Ser_{101}

Bioinformatic analysis indicated that CouN7 is a member of the α,β-hydrolase superfamily, with similarity to CmaE in the coronamic acid biosynthetic pathway [33] and SyrC in the syringomycin pathway [34]. We have recently shown that CmaE [35] and SyrC [36] proceed via covalent catalysis, as do many other members of this enzyme superfamily. To evaluate this mechanistic possibility with CouN7, we determined to use a radioactive surrogate acyl donor, as labeled 5-methylpyrrolyl-2-carboxylate is not readily available. To that end, commercially available [^{14}C]acetyl-CoA could be incubated with the apo form of CouN1 and the purified phosphopantetheinyltransferase, Sfp [37], with resultant [^{14}C]acetyl-*S*-pantetheinyl transfer to generate [^{14}C]acetyl-*S*-CouN1. This served as the acyl donor to CouN7 in brief incubations without an acyl acceptor. The reactions were quenched with SDS and rapidly analyzed by SDS-PAGE and autoradiography. Figure 3A shows that the 10 kDa [^{14}C]acetyl-S-CouN1 is initially radioactive and, within 30 s, transfers [^{14}C]acetyl to CouN7. Labeled CouN7 is detectable for up to 15 min, after which radioactivity disappears. In parallel, the radioactivity disappears from [^{14}C]acetyl-*S*-CouN1. We interpret these data to indicate that CouN7 autoacetylates on an active site residue and, in the absence of acceptor cosubstrate, will be captured by a water molecule for net hydrolysis of the labile acetyl-enzyme species. Enzyme reacetylation will occur through many cycles until all [^{14}C]acetyl-*S*-CouN1 donor molecules have undergone net acetyl transfer to CouN7. Given a ratio of [^{14}C]acetyl-*S*-CouN1/CouN7 of 10:1 in this experiment, at least 10 enzyme acetylation/hydrolytic deacetylation events were being monitored.

To validate that the observed transient labeling of CouN7 by the [^{14}C]acetyl group was the first half reaction on a catalytic pathway, we turned to nonradioactive acetyl-*S*-CouN1 as donor in the presence of acceptor substrate **5**. As shown in Figure 3B, CouN7 addition yields two new HPLC-detectable products growing in over time. The first peak has the mass spectrum of monoacetylcoumermycin 7 (938.3 [M + H]+ calculated, 938.6 observed), and the second peak has the mass spectrum

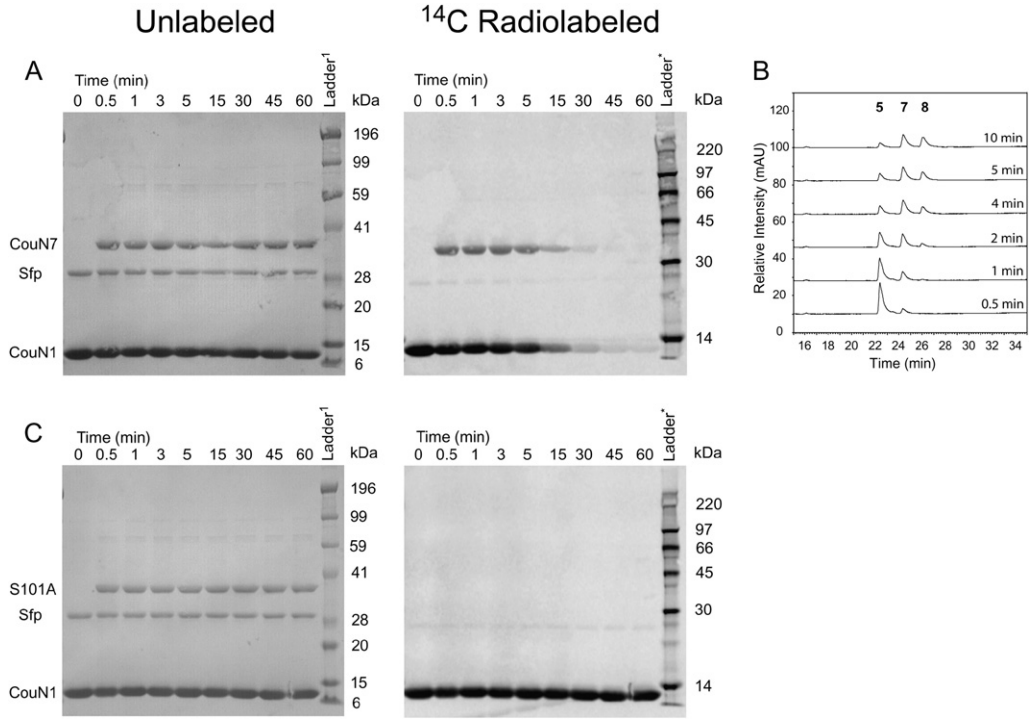


Figure 3. Acylation of Des-5-Methylpyrrolylcoumermycin 5 Proceeds via an Acyl-*O*-CouN7 Intermediate by Using S_{101}

(A) Time course of 250 μM [^{14}C]acetyl-*S*-CouN1 reacting with 25 μM CouN7. Both an unlabeled coomassie-stained gel and a [^{14}C] autoradiography gel are shown.

(B) HPLC time course of reaction containing 171 μM acetyl-*S*-CouN1, 115 μM des-5-methylpyrrolylcoumermycin **5**, and 20 μM CouN7. Products were monitored at 340 nm.

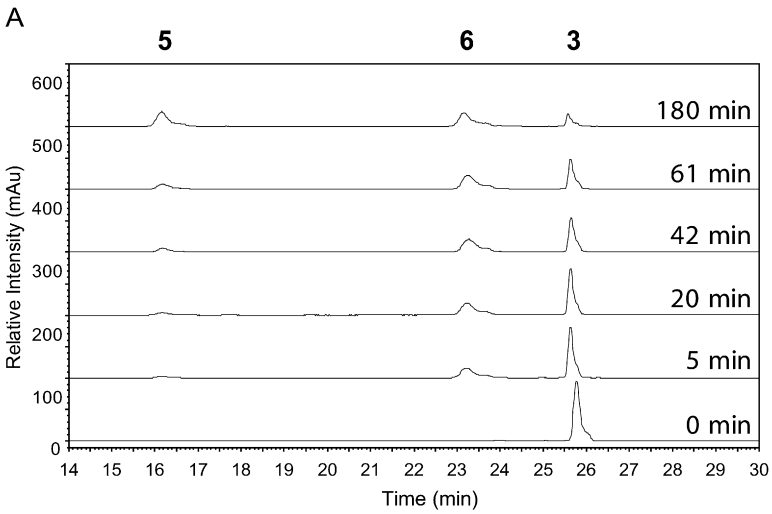
(C) Same as (A), but with the CouN7 S_{101}A mutant. Ladder^{1} is Bio-Rad prestained SDS-PAGE standard, broad range. Ladder* is [^{14}C]methylated proteins, GE Healthcare.

of the bisacetyl variant of coumermycin 8 (980.3 [M + H]+ calculated, 980.5 observed). Therefore, we conclude that the net acetyl transfer to the des-5-methylpyrrolylcoumermycin scaffold is a two-step process and goes via an acetyl-CouN7 covalent enzyme intermediate. Furthermore, CouN7 can catalyze acetyl transfer to both arms of the des-5-methylpyrrolylcoumermycin scaffold, previewing other possible acyl transfers.

Homology with CmaE and SyrC suggests that CouN7 uses the side chain −OH of Ser_{101} as catalytic nucleophile, even though CmaE and SyrC use the thiolate side chain of active site Cys residues as nucleophiles [35, 36]. This difference could be attributed to the eventual nucleophilic acceptor of the acyl moiety being transferred. Whereas CmaE and SyrC catalyze net transthioesterification, CouN7 catalyzes formation of an oxoester from a thioester. The S_{101}A mutant of CouN7 was constructed, expressed, and purified, and when subjected to activity analysis, was catalytically inactive. In particular, when the [^{14}C]acetyl transfer experiment of Figure 3A was repeated with the S_{101}A mutant enzyme, as shown in Figure 3C, there was no transient acetylation of the CouN7 mutant enzyme, nor was there any loss of radioactivity from [^{14}C]acetyl-*S*-CouN1. We conclude that Ser_{101} is indeed part of the CouN7 catalytic apparatus and the likely nucleophile for covalent acyl-enzyme formation in each catalytic turnover. The shuttling of the 5-methylpyrrolyl-2-carbonyl acyl group from thiol donor to alcohol acceptor (3′-OH of noviosyl ring) goes through an enzyme oxoester species.

CouN7 Catalyzes the Back Reaction: Deacylation of Coumermycin A_1

The intermediacy of a covalent acyl-*O*-enzyme intermediate, and the observation that the acetyl-enzyme version was hydrolytically decomposed in the absence of the second substrate, raised the question of whether the normal 5-methylpyrrolyl-2-carbonyl acyl-enzyme might be hydrolytically labile. In turn, given that both the covalent enzyme intermediate and the coumermycin product was a pyrrolyl-2-carboxy ester, we asked if CouN7 would catalyze the reverse acyl transfer as manifested in hydrolysis of coumermycin A_1. Indeed, the data of Figure 4A show HPLC traces that indicate CouN7-mediated conversion of the bis-5-methylpyrrolylcoumermycin A_1 antibiotic **3** first to the monodes-5-methylpyrrolyl **6** and then to the bisdes-5-methylpyrrolyl scaffold **5**. The hydrolytic deacylation follows Michaelis-Menten kinetics, yielding a k_{cat} of 0.054 ± 0.002 min^{-1} and a K_M of 63 ± 9 μM for coumermycin in the back reaction (Figure 4B). A separate set of



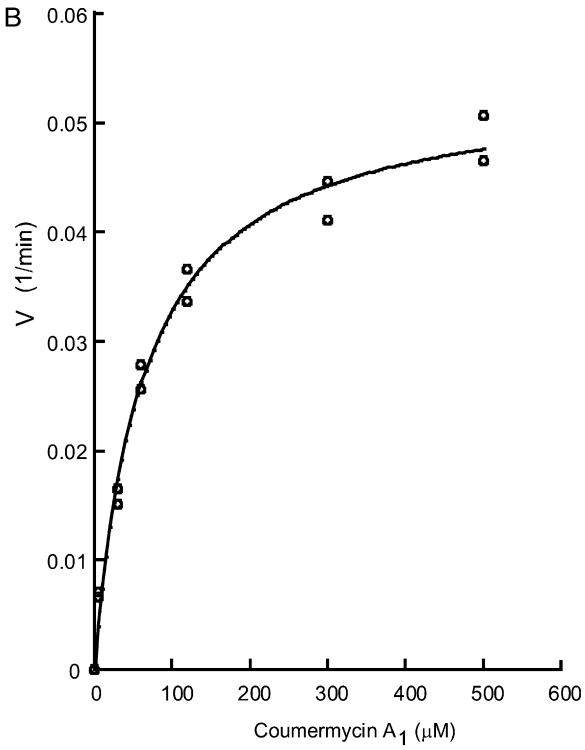


Figure 4. CouN7 Will Deacylate Coumermycin A_1

(A) HPLC time course of reaction containing 50 μM coumermycin A_1 **3**, and 20 μM CouN7. Products were monitored at 340 nm.

(B) Michaelis-Menten kinetic parameters for coumermycin A_1 **3** in the reverse deacylation reaction with 3, 7, or 15 μM CouN7, and various concentrations of coumermycin A_1 **3** ranging from 5 to 500 μM.

experiments showed that CouN7 will not deacylate novobiocin **1** (data not shown), suggesting the carbamoyl group is not an acceptable acyl surrogate for CouN7.

In order to verify that the reverse deacylation proceeds via the same Ser_{101}-*O*-acyl-enzyme intermediate, and was not attributed to a potential secondary editing site on CouN7, we turned again to radioactive labeling experiments. With [^{14}C]acetyl-CoA loaded onto CouN1 by the action of Sfp, a small scale-up reaction was performed to generate mono- and bisacetylated versions of coumermycin, formed by the action of CouN7 on the des-5-methylpyrrolylcoumermycin scaffold **5**. These [^{14}C]-labeled coumermycins were then incubated with CouN7 for various amounts of time, quenched with SDS, and rapidly analyzed by SDS-PAGE and autoradiography. As illustrated in Figure 5, the [^{14}C]acetyl radiolabel is transferred from coumermycin to CouN7 within 30 s. Labeled CouN7 is detectable throughout the 120 min time course, although in lesser amounts as time progresses. In parallel experiments with the CouN7 S_{101}A mutant, no radioactive label is transferred from acetylated coumermycin. Thus, CouN7 deacylates coumermycin via its Ser_{101} active-site residue. The acyl-enzyme species can then reacetylate the coumermycin scaffold or can be captured by a water molecule for net hydrolysis of the labile acetyl-enzyme species.

Two points can be drawn. First, CouN7 will catalyze reversible transfer of the 5-methylpyrrolyl-2-carbonyl

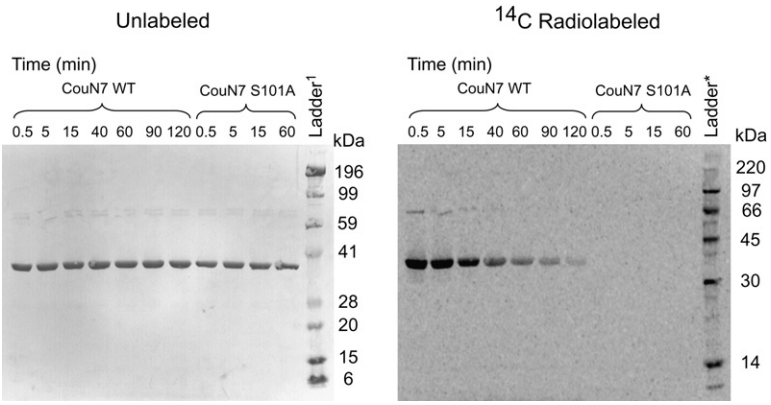


Figure 5. Deacylation of Coumermycin A_1 3 Proceeds via the Same Ser_{101} acyl-*O*-CouN7 Intermediate as the Forward Acylation Reaction

Time course of 50 μM [^{14}C]acetylcoumermycin **7/8** reacting with 25 μM CouN7 or CouN7 S_{101}A. Both an unlabeled coomassie-stained gel and [^{14}C] autoradiography gel are shown. Ladder^1 is Bio-Rad prestained SDS-PAGE standard, broad range. Ladder* is [^{14}C]methylated proteins, GE Healthcare.

acyl group and the 5-methylpyrrolyl-2-carbonyl enzyme intermediate then is susceptible to hydrolysis. Second, the k_{cat} for the back reaction is about 1% the k_{cat} for the forward reaction. It is not yet clear what steps are rate determining in either direction, but at a ratio of 1:100 for coumermycin acylation to net deacylation, the hydrolysis of the acyl-*O*-CouN7 enzyme intermediate should not preclude accumulation of the final antibiotic at the end of the multistep biosynthetic pathway. However, as noted below, the catalytic capacity of CouN7 for the back acyl transfer suggested that additional shuttling reactions could be investigated.

Shuttling of the 5-Methylpyrrolyl-2-Carbonyl Group between Coumermycin and Novobiocin Scaffolds

The hydrolytic back reaction for CouN7-mediated 5-methylpyrrolyl-2-carbonyl transfer from coumermycin A_1 to water suggested that one might be able to intercept the proposed 5-methylpyrrolyl-2-carbonyl-enzyme intermediate with an alternate noviose-containing acceptor. Descarbamoylnovobiocin was readily available and, when incubated with coumermycin A_1 and enzyme, acyl transfer was demonstrable as shown in Figure 6. During the 50 min incubation, almost all the starting coumermycin A_1 **3** is gone after 22 min. As anticipated, the monodeacylated coumermycin scaffold **6** builds up, followed by the bisdesacyl **5**. Meanwhile, a small 5-methylpyrrolylnovobiocin **9** peak is detectable even at 0.5 min, and grows in over the first 30 min of incubation. Under these reaction conditions, 62% ± 1% of the 5-methylpyrrole-2-carbonyl removed from coumermycin A_1 is successfully transferred to descarbamoylnovobiocin by CouN7, the rest presumably being lost to hydrolysis by water. Because CouN7 has a higher affinity for the coumermycin scaffold compared to the novobiocin scaffold, these kinds of incubations were run with large molar excess of the descarbamoylnovobiocin scaffold **4**. Otherwise, it does not outcompete reacylation of monodesacyl **6** and bisdesacyl **5**.

Transfer of an Alternate Acyl Group between the Novobiocin and Coumermycin Scaffolds: 5-Methylthiophene-2-Carbonyl Transfer

As a test for CouN7-dependent transfer of an alternate acyl group between noviosyl acceptors, 5-methylthiophene-2-carbonyl was utilized as a pyrrole-2-carbonyl substitute. We have recently demonstrated that Sfp will utilize the 5-methylthiophene-CoA with apo CouN1 to generate the sulfur-substituted, five-membered heterocyclic acyl donor (M. Fridman, C.J.B., T. Lupoli, D. Kahne, C.T.W., and S.G.-T., data not shown). That molecule could be used for direct transfer by CouN7, either to descarbamoylnovobiocin **4** to yield **10**, or to the des-5-methylpyrrolylcoumermycin scaffold **5** to yield **11** and **12** (Figure 7A, standards). When **10** was incubated with **5** in the presence of enzyme, the HPLC traces of Figure 7A show transfer of the 5-methylthiophene-2-carbonyl group into the coumermycin scaffold. The monoacylated coumermycin scaffold **11** is precursor to a small amount of bisacyl coumermycin product **12**. Under these reaction conditions, 80% ± 5% of the 5-methylthiophene-2-carbonyl removed from 5-methylthiophene novobiocin is successfully transferred to des-5-methylpyrrolylcoumermycin by CouN7, the rest presumably being lost to hydrolysis by water. With optimization of substrates, enzyme concentration, and time, it should be possible to make amounts of mono- and bisacyl coumermycin derivatives, including ones with two distinct acyl groups on each noviosyl arm, for evaluation of any improved antibiotic properties.

DISCUSSION

In recognition of the continuing need for new antibiotics to combat contemporary drug-resistant bacterial pathogens, several research groups have turned back to natural product scaffolds. These include both new molecules, such as platensimycin [38], and previously discovered classes, including the lipodepsipeptides, such as daptomycin [39, 40], ADEPs [41], and mannopeptimycins [42], for reevaluation of clinical utility. We [28, 29, 31, 32, 43–48] and the group of Lutz Heide and colleagues at Tübingen [10–27, 31, 32, 49–52] have been studying the biosynthesis of the aminocoumarin class of antibiotics that target the type II topoisomerase DNA gyrase [1–3]. This enzyme is a validated killing target for bacteria, as exemplified by the widespread use of several synthetic quinolone antibacterial drugs [4, 5]. The quinolones act on the GyrA subunits, while the aminocoumarins, which have not been in wide clinical use, target the GyrB subunits—specifically,

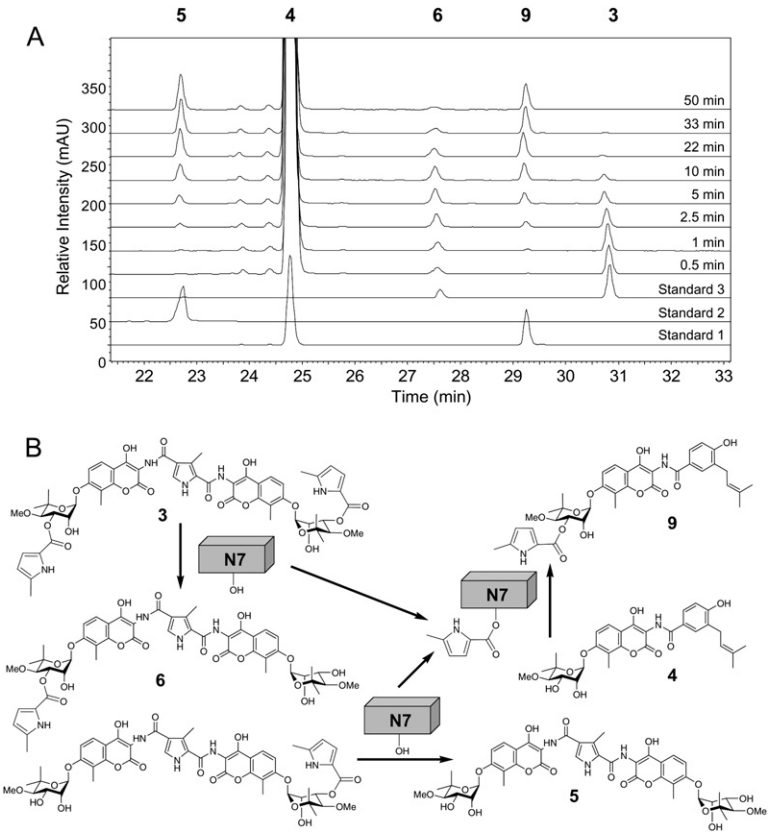


Figure 6. CouN7 Will Shuttle 5-Methylpyrrolyl-2-Carbonyl from Coumermycin A_1 3 to Descarbamoylnovobiocin 4

(A) HPLC time course of reactions containing 50 μM coumermycin A_1 **3**, 200 μM descarbamoylnovobiocin **4**, and 10 μM CouN7. Standard 1 is of descarbamoylnovobiocin **4** and 5-methylpyrrolylnovobiocin **9**. Standard 2 is of des-5-methylpyrrolylcoumermycin **5.** Standard 3 is of mono-5-methylpyrrolylcoumermycin **6** and bis-5-methylpyrrolylcoumermycin **3** generated from des-5-methylpyrrolylcoumermycin **5**. Products were monitored at 340 nm. (B) Scheme depicting the reaction in (A).

the ATPase site involved in driving DNA coiling [1–3]. X-ray structures of the N-terminal 24 kDa fragment of GyrB complexed with either novobiocin or clorobiocin reveal that the acyl substituent on the 3′-OH of the noviosyl ring projects into the GyrB ATPase active site as the key pharmacophore [6–8]. The replacement of the carbamoyl moiety in novobiocin by the 5-methylpyrrolyl-2-carbonyl substituent in clorobiocin (and coumermycin) results in a 10-fold increase in affinity [30]. The noviosyl-aminocoumarin framework can be viewed as a platform for presentation of the 3′-*O*-acyl groups to GyrB.

We and the Heide group have thus been focusing on the biosynthesis and the regioselective installation of the 5-methylpyrrolyl-2-carbonyl moiety in the last stages of aminocoumarin antibiotic maturation [20, 29]. Building on the in vivo evidence of Heide and colleagues, we have recently demonstrated that CouN1 is the free-standing carrier protein domain that, when acylated on the phosphopantetheinyl prosthetic arm, is the acyl donor, and that CouN7 is the 5-methylpyrrolyl-2-carbonyl acyltransferase.

In this study, we have noted that CouN7 can catalyze a slow deacylation of coumermycin A_1, resulting in a net transfer of the 5-methylpyrrolyl-2-carbonyl unit to water to yield the free 5-methylpyrrolylcarboxylate and first mono-5-methylpyrrolylcoumermycin **6**, and subsequently des-5-methylpyrrolylcoumermycin **5**. The k_{cat} of 0.054 min^{-1} is about one one-hundredth the rate of the forward reaction, 5.9 min^{-1}, such that the hydrolytic deacylation reaction is not a concern in the buildup of the mature coumermycin. Nonetheless, we felt that the hydrolytic back reaction capability of CouN7 might be mechanistically diagnostic of an acyl-enzyme intermediate.

Direct demonstration of an acylated CouN7 was obtained with [^{14}C]acetyl-*S*-pantetheinyl CouN1 as surrogate acyl donor. This derived from commercially available [^{14}C]acetyl-CoA, the apo form of the CouN1 carrier proteins, and the phosphopantetheinyltransferase Sfp [37], which we have previously used to install 5-methylpyrrolyl-2-carbonyl- and pyrrolyl-2-carbonyl-*S*-pantetheinyl units on CouN1 [29]. There is clear evidence from SDS-PAGE analysis that a [^{14}C]acetyl-CouN7 forms transiently and then transfers the acetyl group to the des-5-methylpyrrolylcoumermycin scaffold to yield first the monoacetylcoumermycin **7**, and then the bisacetylcoumermycin **8** variants. Thus, the acetyl-CouN7 form of the enzyme is chemically competent as a reaction intermediate. Bioinformatic analysis with other α,β-hydrolases suggests the side chain –OH of Ser_{101} as candidate for catalytic nucleophile. The $S_{101}A$ mutant was indeed catalytically inactive and could not be acetylated. Consistent with the mechanistic prediction, we thus identify the acyl-enzyme in normal turnover as the 5-methylpyrrolyl-2-carbonyl-*O*-Ser_{101}-CouN7.

The fact that CouN7 catalysis proceeds via covalent acyl-enzyme formation suggested that it might be possible to transfer the itinerant acyl group between different noviosyl-aminocoumarin scaffolds. This was first

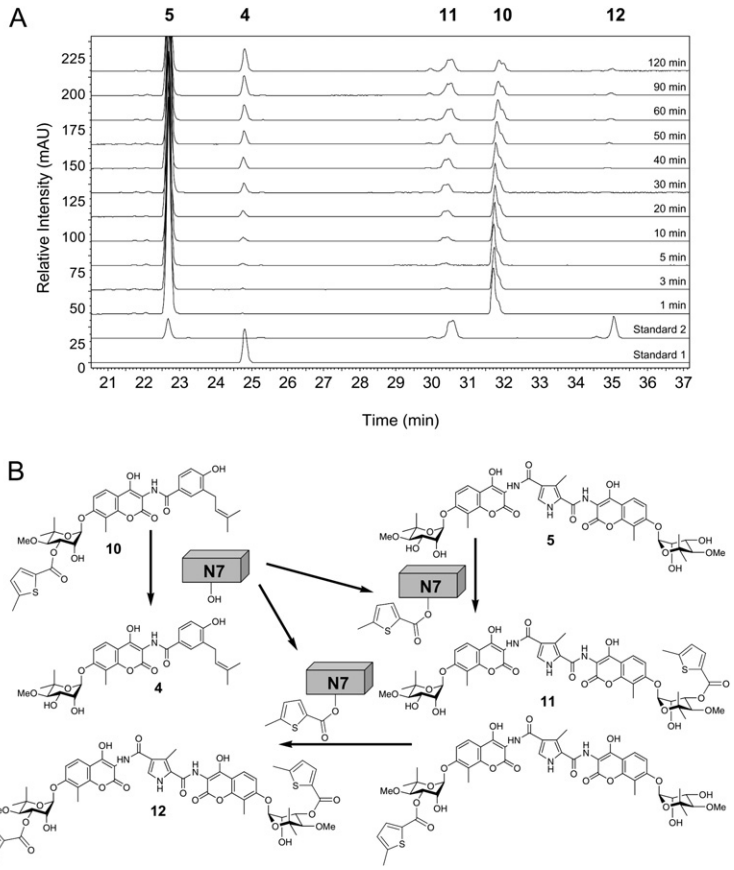


Figure 7. CouN7 Will Shuttle 5-Methylthiophene-Carbonyl from 5-Methylthiophene-Novobiocin 10 to Des-5-Methylpyrrolylcoumermycin 5 and Mono-5-Methylthiophene-Coumermycin 11

(A) HPLC time course of reactions containing 50 μM 5-methylthiophene-novobiocin **10**, 120 μM des-5-methylpyrrolylcoumermycin **5**, and 20 μM CouN7. Standard 1 is of descarbamoylnovobiocin **4**. Standard 2 is of mono-5-methylthiophene-coumermycin **11** and bis-5-methylthiophene-coumermycin **12** generated from des-5-methylpyrrolylcoumermycin **5**. Products were monitored at 340 nm.

(B) Scheme depicting the reaction in (A).

validated by starting with coumermycin A_1 **3** and descarbamoylnovobiocin **4** (free noviosyl 3′-OH) as the potential acceptor. Addition of CouN7 led to a time-dependent deacylation of coumermycin A_1 and concomitant formation of the 5-methylpyrrolylnovobiocin **9** product, reflecting a successful competition by the novobiocin scaffold for the 5-methylpyrrolyl-*O*-Ser_{101}-CouN7.

In terms of potential utility for creating coumermycin (and clorobiocin) analogs with altered 3′-*O*-acyl pharmacophores and consequent distinctions in antibiotic properties, transfer of other acyl groups between scaffolds would be of interest. We have recently investigated CouN7 for permissivity for acyl group transfer by starting from several heterocyclic-2-carboxylates, conversion to the acyl-CoAs, installation as acyl-*S*-pantetheinyl-CouN1 derivatives, and then transfer to the descarbamoylnovobiocin scaffold (M. Fridman, C.J.B., T. Lupoli, D. Kahne, C.T.W., and S.G.-T., data not shown). The 5-methylthiophene-2-carboxylate in which the nitrogen of the pyrrole ring has been replaced by sulfur is a prototypically good substrate, allowing accumulation of the 5-methylthiophenyl derivative of novobiocin. Staring from this variant, 5-methylthiophene-novobiocin **10**, and the des-5-methylpyrrolylcoumermycin **5**, CouN7 transfers the 5-methylthiophene-2-carbonyl sequentially to the 3′-OH of the noviosyl arms in the coumermycin scaffold. It is likely that bisacyl coumermycins, including those with different acyl moieties on each of the novioses in the two arms of coumermycin, can be constructed by such enzymatic shuffling of acyl groups from acyl-CoAs via the CouN1 carrier protein route.

Our findings demonstrating shuffling of acyl moieties between distinct aminocoumarin scaffolds by the acyltransferase CouN7 parallel those recently reported for glycosyltransferases in the vancomycin and calicheamicin biosynthetic systems [53]. In that study, it was shown that glycosyltransferases too are reversible, and can deglycosylate their products to form NDP sugars, which in turn can be transferred to new scaffolds. The reversibility of tailoring enzymes within natural product biosynthetic systems may be a general phenomenon, and could possibly be exploited for the combinatorial synthesis of novel compounds.

CouN7 and its close congener, CloN7, in clorobiocin assembly join two other tailoring enzymes recently identified in NRPS assembly lines. CmaE shuttles an *allo*-Ile group between carrier protein domains in coronamic acid biogenesis [33], while SyrC moves a 4-chloro-Thr between pantetheinyl arms of SyrB1 and SyrE in maturation of the phytotoxin syringomycin [36]. While CmaE and SyrC use active-site Cys thiolate side chains as nucleophiles to make aminoacyl-*S*-enzyme intermediates in the net transthiolations, CouN7 uses an acyl-*O*-Ser enzyme intermediate and transfers the migrating acyl moiety to an oxygen nucleophile in the coumermycin scaffold. It will be of interest to ascertain other assembly lines with shuttle enzymes

and to determine the practical utility for shuffling of other assembly lines in natural product biosynthetic diversification efforts.

SIGNIFICANCE

Bacterial DNA gyrase is targeted by the synthetic quinolone antibacterial agents on the GyrA subunits and the natural product aminocoumarins on the GyrB subunits in the ATP binding site. The bicyclic aminocoumarin scaffolds of clorobiocin and coumermycin present an L-noviosyl sugar substituted at the 3′-OH with a 5-methylpyrrolyl-2-carbonyl acyl moiety as the pharmacophore. This acyl group is installed at the end of the biosynthetic pathway by the acyltransferases, ClоN7/CouN7. Here, we show that CouN7 catalyzes a slow hydrolytic deacylation of the 3′-*O*-acyl groups on both arms of coumermycin via an acyl-*O*-Ser$_{101}$-CouN7 intermediate. The acyl-*O*-CouN7 enzyme intermediate can be intercepted by descarbamoylnovobiocin 4 to shuttle the 5-methylpyrrolyl-2-carbonyl group between coumarin scaffolds. Also, use of an alternate acyl group, such as the 5-methylthiophene-2-carbonyl moiety presented on the pantetheinyl arm of the CouN1 donor protein or on the 3′-OH of L-noviose on the novobiocin scaffold, allows CouN7-mediated mono- and bis-acylation of the des-5-methylpyrrolylcoumermycin scaffold 5. Thus, CouN7 should be useful for shuttling different acyl groups between the noviosyl-3′-OH units of different aminocoumarins to generate variant pharmacophores in this natural product antibiotic class.

EXPERIMENTAL PROCEDURES

Materials and General Methods

Standard recombinant DNA, molecular cloning, and microbiological procedures were performed as previously described [54]. Competent TOP10 and BL21 (DE3) *E. coli* strains were from Invitrogen. Oligonucleotide primers were from Integrated DNA Technologies. Phusion DNA polymerase, restriction enzymes, and T4 DNA ligase were from New England Biolabs. Plasmid pET28a was from Novagen. DNA sequencing to verify PCR fidelity was performed on double-stranded DNA by the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). Plasmid DNA preparation was performed with the Qiaprep kit from Qiagen. Gel extraction of DNA fragments, as well as restriction endonuclease cleanup, were done with the GFX kit from GE Healthcare. Ni-NTA Superflow resin was from Qiagen. SDS-PAGE gels were from Invitrogen. Protein samples were concentrated with a 10KMWCO Amicon Ultra device from Millipore, and final protein concentrations were calculated with the protein's absorbance at 280 nm and the predicted molar extinction coefficient.

Radio-HPLC was performed on a Beckman Coulter System Gold instrument equipped with a β-Ram module 3 radioisotope detector (IN/US Systems). LCMS identification was carried out on a Shimadzu LCMS-QP8000α equipped with two LC-10AD*VP* liquid chromatography pump modules, an SPD-10AV*VP* UV-vis detector, an SIL-10AD*VP* autosampler module, and a Vydac C18 Mass Spec column (5 μm, 2.1 × 250 mm). Analytical HPLC was performed with a Beckman Coulter System Gold instrument. Radiolabeled SDS-PAGE gels were exposed to a BAS-IIIs image plate for approximately 24 hr and subsequently read by a Typhoon 9400 variable mode imager (GE Healthcare) followed by analysis with ImageQuant Software.

[^{14}C]acetyl-CoA (56 mCi/mmol) was purchased from GE Healthcare. 5-Methylpyrrolyl-2-carbonyl-CoA [29], 5-methylthiophene-2-carbonyl-CoA and descarbamoylnovobiocin (M. Fridman, C.J.B., T. Lupoli, D. Kahne, C.T.W., and S.G.-T., data not shown), and des-5-methylpyrrolylcoumermycin (CouLM product + CouP) [32] were obtained as previously descibed. Coumermycin A$_1$ was purchased from Sigma and novobiocin was purchased from Calbiochem.

Cloning and CouN7 S$_{101}$A

CouN7 S$_{101}$A was obtained by the splicing by overlap extension method [55] with pCouN7-pET28a [29] as template. The template was amplified with the following primers in the first round (underlined, restriction site; italics, mutation): (a) 5′-GCATGACT<u>CATATG</u>GCGAACCACCGACCGG-3′ and 5′-CAGCGCGAG*C*G*C*GGTCACACCGCCTCCCGCGCTGGCGAAC-3′; (b) 5′-GCCGGGCCG*GCG*TACTTGTTCGCCAGCGCGGGAGGCGGTG-3′ and 5′-CGT<u>GAATTC</u>TTAGGGACCGTTGTGCGAGCGC-3′. The a and b fragments were mixed and then amplified with the first forward primer from the a fragment and the second reverse primer from the b fragment. The final PCR product was digested with *Nde*I/*Hin*dIII and ligated into a similarly digested pET28a vector.

Protein Expression

CouN1, CouN7 wild-type, and CouN7 S$_{101}$A mutant proteins were expressed and purified as described previously [29]. The mutant protein expressed at 20 mg/l and behaved identically to the wild-type during isolation.

Characterization of CouN7 Activity on Des-5-Methylpyrrolylcoumermycin 5

The initial reaction time course to determine the ability of CouN7 to acylate des-5-methylpyrrolylcoumermycin was performed as follows. The CouN1 loading reaction (50 μl) contained 10 mM $MgCl_2$, 75 mM Tris (pH 7.5), 1 mM TCEP (pH 7), 150 μM CouN1, 150 μM 5-methylpyrrolyl-CoA, and 5 μM Sfp, and proceeded for 1 hr at RT. The reaction to produce des-5-methylpyrrolylcoumermycin (25 μl) contained 10% DMSO, 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 20 μM CouLM product, 500 μM SAM, 1 mg/ml BSA, and 10 μM CouP, and proceeded for 6 hr at RT. The two reactions were then mixed, initiated with 12.5 μl of 149 μM CouN7, and quenched with 150 μl of methanol at desired time points. The quenched reaction was placed at −20°C for 20 min, centrifuged at 13,000 rpm to pellet protein, and the supernatant was added to 50 μl of 1 M ammonium acetate (pH 3.5). HPLC analysis was performed on a Vydac C18 small-pore column (250 × 4.6 mm) with a gradient of 15%–100% acetonitrile over 30 min, starting with 0.1% TFA in H_2O. Products were monitored at 340 nm.

Kinetics were performed as follows. A 40 μM solution of des-5-methylpyrrolylcoumermycin was made by incubating 10% DMSO, 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 40 μM CouLM product, 500 μM SAM, 1 mg/ml BSA, and 25 μM CouP for 3 hr at RT. Reactions for priming CouN1 contained 10 mM $MgCl_2$, 75 mM Tris (pH 7.5), 1 mM TCEP (pH 7), 150 μM CouN1, 150 μM 5-methylpyrrolyl-CoA, and 5 μM Sfp, and proceeded for 1 hr at RT. The final 75 μl reaction contained 50 μl of the CouN1 priming reaction, 0.1 μM CouN7, and various concentrations of des-5-methylpyrrolylcoumermycin (0.05, 0.1, 0.3, 0.75, 2, 5, and 12.5 μM). Reactions were quenched and analyzed as described previously here.

Reactions to visualize the acyl-enzyme intermediate were done at RT and contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 250 μM CouN1, 5 μM Sfp, 375 μM unlabeled acetyl-CoA, 125 μM [^{14}C]acetyl-CoA, and, after 1 hr to allow for CouN1 loading, 25 μM of CouN7(S$_{101}$A) was added. At various time points, 5 μl of sample was quenched into 10 μl of 2× SDS sample buffer (125 mM Tris [pH 6.8], 41% SDS, 20% glycerol, 0.01% Brilliant Blue) and loaded onto a 12% NuPAGE Bis-Tris gel. After running, the gel was transferred to a PVDF membrane (Bio-Rad) before phosphorimaging.

Reactions to produce acetylated coumermycin contained the following. A 345 μM solution of des-5-methylpyrrolylcoumermycin was

made by incubating 10% DMSO, 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 345 μM CouLM product, 5 mM SAM, 1 mg/ml BSA, and 50 μM CouP for 3 hr at RT. Reactions for priming CouN1 contained 10 mM $MgCl_2$, 75 mM Tris (pH 7.5), 1 mM TCEP (pH 7), 300 μM CouN1, 400 μM acetyl-CoA, and 10 μM Sfp, and proceeded for 1 hr at RT. The final 50 μl acetylation reaction contained 28.52 μl of the CouN1 priming reaction, 16.67 μl of the 345 μM des-5-methylpyrrolylcoumermycin, 20 μM CouN7, and 10% DMSO. A 4 μl aliquot of the reaction was quenched into 150 μl methanol, 71 μl of water was added, and analysis was conducted by HPLC as described previously here.

Characterization of CouN7-Mediated Deacylation of Coumermycin A_1

Kinetics of deacylation were carried out as follows. Reactions contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 1 mg/ml BSA, either 3, 7, or 15 μM CouN7, and various amounts of coumermycin A_1, ranging between 5 and 500 μM. At desired time points, 75 μl reactions were quenched and analyzed as described previously here.

To produce [^{14}C] labeled mono- and bisacetylcoumermycin, a small scale up was performed. A 345 μM solution of des-5-methylpyrrolylcoumermycin was made by incubating 10% DMSO, 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 345 μM CouLM product, 5 mM SAM, 1 mg/ml BSA, and 50 μM CouP for 3 hr at RT. Reactions for priming CouN1 contained 10 mM $MgCl_2$, 75 mM Tris (pH 7.5), 1 mM TCEP (pH 7), 300 μM CouN1, 233 μM [^{14}C]acetyl-CoA, 250 μM unlabeled acetyl-CoA, and 10 μM Sfp, and proceeded for 1 hr at RT. The final 200 μl reaction contained 10% DMSO, 115 μM of des-5-methylpyrrolylcoumermycin, 114 μl of the CouN1 priming reaction, and 20 μM CouN7. After 20 min, the reaction was quenched into 400 μl methanol and worked up as usual. The mono- and bisacetylated products were purified by HPLC, evaporated to dryness, and redissolved in DMSO for future use.

Reactions to visualize the acyl-enzyme intermediate contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 25 μM CouN7(S_{101}A), 50 μM [^{14}C]acetylcoumermycin **7**/**8**, and 10% DMSO. At various time points, 5 μl of sample was quenched into 10 μl of 2× SDS sample buffer (125 mM Tris [pH 6.8], 41% SDS, 20% glycerol, 0.01% Brilliant Blue) and loaded onto a 12% NuPAGE Bis-Tris gel. After running, the gel was transferred to a PVDF membrane (Bio-Rad) before phosphorimaging.

Shuttling of Acyl Substituents between Scaffolds

Reactions to demonstrate shuttling of 5-methylpyrrole between coumermycin A_1 and descarbamoylnovobiocin **4** contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 1 mg/ml BSA, 200 μM descarbamoylnovobiocin, 3 μM coumermycin A_1, 7.5% DMSO, and 10 μM CouN7. At desired time points, the reactions were quenched and analyzed by HPLC as described previously here, except that a Phenomenex 5 μ C_{18} Luna (250 × 4.6 mm) column was used instead. Standards for 5-methylpyrrolylnovobiocin **9** and mono-5-methylpyrrolylcoumermycin **6** began by priming N1 with 5-methylpyrrolyl-CoA. Reactions contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 500 μM 5-methylpyrrolyl-CoA, 400 μM CouN1, and 10 μM Sfp, and proceeded for 1 hr at RT. Final acylation reactions contained 50 μl of the N1 priming reaction, 10% or 5% DMSO, 10 μM des-5-methylpyrrolylcoumermycin or 200 μM descarbamoylnovobiocin, 1 mg/ml BSA, and 3 or 25 μM CouN7, respectively. The coumermycin reaction was quenched at 15 min, and the novobiocin reaction was quenched at 2 hr.

The scale-up reaction for producing 5-methylthiophene-novobiocin **10** contained 10 mM $MgCl_2$, 75 mM Tris (pH 7.5), 1 mM TCEP (pH 7), 2.5 mM 5-methylthiophene-CoA, 1.5 mM CouN1, and 20 μM Sfp (150 μl). After priming for 1 hr at RT, 5% DMSO, 125 μM descarbamoylnovobiocin, 1 mg/ml BSA, and 125 μM CouN7 was added to a final volume of 225 μl, and the reaction was quenched after 2 hr as described previously here. The product was purified by HPLC, evaporated to dryness, and redissolved in DMSO for future use.

Reactions to demonstrate shuttling of 5-methylthiophene between novobiocin and des-5-methylpyrrolylcoumermycin contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 1 mg/ml BSA, 50 μM 5-methylthiophene-novobiocin, 120 μM des-5-methylpyrrolylcoumermycin, and 20 μM CouN7. At desired time points, the reactions were quenched and analyzed by HPLC as described previously here. Standards for mono- and bis-5-methylthiophene-coumermycin began by priming N1 with 5-methylpyrrolyl-CoA. Reactions contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM TCEP (pH 7), 400 μM 5-methylthiophene-CoA, 300 μM CouN1, 10 μM Sfp, and proceeded for 1 hr at RT. Final acylation reactions contained 6.84 μl of the N1 priming reaction, 10% DMSO, 115 μM des-5-methylpyrrolylcoumermycin, and 20 μM CouN7. The reaction was quenched at 15 min.

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