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Characterization of the Aminocoumarin Ligase SimL from the Simocyclinone Pathway and Tandem Incubation with NovM,P,N from the Novobiocin Pathway†

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ABSTRACT: Simocyclinone D_8 consists of an anguicycline *C*-glycoside tethered by a tetraene diester linker to an aminocoumarin. Unlike the antibiotics novobiocin, clorobiocin, and coumermycin A_1, the phenolic hydroxyl group of the aminocoumarin in simocyclinone is not glycosylated with a decorated noviosyl moiety that is the pharmacophore for targeting bacterial DNA gyrase. We have expressed the *Streptomyces antibioticus* simocyclinone ligase SimL, purified it from *Escherichia coli*, and established its ATP-dependent amide bond forming activity with a variety of polyenoic acids including retinoic acid and fumagillin. We have then used the last three enzymes from the novobiocin pathway, NovM, NovP, and NovN, to convert a SimL product to a novel novobiocin analogue, in which the 3-prenyl-4-hydroxybenzoate of novobiocin is replaced with a tetraenoate moiety, to evaluate antibacterial activity.

The aminocoumarin antibiotics novobiocin **1**, clorobiocin **2**, and coumermycin A_1 **3**, produced by various *Streptomyces* species (Figure 1), contain a bicyclic 3-amino-4,7-dihydroxycoumarin ring system, which serves as an essential scaffold for targeting them to the bacterial type II topoisomerases DNA gyrase and topoisomerase IV (*1*−*5*). Coumermycin A_1 is a pseudosymmetric dimer, containing elements of novobiocin (the 8-methylaminocoumarin) and clorobiocin (the 3′-*O*-methylpyrrolyl acyl group). Cocrystals of the N-terminal 24 kDa subfragment of the DNA gyrase B subunit (GyrB)[1] with **1** and **2** (*1*, *2*) reveal that this family of antibiotics uses the aminocoumarin as a planar scaffold to present the decorated 4′-*O*-methyl-3′-*O*-acyl noviosyl moiety as the pharmacophore for inhibiting ATP hydrolysis in GyrB. These decorations of the L-deoxy sugar noviose include 4′-O-methylation and 3′-O-acylation with either a carbamoyl or methylpyrrolyl group. This 3′-*O*-acyl substituent is in intimate contact with GyrB active site residues and/or bound water molecules.

Recently, another antibiotic containing the conserved aminocoumarin moiety, simocyclinone D **4** from *Streptomyces antibioticus*, has been discovered (*6*, *7*). Simocyclinone D is a structurally unique antibiotic containing an aromatic anguicycline polyketide nucleus, the deoxy sugar D-olivose, and either the 8-chloro or 8-desmethyl version of the 3-amino-4,7-dihydroxycoumarin (*8*). The anguicycline *C*-glycoside and the aminocoumarin elements are linked by a tetraene dicarboxylic acid moiety. Simocyclinone possesses antimicrobial activity against Gram-positive bacteria, as well as exhibiting cytostatic effects against human tumor cell lines (*6*). However, its lack of a decorated noviosyl moiety at the 7-hydroxy position of the aminocoumarin scaffold suggests a molecular mechanism of action different than that of **1** and **2** given the X-ray information on GyrB complexes noted above for **1** and **2** (*1*, *2*). Indeed, recent studies reveal that simocyclinone D8 is a potent inhibitor of gyrase, albeit through a novel mode of action by preventing the initial binding of gyrase to DNA (Anthony Maxwell, personal communication, John Innes Centre, Norwich). Significantly, the aminocoumarin moiety of simocyclinone D is crucial to this potent inhibitory activity.

Further inspection of the aminocoumarin natural products **1**−**4** reveals three distinct types of carboxylic acid elements in amide linkage to the 3-amino group of the aminocoumarin rings. While novobiocin and clorobiocin utilize a 3-prenyl-4-hydroxybenzoate, the two aminocoumarins in coumermycin are linked by the two carboxylates of 3-methylpyrrole-2,4-dicarboxylic acid. In simocyclinone D, again an organic diacid is coupled, but in this case it is the $\Delta^{2,4,6,8}$ tetraene C_{10} diacid, in which one end is ligated to the aminocoumarin and the other is tethered to the polyketide glycoside.

The biosynthetic gene clusters for **1**−**4** (*9*−*12*) have been sequenced and the ATP-dependent amide bond forming ligases identified. NovL, CloL, and CouL have been purified, and their ligase activity has been validated (*13*−*15*), providing the assurance that SimL is indeed the complementary simocyclinone ligase for simocyclinone biosynthesis. Previous efforts to introduce diversity in the aminocoumarins using the NovL, CloL, and CouL ligases have been limited by their specificity for benzoic acid or pyrrolecarboxylic acid sub-

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[1] Abbreviations: GyrB, DNA gyrase B subunit; IPTG, isopropyl *β*-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; RP-HPLC, reverse-phase high-performance liquid chromatography; SAM, *S*-adenosylmethionine; MIC, minimum inhibitory concentration.

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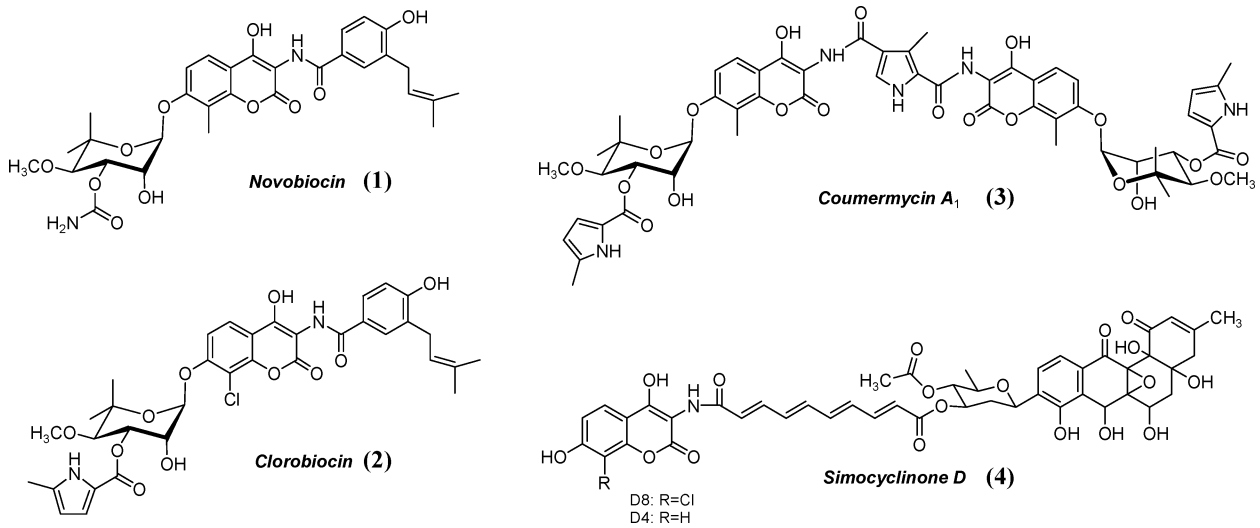


FIGURE 1: The aminocoumarin antibiotics.

strates (*16*, *17*). SimL, however, activates a structurally distinct polyunsaturated carboxylic acid ester moiety, making it a potentially useful tool in the combinatorial biosynthesis of novobiocin analogues.

In this study we have undertaken the expression and overproduction of the *Streptomyces antibioticus* SimL in *Escherichia coli*. We have tested several polyenoic acids as substrates for SimL ligation to the 8-methylaminocoumarin, available from selective degradation of novobiocin. We have also then examined if a SimL ligation product could be further processed by the last three enzymes of the noviobiocin pathway, NovM, NovP, and NovN (*18*, *19*), to produce a decorated noviosyl-containing novobiocin analogue to evaluate for antibacterial activity.

MATERIALS AND METHODS

Cloning of SimL. The gene encoding *simL* was PCR amplified from a pQE70 expression vector containing the *simL* gene cloned from *S. antibioticus* (obtained from Lutz Heide, Tübingen). PCR amplification of a C-terminally histidine-tagged SimL construct, pSimL-C, was accomplished using the forward primer simL-1 (5′-GAGGAGAAATTACATATGGAAGG CAACGAGCAC-3′) and the reverse primer simL-2 (5′-GTGATGGTGATGAAGCTTTTCG CCATGGGTGGC-3′). These primers introduced the respective *Nde*I and *Hin*dIII restriction sites (underlined above). PCR amplification was performed with Pfu Turbo polymerase (Stratagene). Similarly, the PCR amplification of an N-terminally histidine-tagged SimL construct, pSimL-N, was accomplished using the same forward primer simL-1 and the reverse primer simL-3 (5′-CTAATTAAGCTTAGTGATGGTGATGGTGATG ATCATTCGCC-3′), introducing the same restriction sites. In both constructs, the forward simL-1 primer also changed the *simL* TTG start codon to an ATG start codon. The PCR products were gel-purified, digested with *Nde*I and *Hin*dIII, and ligated into linearized pET37b and pET16b vectors (Novagen) to give the C-terminal His_8-tagged pSimL-C-pET37b and the N-terminal His_{10}-tagged construct pSimL-N-pET16b.

Overexpression and Purification of SimL. The pSimL-C-pET37b and pSimL-N-pET16b expression constructs were transformed into *E. coli* BL21(DE3) competent cells (Invitrogen) for protein overproduction. Transformants harboring the desired constructs were grown at 25 °C in LB supplemented with 50 μg/mL kanamycin (for pSimL-C-pET37b) and 100 μg/mL ampicillin (for pSimL-N-pET16b) to an OD_{600} of 0.6, then induced with IPTG to a final concentration of 60 μM, and grown for an additional 14 h at 25 °C. The cells were harvested by centrifugation (15 min at 6000*g*) and frozen at −80 °C. Thawed cells were resuspended in buffer A [25 mM Tris-HCl (pH 8.0), 400 mM NaCl, 2 mM imidazole, and 10% glycerol] and lysed by French press (three passes at 15000 psi), and the resultant cell debris was removed by centrifugation (30 min at 10000*g*). The supernatant was incubated with 3 mL of Ni-NTA resin (Qiagen) for 2 h at 4 °C. The recovered resin was washed with 50 mL of buffer A and packed into a column, and the protein was eluted using a stepwise gradient of 5−500 mM imidazole. Fractions containing the target protein (judged by SDS−PAGE) were pooled and dialyzed against buffer B [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 10% glycerol] overnight. The protein was dialyzed a second time in buffer C [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM TCEP, and 10% glycerol], concentrated, flash frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined spectrophotometrically at 280 nm using the calculated molar extinction coefficient (51350 M^{-1} cm^{-1}).

Preparation of Decatetraenedioic Acid. To a 1 N NaOH solution (100 mL) was added 25 g of Fumidil-B (Mann-Lake), and the resultant solution was stirred for 24 h. The solution was washed three times with 100 mL portions of ether to remove fumagillol and uncleaved fumagillin. The solution was acidified to pH 1 with 6 N HCl, and a bright yellow precipitate was collected by filtration to give the pure diacid in 82% yield [ESI for $C_{10}H_{10}O_4$: calcd 194.1, obsd 193.0 $(M - H)^-$]. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.20 (br s, 2H, COOH, 7.25 (dd, 2H, J = 15.5 Hz, J = 11.0 Hz), 6.80 (m, 2H), 6.62 (m, 2H), 5.99 (d, 2H, J = 15 Hz).

Preparation of Decatetraenedioic Monomethyl Ester. Decatetraenedioic acid (20 mg) was dissolved in ∼0.6 mL of CH_2Cl_2, 2 equiv of (trimethylsilyl)diazomethane (in hexanes) was added, and the reaction was stirred at room temperature for 24−48 h. The reaction was purified by

preparatory RP-HPLC [linear gradient of 30% (H_2O, 0.1% TFA) to 60% CH_3CN over 30 min]. Fractions containing the diester product were pooled, concentrated under reduced pressure, and lyophilized. Decatetraenedioic dimethyl ester formation was confirmed by LC-MS [ESI for $C_{12}H_{14}O_4$: calcd 222.1, obsd 221.1 $(M - H)^-$] and isolated at 43% yield. The diester (5 mg) was dissolved in ~0.5 mL of THF/H_2O (5:1), 1 equiv of LiOH was added, and the solution was stirred at room temperature for 1−2 h. The reaction was monitored for decatetraenedioic monomethyl ester formation by analytical RP-HPLC and subsequently purified by preparatory RP-HPLC. Fractions containing the monoester product were pooled, evaporated, and concentrated under vacuum (Speedvac). Decatetraenedioic monomethyl ester was isolated at 20% yield, and the product was confirmed by LC-MS [ESI for $C_{11}H_{12}O_4$: calcd 208.1, obsd 207.1 $(M - H)^-$].

Characterization of SimL. Preliminary assays to reconstitute SimL activity contained 1 mM aminocoumarin **5** (*17*, *20*) and 1 mM carboxylic acid substrate and were carried out in buffer containing a final concentration of 75 mM Tris-HCl (pH 8.0), 5 mM ATP, 10 mM $MgCl_2$, 1 mg/mL bovine serum albumin (BSA), and 10% DMSO. Reactions were initiated with SimL and quenched at specific time points with an equal volume of methanol at 4 °C. The quenched reactions were incubated at −20 °C for 20 min and centrifuged to remove precipitated protein (5 min, 13000 rpm). The supernatant of each reaction was analyzed by analytical RP-HPLC [linear gradient of 100% (H_2O, 0.1% TFA) to 100% CH_3CN over 20 min; then 100% CH_3CN for 15 min]. Product formation was confirmed by LC-MS. Carboxylic acid substrates tested include *all-trans*-2,4,6,8-decatetraenoic acid (Toronto Research Chemicals), fumagillin (Sigma), *trans*-retinoic acid (Sigma), 13-*cis*-retinoic acid (Sigma), sorbic acid (Sigma), 2,3,6-octatrienoic acid (Sigma), *trans*,*trans*-muconic acid (Sigma), decanoic acid (Sigma), 10-hydroxydecanoic acid (Sigma), 3-methylpyrrole-2,4-dicarboxylic acid, 3-prenyl-4-hydroxybenzoic acid, decatetraenedioic acid, decatetraenedioic acid monomethyl ester, *N*-biotinyl-8-aminocaprylic acid, and *N*-biotinyl-12-aminododecanoic acid.

For the determination of kinetic parameters for the carboxylic acids accepted by SimL, the concentration of the aminocoumarin substrate was kept constant at 200 μM while the concentration of the acid substrates was varied in the above-described reaction buffer. Each reaction was analyzed as described above, and the product concentration was calculated by comparison with the product standard curves described below. For the decatetraenoic acid reactions, the concentration was varied from 0.1 to 2 mM, initiated with 100 nM SimL, quenched at 10 min, and monitored at the product λ_{max} of 370 nm. For the fumagillin reactions, the concentration was varied from 0.01 to 2.5 mM, initiated with 200 nM SimL, quenched at 7 min, and monitored at 365 nm. For the *trans*-retinoic acid reactions, the concentration was varied from 10 to 250 μM, initiated with 150 nM SimL, quenched at 5 min, and monitored at 390 nm. For the decatetraenedioic acid monomethyl ester reactions, the concentration was varied from 0.25 to 3 mM, initiated with 500 nM SimL, quenched at 45 min, and monitored at 360 nm. All described determinations of K_m and k_{cat} were carried out in triplicate.

For the determination of kinetic parameters for the aminocoumarin ring, the concentration of fumagillin was kept constant at 2 mM while the concentration of aminocoumarin was varied from 2 to 250 μM in the above-described reaction buffer. The reactions were initiated with 100 nM SimL, quenched after 5 min, and analyzed by RP-HPLC while being monitored at 365 nm.

Preparation of SimL Products. The milligram scale enzymatic synthesis of four SimL reaction products (aminocoumarin **5** coupled to either decatetraenoic acid, fumagillin, *trans*-retinoic acid, or decatetraenedioic monomethyl ester) was undertaken as follows. Three separate 5 mL reactions containing 1 mM aminocoumarin, 75 mM Tris-HCl (pH 8.0), 1 mg/mL BSA, 10 mM $MgCl_2$, 5 mM ATP, 10% DMSO, and a given carboxylic acid substrate [either 1 mM 2,4,6,8-decatetraenoic acid or 1 mM fumagillin, 300 μM *all-trans*-retinoic acid or ~200 μM decatetraenedioic acid monomethyl ester] were initiated by the addition of SimL-C-His to a final concentration of 2 μM and were allowed to proceed at ambient temperature for 18−48 h. Reaction progress was monitored by RP-HPLC. The crude reaction mixture was adjusted to pH 6 by the addition of 1 M MES, pH 6, and desalted and concentrated by passage over a C18 SepPak (900 mg bed) (Maxi-Clean Cartridge, Alltech) conditioned with 75 mM MES, pH 6. Following loading of the crude reaction product, the SepPak was washed with 75 mM MES, pH 6 (10 mL), followed by water (10 mL). The crude reaction product was eluted from the SepPak using DMSO (5 mL). The product was further purified by preparatory RP-HPLC using the conditions described above. Fractions containing the desired product were pooled and concentrated under reduced pressure, yielding yellowish solids. Products were confirmed by LC-MS: decatetraenoic acid SimL product **7** [ESI for $C_{20}H_{19}NO_5$: calcd 353.13, obsd 352.1 $(M - H)^-$], fumagillin SimL product [ESI for $C_{36}H_{41}NO_{10}$: calcd 647.27, obsd 646.3 $(M - H)^-$], *trans*-retinoic acid SimL product [ESI for $C_{30}H_{35}NO_5$: calcd 489.3, obsd 488.2 $(M - H)^-$], decatetraenedioic acid monomethyl ester SimL product [ESI for $C_{21}H_{19}NO_7$: calcd 397.12, obsd 396.1 $(M - H)^-$]. The decatetraenoic acid SimL product **7** was also confirmed by ^1H NMR (500 MHz, DMSO-d_6): δ 7.54 (d, 1H, J = 8.3 Hz, coum CH), 7.22 (dd, 1H, J = 14.5 Hz, J = 11.5 Hz, H-3), 6.83 (d, 1H, J = 8.3 Hz, coum CH), 6.73 (dd, 1H, J = 14.5 Hz, J = 11.5 Hz, H-5), 6.44 (m, 3H, H-2, H-4, H-7), 6.31 (dd, 1H, J = 15.2 Hz, J = 11.2 Hz, H-6), 6.18 (dd, 1H, J = 15.2 Hz, J = 11.2 Hz, H-8), 5.88 (m, 1H, H-9), 2.14 (s, 3H, coum Me), 1.79 (d, 3H, H_3-10). Standard curves were generated for each product using analytical reverse-phase HPLC over specific concentration ranges: decatetraenoic acid SimL product (0.009−0.4 nmol), fumagillin SimL product (0.009−1.08 nmol), *trans*-retinoic SimL product (0.025−2 nmol), and the decatetraenedioic monomethyl ester SimL product (0.25−5 nmol).

*Preparation of Novel Novobiocin Analogue **9** via Four-Enzyme Tandem Incubation.* For the generation of compound **9**, NovM, NovP, and NovN were purified as previously described (*18*, *19*). A reaction mixture (50 μL) contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 1 mg/mL BSA, 10% DMSO, 100 μM decatetraenoic acid, and 100 μM aminocoumarin. SimL was added to a final concentration of 1 μM, and the reaction mixture was incubated at room temperature for 12 h. TDP-L-noviose (*18*)

(final concentration = 150 μM) and NovM (final concentration = 1 μM) were added, and the reaction mixture was incubated for an additional 12 h at room temperature. *S*-Adenosylmethionine (SAM) (final concentration = 500 μM) and NovP (final concentration = 1 μM) were added, and the reaction mixture was incubated overnight at room temperature. Finally, carbamoyl phosphate (final concentration = 500 μM) and NovN (1 μM) were added, and the reaction mixture was incubated for an additional 12 h at room temperature. The reaction was then quenched with methanol (100 μL) and incubated at 4 °C for 20 min. The supernatant was analyzed by reverse-phase HPLC and LC-MS.

Milligram Scale Synthesis of Novobiocin Analogue ***9.*** For the preparation of novobiocin analogue **9**, aminocoumarin **5** (2.8 mg, 11.4 μmol) and decatetraenoic acid **6** (1.4 mg, 8.6 μmol) were each dissolved in DMSO (0.25 mL) and added to a Tris-HCl-buffered solution (9.5 mL, pH 7.5) containing DMSO (0.45 mL, 10% v/v). ATP and $MgCl_2$ were added to a final concentration of 5 and 10 mM, respectively, followed by the addition of BSA (9.5 mg, final concentration = 1 mg/mL). SimL was added to a final concentration of 3 μM, and the reaction was incubated at ambient temperature for 24 h. The reaction was monitored by reverse-phase HPLC [linear gradient of 100% (H_2O, 0.1% TFA) to 100% CH_3CN over 30 min]. When ligation was complete (as determined by HPLC), TDP-L-noviose (5.3 mg, final concentration = 1 mM) was added followed by the addition of NovM (final concentration = 500 nM). The reaction mixture was incubated overnight at room temperature. Reverse-phase HPLC analysis indicated that glycosylation was complete.

The reaction mixture was diluted to 35 mL by the addition of 10% DMSO in 75 mM Tris-HCl, pH 7.5 (25.5 mL). NovP was added to a final concentration of 3 μM followed by the addition of SAM (10 mg, final concentration = 2 mM). The reaction was incubated overnight at room temperature. Completion of methylation by NovP was confirmed by HPLC.

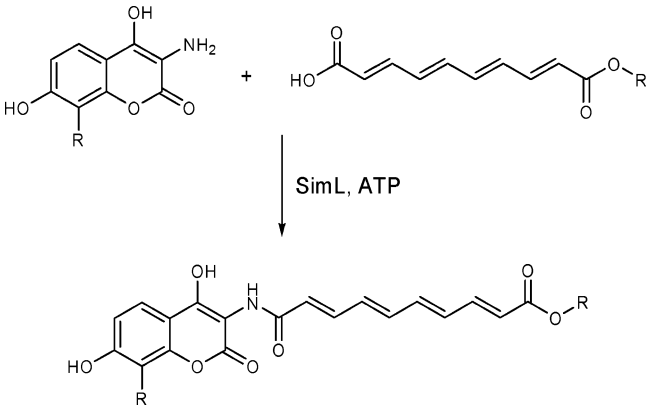
Carbamoyl phosphate (5.5 mg, final concentration = 1 mM) was added followed by the addition of NovN to a final concentration of 1 μM. The reaction was incubated at room temperature for 24 h. Complete conversion to novobiocin analogue **9** was confirmed by HPLC and LC-MS [ESI for $C_{29}H_{34}N_2O_{10}$: calcd 570.2, obsd 569.3 $(M - H)^-$].

The crude reaction mixture was concentrated by C18 SepPak as described above. Fractions containing product **9** (as determined by HPLC) were combined, and the product was purified by preparatory reverse-phase HPLC [gradient of 100% (H_2O, 0.1% TFA) to 100% CH_3CN over 30 min]. Lyophilization of the product-containing fractions afforded novobiocin analogue **9** (3.3 mg, 68% yield) as a bright yellow solid. High-resolution mass spectroscopy data for analogue **9** was obtained at the Mass Spectroscopy Facility at the Department of Chemistry and Chemical Biology at Harvard University [ESI for $C_{29}H_{34}N_2O_{10}$: calcd 570.2291, obsd 571.2296 $(M + H)^+$].

Determination of Antibacterial Activity of Novobiocin Analogue ***9.*** Twenty-two hour minimum inhibitory concentrations (MICs) were determined against strains grown in brain-heart-infusion broth in a microdilution format according to NCCLS guidelines (*21*). The following strains were used for MIC determination: *Enterococcus faecium*, 49624; *E. faecium*, resistant (VanA), CL4931; *Enterococcus faecalis*, 29212; *E. faecalis*, resistant (VanB), CL4877; *Staphylococcus aureus*, 29213.

For the determination of gyrase inhibitory activity, a "relaxation kit" was obtained from John Innes Enterprises Ltd. (John Innes Centre, Norwich, U.K.), and assays were performed according to the manufacturer's instructions.

Scheme 1: Amide Bond Forming Reaction Catalyzed by SimL



RESULTS

Expression and Purification of SimL. Based on sequence similarity to novobiocic acid synthetase (NovL), specifically the presence of conserved motifs involved in nucleotide binding, PP_i release, and adenylation of the carboxylate substrate moiety, SimL was presumed to be the complementary ligase in simocyclinone biosynthesis. Alignment of the four aminocoumarin amide bond forming ligases reveals that SimL is the most distant ligase, exhibiting 52% similarity with the novobiocin ligase, NovL, as compared with CouL and CloL (the coumermycin and clorobiocin ligases) which exhibit 87% and 92% similarity to NovL, respectively. This difference can most likely be attributed to the variation in carboxylic acid substrates recognized by their cognate enzymes. While NovL, CloL, and CouL activate benzoic acid and pyrrolecarboxylic acid rings, SimL activates a polyunsaturated carboxylic acid moiety (Scheme 1).

The 57.0 kDa SimL ligase was heterologously expressed and purified to homogeneity by Ni(II) affinity chromatography as both a C-terminally His_8-tagged and an N-terminally His_{10}-tagged protein with yields of 9 and 3 mg/L, respectively. In addition to the higher yield, the C-terminally tagged SimL gave approximately 4-fold higher activity and was therefore used in all of the experiments described herein (Figure 2).

Characterization of SimL Activity. The in vitro reconstitution of recombinantly expressed SimL was accomplished using the aminocoumarin moiety and a variety of carboxylic acid substrates. Ligase activity was found to be dependent on the presence of ATP, as well as divalent cations such as Mg^{2+} and Mn^{2+}. The pH optimum was determined to be 8.0 in Tris-HCl buffer.

Initial confirmation of ligase activity was accomplished by incubation of SimL with aminocoumarin **5** and decatetraenoic acid **6** (data not shown). The appearance of product was accompanied by a corresponding decrease in the free carboxylate substrate, and the reaction was monitored by RP-HPLC and confirmed by LC-MS [ESI for $C_{20}H_{19}NO_5$: calcd

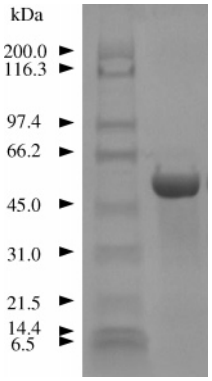


FIGURE 2: Overproduction and purification of C-terminal His-8 SimL from *E. coli*.

353.1, obsd 352.1 $(M - H)^-$]. Similar reaction profiles are also observed for the other two most active carboxylate substrates (data not shown): fumagillin [ESI for $C_{36}H_{41}NO_{10}$: calcd 647.27, obsd 646.3 $(M - H)^-$] and *trans*-retinoic acid [ESI for $C_{30}H_{35}NO_5$: calcd 489.25, obsd 488.2 $(M - H)^-$]. Product formation is also observed for 2,4,6-octatrienoic acid [ESI for $C_{18}H_{17}NO_5$: calcd 327.1, obsd 326.2 $(M - H)^-$] and sorbic acid [ESI for $C_{16}H_{15}NO_5$: calcd 301.1, obsd 300.0 $(M - H)^-$] although at decreased rates (data not shown). The length of the unsaturated chains in octatrienoic and sorbic acids may account for the decreased product formation observed. No reaction is observed for the *trans*,*trans*-muconic acid substrate.

Unexpectedly, SimL also readily accepts saturated acyl chains. SimL ligated product is observed for the following substrates: decanoic acid [ESI for $C_{20}H_{27}NO_5$: calcd 361.2, obsd 360.2 $(M - H)^-$], 10-hydroxydecanoic acid [ESI for $C_{20}H_{27}NO_6$: calcd 377.2, obsd 376.2 $(M - H)^-$], *N*-biotinyl-8-aminocaprylic acid [ESI for $C_{28}H_{38}N_4O_7S$: calcd 574.3, obsd 573.2 $(M - H)^-$], and *N*-biotinyl-12-aminododecanoic acid [ESI for $C_{32}H_{46}N_4O_7S$: calcd 630.3, obsd 629.3 $(M - H)^-$].

On the other hand, SimL is unable to catalyze amide bond formation for either the NovL or CouL substrates 3-prenyl-4-hydroxybenzoic acid and 3-methylpyrrole-2,4-dicarboxylic acid. In addition, neither NovL nor CouL exhibits any activity on any of the acyl SimL substrates tested here. SimL, therefore, shows unusual promise for promiscuity for the acyl partner in ligation to aminocoumarins.

Substrate Specificity of SimL. Since authentic 8-chloro-aminocoumarin is not available, the 8-methylaminocoumarin moiety (derived from novobiocin) was used as a substrate for SimL. As reported in in vivo experiments (*12*), the methylcoumarin is readily accepted by SimL as a substrate. The kinetic parameters for SimL were defined for this aminocoumarin substrate by holding the concentration of fumagillin constant at 2 mM. Addition of SimL yields a typical hyperbolic saturation curve over varying aminocoumarin ring concentrations, resulting in a K_m of 5.4 ± 1.8 μM for the aminocoumarin moiety. The turnover rate is measured to be 13.4 ± 1.4 min^{-1}. Enzyme inhibition is observed when the aminocoumarin concentration is greater than 1 mM.

Kinetic parameters were determined for three of the alternative carboxylic acids accepted by SimL. For fumagillin, decatetraenoic acid, and *trans*-retinoic acid, kinetic measurements were made while the concentration of the aminocoumarin moiety was held constant at 200 μM. Fumagillin exhibits a K_m of 463 ± 52 μM and a k_{cat} of 16.5 ± 2.5 min^{-1} (Figure 3, Table 1), which agree well with the maximal rate for the aminocoumarin measured in parallel experiments. For the decatetraenoic acid substrate, since enzyme inhibition is observed at concentrations exceeding 2.0 mM, a Michaelis−Menten curve fit was performed for data up to 2.0 mM, yielding an apparent K_m of 2250 ± 410 μM, about 5-fold greater than fumagillin, and a k_{cat} of 16.6 ± 3.0 min^{-1}. Retinoic acid has a k_{cat} of 32.4 ± 3.0 min^{-1} and a K_m of 31.7 ± 7.2 μM, about 10-fold lower than decatetraenoic acid (Table 1).

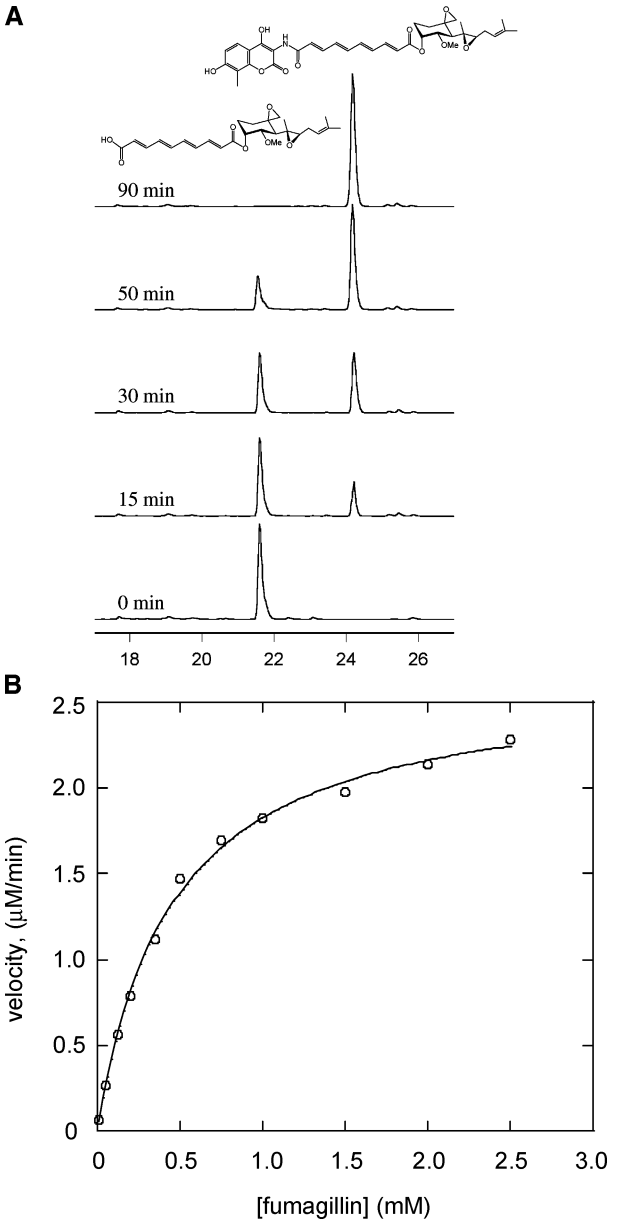
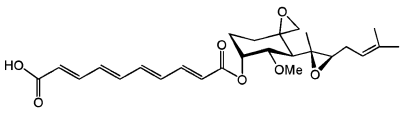
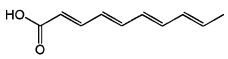
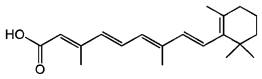


FIGURE 3: Characterization of SimL activity with aminocoumarin **5** and fumagillin. (A) Time course of the SimL amide bond forming reaction of aminocoumarin and fumagillin monitored by RP-HPLC. (B) Michaelis−Menten plot for measurement of kinetic parameters for the fumagillin substrate.

The identity of the authentic SimL carboxylate substrate is unknown. The presence of various simocyclinone intermediates in *S. antibioticus*, such as simocyclinone B (anguclinone moiety linked to the D-olivose sugar) and simocyclinone C (D-olivose linked to the decatetraenoic acid and

Table 1: Summary of Kinetic Parameters for SimL Carboxylic Acid Substrates

Substrate	k_{cat} (min^{-1})	K_m (μM)
	16.5 ± 2.5	463 ± 52
	16.6 ± 3.0	2250 ± 410
	32.4 ± 3.0	31.7 ± 7.2

angucyclinone moieties) (*8*), suggests that the ligation of the aminocoumarin ring to form simocyclinone D may be the final biosynthetic step. The reconstitution of SimL has provided the first opportunity to test in vitro enzymatic substrate specificity. Two possible carboxylate substrates for SimL include the decatetraenoic diacid polyketide moiety alone or this moiety linked to the glycosylated angucyclinone ring system. Since the latter is unavailable, the decatetraenedioic acid was prepared and tested as a potential SimL substrate. No product formation is observed in incubations of the diacid with SimL (data not shown). In contrast, substitution of one of the carboxylic acids with an ester to form decatetraenedioic acid monomethyl ester yields a model substrate that is acted on by SimL. In preliminary studies the k_{cat}/K_m catalytic efficiency ratio (0.5 mM^{-1} min^{-1}) (data not shown) was comparable to that of the decatetraenoate (0.7 mM^{-1} min^{-1}, from Table 1).

*Formation of Novel Novobiocin Analogue **9** via Four-Step Tandem Enzyme Incubation.* Previous studies aimed at varying the ring A acyl moiety of novobiocin using the characterized ligases NovL and CouL have been limited by the substrate specificity these enzymes exhibit for 3′-substituted benzoic acid and pyrroledicarboxylic acid rings (*16*). The reconstitution of SimL presents an opportunity to test whether novel novobiocin analogues containing the polyunsaturated acyl groups found in simocyclinone could be made via the tandem action of the SimL ligase, the NovM glycosyltransferase, and the sugar tailoring enzymes NovP and NovN (*18*, *19*). Preliminary results were promising, as an authentic sample of simocyclinone D8 (*22*) could be glycosylated with TDP-L-noviose and NovM (data not shown). In addition, coincubations of the SimL ligase with the NovM glycosyltransferase (with aminocoumarin, decatetraenoic acid, and TDP-L-noviose) yielded the noviosylated SimL product **8** [ESI for $C_{27}H_{31}NO_9$: calcd 513.2, obsd 512.2 $(M - H)^-$] (data not shown).

Prompted by these initial results, we initiated a four-enzyme tandem incubation with aminocoumarin, decatetraenoic acid, ATP, SAM, carbamoyl phosphate, SimL, NovM, NovP, and NovN. Following sequential addition and overnight incubation of each enzyme to maximize the amount of intermediate formed after each phase, the supernatant was analyzed by reverse-phase HPLC and LC-MS (Figure 4B). Confirmation of formation of novobiocin analogue **9** was accomplished by LC-MS [ESI for $C_{29}H_{34}N_2O_{10}$: calcd 570.2, obsd 569.3 $(M - H)^-$]. The presence of intermediates arising from incomplete methylation [NovM product **8**; ESI for

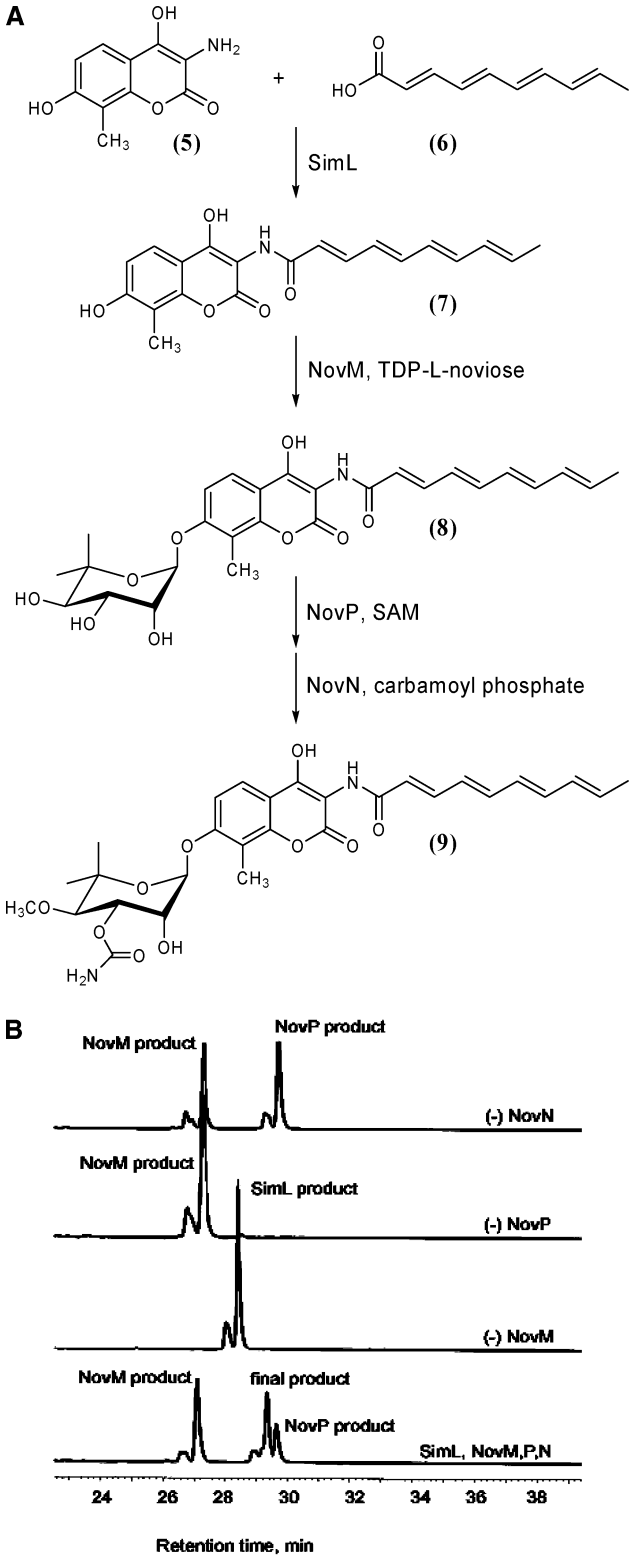


FIGURE 4: Preparation of novel novobiocin analogue **9** via four-enzyme tandem incubation. (A) SimL-, NovM-, NovP-, and NovN-mediated conversion of aminocoumarin **5** and decatetraenoic acid **6** to novel novobiocin analogue **9**. (B) Conversion of SimL product **7** to novobiocin analogue **9** monitored by RP-HPLC.

$C_{27}H_{31}NO_9$: calcd 513.2, obsd 512.4 $(M - H)^-$] and carbamoylation [NovP product; ESI for $C_{28}H_{33}NO_9$: calcd 527.2, obsd 526.3 $(M - H)^-$] was also detected by LC-MS. These results confirm that the generation of novel novobiocin

analogues via a four-enzyme tandem incubation is possible and that the combinatorial biosynthesis of such compounds using SimL is feasible.

Novobiocin analogue **9** was prepared enzymatically on a scale sufficient for evaluation of biological activity, with an overall yield of 68% after four tandem enzyme incubations. MIC assays performed on this compound compared with authentic novobiocin showed that this analogue possesses no noticeable antibacterial activity on all of the strains of *Enterococci* tested but exhibits a MIC value of 50 μg/mL for *S. aureus*, 29213. Novobiocin showed anticipated MIC values for these strains: 0.8 μg/mL for *E. faecium*, 49624; 0.8 μg/mL for *E. faecium*, resistant (VanA), CL4931; 12.5 μg/mL for *E. faecalis*, 29212; 12.5 μg/mL for *E. faecalis*, resistant (VanB), CL4877; 0.4 μg/mL for *S. aureus*, 29213 (*17*). Since this result yields no information on the specific cause of this inactivity, the ability of novobiocin analogue **9** to inhibit DNA gyrase was tested. In this assay, novobiocin exhibits an IC_{50} of 0.6 μM, which is similar to the IC_{50} of 0.9 μM previously reported for novobiocin using the same gyrase assay kit (*23*). Novobiocin analogue **9** exhibits an IC_{50} of 4.3 $\pm$ 0.2 μM, about a 7-fold increase over novobiocin.

DISCUSSION

The aminocoumarin antibiotics novobiocin **1**, clorobiocin **2**, and coumermycin A_1 **3** (Figure 1), produced by various *Streptomyces* species, use a decorated 3′-*O*-acyl-4′-*O*-methylnoviosyl moiety as the pharmacophore for inhibiting ATP hydrolysis in DNA gyrase and topoisomerase IV. The noviose moiety is presented by 3-amino-4,7-dihydroxycoumarin, a scaffold which is essential for the binding of these compounds to the B subunits of gyrase (GyrB). Simocyclinone D **4**, produced by *S. antibioticus*, is a recently discovered antibiotic that contains this conserved aminocoumarin moiety. However, simocyclinone is a structurally unique hybrid antibiotic containing two polyketide moieties, an anguicyclinone and a decatetraene dicarboxylic acid, linked by the deoxy sugar D-olivose. This anguicycline *C*-glycoside tetraene half-ester is linked to the aminocoumarin moiety via an amide bond. Unlike **1**−**3**, there is no decorated noviosyl moiety at the 7-hydroxy position of the aminocoumarin. The fact that simocyclinone exhibits antimicrobial activity (*6*) suggests that its mechanism of action may differ from that of the other aminocoumarin antibiotics. Indeed, recent studies confirm that simocyclinone D8 inhibits DNA gyrase through a novel mode of action (Anthony Maxwell, personal communication, John Innes Centre, Norwich). This makes simocyclinone an attractive molecule to study from both a biosynthetic and a combinatorial perspective.

The recent sequencing of the simocyclinone biosynthetic gene cluster (*12*) makes it possible to characterize its amide bond synthetase, SimL, a key enzyme in simocyclinone D assembly. The ATP-dependent amide bond forming ligases from the novobiocin, clorobiocin, and coumermycin A_1 clusters have been purified, and their ability to activate either benzoic acid or pyrrolecarboxylic acid rings has been demonstrated. The complementary simocyclinone ligase, SimL, on the other hand, activates a structurally distinct polyunsaturated carboxylic acid ester moiety.

The goal of this study has been to validate the role of SimL as the simocyclinone amide bond forming ligase and to assess its specificity in order to explore its potential as a tool in the combinatorial biosynthesis of novobiocin analogues. We began by overexpressing SimL-C-His in *E. coli* in soluble form and purifying it to homogeneity. The natural nucleophilic substrate in simocyclinone is 3-amino-8-demethyl-4,7-dihydroxycoumarin, where the amino group is the attacking moiety. To assess SimL activity in vitro, we used the corresponding 8-methylaminocoumarin **5** as an analogue of the physiologic nucleophilic substrate, which was available from degradation of novobiocin (*20*). SimL acts by first activating the C_1 carboxylate of the tetraenedioate in the presence of ATP to form an acyl-AMP intermediate, the electrophile which is captured by the aminocoumarin. Since the full right-hand fragment of simocyclinone D was not available to test as a substrate, decatetraenoic acid (a simple substitution of COOR with CH_3) was used and confirmed the activity of SimL as the simocyclinone amide bond forming ligase with a k_{cat} of 16.6 $\pm$ 3.0 min^{-1} and a K_m of 2250 $\pm$ 410 μM (Table 1).

Kinetic parameters were determined for alternative carboxylic acids accepted by SimL and presage broad tolerance for the carboxylic acid moieties. The natural product, fumagillin, a commercially available tetraenedioate half-ester, exhibits a k_{cat} of 16.5 $\pm$ 2.5 min^{-1} and a 5-fold lower K_m (463 $\pm$ 52 μM) than the simple tetraenoate (Figure 3, Table 1). In the parallel experiment, the aminocoumarin exhibits a similar k_{cat} of 13.4 $\pm$ 1.4 min^{-1} and a K_m of 5.4 $\pm$ 1.8 μM. Remarkably, retinoic acid was found to be the best of the three carboxylic acid substrates, with a k_{cat} of 32.4 $\pm$ 3.0 min^{-1} and a K_m of 31.7 $\pm$ 7.2 μM. SimL could also use the shorter chain C_8 and C_6 polyenoates, albeit at lower efficiency, with a requirement for a trans stereochemistry in the unsaturated fatty acid chain, as no reaction was observed for any cis-configured chains tested. The decatetraenedioic free acid is not a substrate, consistent with biosynthetic expectations that SimL acts at the last step of the assembly line, ligating the anguicycline olivose tetraenoic acid as the $RCOO^-$ substrate. SimL also accepts saturated acyl chains, including decanoate, 10-hydroxydecanoate, and even the biotin-substituted ω-amino C_8 and C_{12} acids.

Previous tests of cross substrate specificity between aminocoumarin ligases demonstrated that while neither NovL nor CloL accepts the CouL substrate (3-methylpyrrole-2,4-dicarboxylic acid), CouL showed moderate activity toward the NovL substrate (3-prenyl-4-hydroxybenzoic acid) (*14*). Tests of ligase activity on the NovL and CouL RCOOH substrates reveal that SimL is unable to catalyze amide bond formation for either of these substrates. Similarly, neither NovL nor CouL is able to catalyze the formation of an amide bond between the aminocoumarin and any of the acyl SimL substrates tested here. SimL is clearly the preferred ligase catalyst for assembling variant members of the aminocoumarin antibiotic class.

As an initial test of the utility of SimL to generate novel ligation products as substrates for the late stage coumarin antibiotic biosynthetic pathway enzymes, a tandem four-enzyme reaction sequence was carried out. Starting with tetraenoic acid **6** and 8-methylaminocoumarin **5**, SimL generated the amide product **7**. Then the last three enzymes of the novobiocin pathway, NovM, NovP, and NovN, were

used to further modify the SimL product. Compound **7** is accepted by NovM for noviosylation of the phenolic OH to yield compound **8**. In turn, NovP creates the *O*-methyl linkage at the 4′-hydroxyl of the noviosyl moiety, and then NovN adds a carbamoyl group to the 3′-hydroxyl to create **9** with the methylation and acylation required in the noviosyl pharmacophore of novobiocin. Authentic novobiocin differs from **9** in having a prenyl hydroxybenzoate as the acid component instead of the tetraenoate acyl group.

Compound **9** was tested in MIC assays and has no detectable antibacterial activity against all tested *Enterococci* under conditions where novobiocin displays anticipated antibacterial activity. Some inhibition was observed, however, with *S. aureus* (MIC = 50 μg/mL), albeit at levels 100-fold less potent than novobiocin. To determine if the lack of activity against whole cells is due to the failure of the decatetraenoyl analogue of novobiocin to recognize and inhibit DNA gyrase or is a result of poor bacterial cell penetration, in vitro DNA gyrase IC_{50} data were obtained and compared with novobiocin and coumermycin A_1. Novobiocin is found to have an IC_{50} of 0.6 μM in this assay, and coumermycin A_1 has an IC_{50} of 0.2 μM, a 3-fold difference consistent with previous reports (*24*). Novobiocin analogue **9** exhibits an IC_{50} of 4.3 $\pm$ 0.2 μM, about a 7-fold increase over novobiocin. The retention of measurable DNA gyrase activity suggests that poor penetration may be the issue with **9**, and the combination of MIC and DNA gyrase IC_{50} assays should allow SAR optimization in SimL tandem incubations.

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