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Chemical Perturbation of Secondary Metabolism Demonstrates Important Links to Primary Metabolism

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SUMMARY

Bacterially produced secondary metabolites are used as antibiotics, anticancer drugs, and for many other medicinal applications. The mechanisms that limit the production of these molecules in the laboratory are not well understood, and this has impeded the discovery of many important compounds. We have identified small molecules that remodel the yields of secondary metabolites in many actinomycetes and show that one set of these molecules does so by inhibiting fatty acid biosynthesis. This demonstrates a particularly intimate relationship between this primary metabolic pathway and secondary metabolism and suggests an approach to enhance the yields of metabolites for discovery and biochemical characterization.

INTRODUCTION

The secondary metabolites produced by bacteria exhibit potent biological activities and have been developed extensively for antimicrobial, anticancer, and other vital therapeutic applications. Most of these molecules are polyketides and nonribosomal peptides and are produced by biochemical pathways that are encoded in discrete islands within the genome. The genes encoded in these islands normally include all the metabolite-specific biochemical steps; however, certain precursor molecules and other resources are drawn from primary metabolism (Martín and Liras, 2010; Olano et al., 2008).

An outstanding source of natural products and drug leads is the actinobacteria. One of these, *Streptomyces coelicolor*, is a superb model system for secondary metabolism due to its production of pigmented secondary metabolites, including the blue polyketide actinorhodin (Okamoto et al., 2009), and the red prodiginines, undecylprodiginine and streptorubin B (Mo et al., 2008). These molecules can be readily detected visually and quantified spectrophotometrically.

Understanding the metabolic and regulatory mechanisms that determine the yields of secondary metabolites from producer organisms is important for the discovery and exploitation of these molecules. A major outcome of genome sequencing has been the discovery that each streptomycete chromosome encodes many more secondary metabolites than can be detected from routine laboratory culture (Bentley et al., 2002; Nett et al., 2009). Efforts to enhance output have focused on the engineering of strains to overexpress pathway-specific (Laureti et al., 2011) and pleiotropic regulators (McKenzie et al., 2010) of secondary metabolism and on moving biosynthetic gene clusters into engineered overproduction strains (Gomez-Escribano and Bibb, 2011; Komatsu et al., 2010). These approaches have been successful but are technically cumbersome and not amenable to high throughput.

In this work we report the discovery of 19 small molecules that remodel the secondary metabolite output of the streptomycetes. We show that one set of these molecules targets fatty acid biosynthesis, demonstrating that the relationship between primary and secondary metabolism is one limit on secondary metabolite yields.

RESULTS

Small Molecule Probes of Secondary Metabolism

To identify small molecules that perturb secondary metabolism, we screened the Canadian Compound Collection of 30,569 small molecules for their ability to alter the pigmentation of *S. coelicolor* colonies during growth on solid medium. The compounds were screened at 10 μM in duplicate, colonies were examined daily over a 10 day period (see Table S1 and Figure S1 available online), and we focused in particular on hits that caused elevated or precocious production of actinorhodin. A total of 112 primary hits were identified, and of these, 19 compounds were selected for follow-up on the basis of potency, reproducible behavior, and the absence of pronounced antimicrobial activity.

Four of the 19 molecules, ARC2, ARC3, ARC4, and ARC5 (referred to as the ''ARC2 series'' for *a*ntibiotic-*r*emodeling *c*ompounds; Figure 1A), had similar structures and stimulated blue pigmentation during growth on solid medium. Spectrophotometric analysis of liquid cultures demonstrated that the ARC2 series also reduced yields of the prodiginines (Figure 1B). Using LC-MS analysis, we found that ARC2 induced changes in the production of two other *S. coelicolor* secondary metabolites. Yields of the germicidins (Aoki et al., 2011) (germicidin B/C 183.107 $[M+H]^+$ and germicidin A 197.125 $[M+H]^+$) were enhanced ~3-fold by ARC2 (Figure 1C); whereas yields of the daptomycin-like calcium-dependent antibiotic (CDA) (1495.485 $[M+H]^+$) (Hojati et al., 2002; Lewis et al., 2011) were reduced

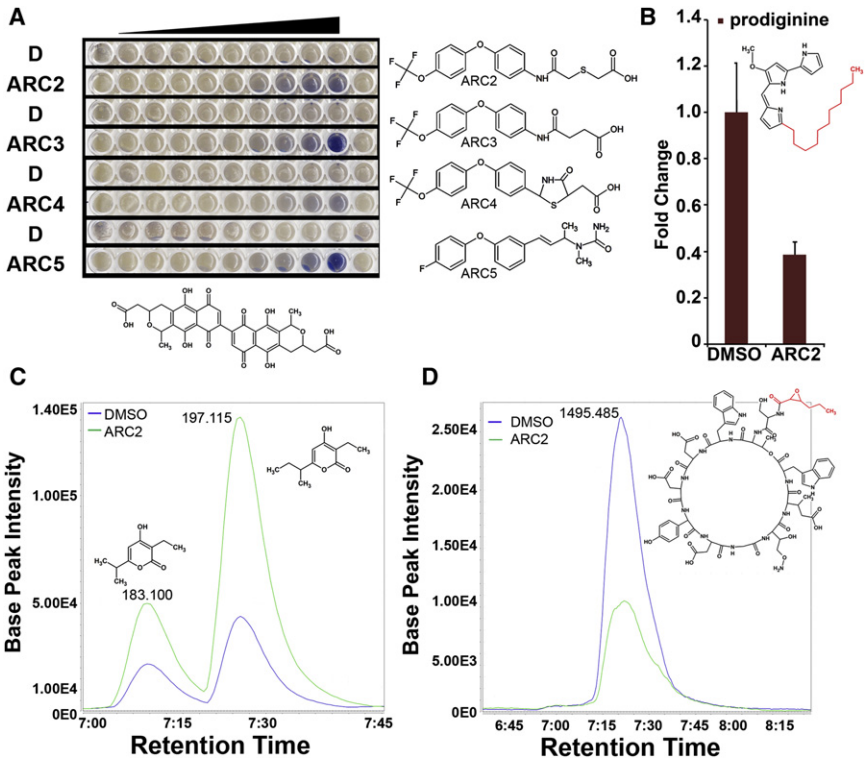

Figure 1. The ARC2 Series of Actinorhodin-Inducing Molecules

(A) The structures of the ARC2 series and their effect on blue pigmentation of *S. coelicolor* colonies. Molecules were added at 0.39, 0.78, 1.56, 3.13, 6.25 12.5, 25, 50, and 100 μM to solid medium along with *S. coelicolor* spores. The DMSO solvent-only controls are indicated (D) and in outer wells (1 and 11) of compound rows. The plate was photographed following 3 days of growth.

(B) Spectrophotometric analysis of prodiginine yields in *S. coelicolor* M145 grown in the presence of 25 μM ARC2 or with a solvent-only control (DMSO) after 3 days of growth in liquid R5M ±SD.

(C) The effects of 10 μM ARC2 on germicidin production (germicidin B/C 183.107 $[M+H]^+$ and germicidin A 197.125 $[M+H]^+$) were assessed using LC-MS analysis after 7 days of growth on solid R5M.

(D) The production of CDA (1495.485 $[M+H]^+$ for CDA 4a) was assessed in the presence of DMSO or 10 μM ARC2 using LC-MS after 7 days of growth on solid R5M.

FabI-dependent fatty acid moieties of prodiginine (B) and CDA (D) are shown in red.

See also Figure S1 and Table S1.

~2-fold (Figure 1D). The ARC2 series can therefore pleiotropically remodel secondary metabolism in *S. coelicolor*.

These effects led us to investigate the action of ARC2 on secondary metabolism in other bacteria. We added ARC2 to cultures of *Kutzneria* sp. 744, *S. pristinaespiralis* ATCC 25486, and *Streptomyces peucetius* 27952 and observed their small molecule output using LC-MS (Figure 2). ARC2 enhanced yields of desferrioxamine B and E (Barona-Gómez et al., 2006) (561.362 and 601.360 $[M+H]^+$, respectively) in *S. pristinaespiralis* (Figure 2A), doxorubicin (544.196 $[M+H]^+$), baumycin (674.258 $[M+H]^+$), and three unknown molecules (417.103 $[M+H]^+$, 433.097 $[M+H]^+$, and 615.356 $[M+H]^+$) in *S. peucetius* (Lomovskaya et al., 1998) (Figure 2B), and an unknown metabolite (252.175 $[M+H]^+$) in *Kutzneria* (Figure 2C). Yields of some secondary metabolites were unchanged: for example, ARC2 had no effect on yields of pristinamycin IIA (526.259 $[M+H]^+$) in *S. pristinaespiralis* (Mast et al., 2011). These data suggest that the molecular target and mechanism of ARC2 are conserved in these diverse actinomycetes.

Structural Determinants of ARC2 Activity

To gain further insight into the structural features of the ARC2 series, we generated a structure-activity library by sourcing related commercial molecules (compounds 1–8) and by chemical synthesis (compounds 9–32) and exploring the capacity of these molecules to alter secondary metabolism in *S. coelicolor* (Figure 3A). Changes to the core structure (bottom-left panel in Figure 3A) such as the removal of ring A (compound 6) or replacement of the ether linkage between rings A and B with a ketone (compound 5) eliminated the enhancement of blue pigmentation. Insertion of an extra carbon between rings A and B similarly abolished activity (compound 7). These data suggested that the core shape of the molecules and rotational freedom around the ether linkage are important for biological activity.

The tails on ring B were the most variable feature (R_4 and R_5) (top-right panel in Figure 3A); alterations introduced in the structure-activity analysis had similar variable effects on biological activity. We investigated 13 substitutions at R_4 and R_5 in the context of a functional ring A (a Cl at R_1; see below) and found that most variants exhibited relatively weak actinorhodin-stimulating activity or were inactive. One variant that proved functional was an acetamide group at R_4 (Figure 3A, compound 16 in top-left panel).

We similarly generated compounds having varied substitutions on ring A in the context of an acetamide substitution at position R_4 of ring B (top-left panel in Figure 3A)—variation on this ring had a greater influence on function. For example we found that the absence of an electron-withdrawing group at R_1 compromised biological activity (compound 18). The electron-withdrawing group could not be moved to R_2 or R_3 without reducing or eliminating activity (compounds 20, 21, and 22). The nature of this electron-withdrawing group was also important. For halogen substitutions, Br > Cl > F (compare compounds 9, 10, 16, and 17 in Figure 3A). Although halogens were most active, other electron-withdrawing substitutions at R_1 were also tolerated, including $N(CH_3)_2$ and OCH_3 (compare compounds 11, 12, and 15 in top-left panel of Figure 3A). The only substitution that did not agree with this rule was CH_3, which proved functional (compound 11).

This structure-activity analysis generated several molecules that were active at lower concentrations than ARC2 (compounds

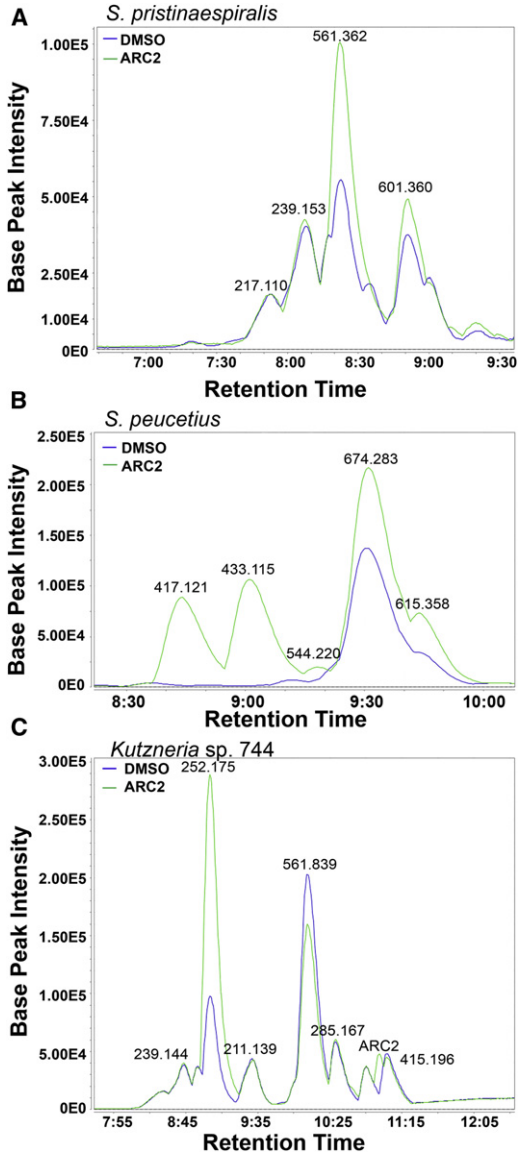

Figure 2. ARC2-Enhanced Yields of Secondary Metabolites

The effect of 10 μM ARC2 on secondary metabolite output was assessed in (A) *S. pristinaespiralis*, (B) *S. peucetius*, and (C) *Kutzneria* sp. 744 using LC-MS analysis. All strains were grown for 7 days in the presence of either DMSO or 10 μM ARC2. Enhanced yields of desferrioxamine B and E (561.362 and 601.360 $[M+H]^+$, respectively) in *S. pristinaespiralis* (A), doxorubicin (544.220 $[M+H]^+$), baumycin (674.283 $[M+H]^+$), and three unknown metabolites (417.121, 433.115, 615.356 $[M+H]^+$) in *S. peucetius* (B) and an unknown metabolite (252.175 $[M+H]^+$) in *Kutzneria* (C) were observed.
See also Figure S2.

10, 13, 15, 16, and 17; see left panel of Figure 3A) and confirmed that the important features for activity were two benzene rings joined by an ether linkage with, in most cases, an electron-withdrawing group at the R_1 position. As was the case with the initial hits of the ARC2 series, we observed a correlation between enhanced yields of γ-actinorhodin and reduced yields of the prodiginines during liquid culture (Figure 3B). The conserved structural features of the active molecules and related phenotypic effects are consistent with a shared molecular mechanism of action for the ARC2 series.

The ARC2 Series Target Fatty Acid Biosynthesis

Structural analysis of the ARC2 series suggested a possible molecular target for the series. Several of the most active molecules (e.g., compounds 16 and 17) were structurally similar to the known antimicrobial agent triclosan, an inhibitor of fatty acid synthesis (Figure 4A). Triclosan is a covalent inhibitor of the FabI enoyl reductase, which catalyzes the final and rate-limiting step in fatty acid biosynthesis (Bergler et al., 1996). We were intrigued by this similarity because fatty acid biosynthesis shares the precursors acetyl-CoA and malonyl-CoA with polyketide biosynthesis. The ARC2 series might therefore shunt resources from primary metabolism thereby enhancing the yields of some polyketides. Indeed, of the five metabolites that we were able to identify that were enhanced in yield by ARC2, four were polyketides that depend on the cellular acyl-CoA pool: actinorhodin, doxorubicin, baumycin, and germicidin. Desferrioxamine belongs to the nonpeptide hydroxymate siderophores, and at least one step in its biosynthesis is dependent on acetyl-CoA as well (Barona-Gómez et al., 2004). Furthermore, the two metabolites that were reduced in yields by ARC2, the prodiginines and CDA, both contain fatty acid moieties (highlighted with red on the structures in Figures 1B and 1D) that are generated via primary metabolism and require the enoyl reductase activity of FabI (Hojati et al., 2002; Williamson et al., 2006). FabI exhibits significant end-to-end sequence similarity and is highly conserved in both its catalytic center and triclosan-binding residues (see Figure S2) (Gago et al., 2011; Lu and Tonge, 2008). This could be consistent with the broad spectrum of these molecules to influence secondary metabolism in diverse organisms. We have addressed this hypothesis in several ways.

First, we explored triclosan itself, finding that it exhibited a potent antimicrobial effect on *S. coelicolor* growth in the 1–50 μM range where the ARC2 series influenced pigmentation (the minimum inhibitory concentration was 3.13 μM). However, when we reduced the triclosan concentration below the MIC, we observed a clear stimulation of blue pigmentation (Figure 4B). This led us to synthesize three additional compounds containing the 2-phenoxyphenol core of triclosan (compounds 33, 34, and 35) (Figure 4A), each of which has been shown previously to inhibit FabI (Tipparaju et al., 2008). Compound 33 inhibited *S. coelicolor* growth with an MIC of 25 μM; compounds 34 and 35 were less-potent antimicrobials. Importantly, all three triclosan/ARC2 chimeras stimulated blue pigmentation of *S. coelicolor* colonies at concentrations that were subinhibitory for growth (Figure 4B). The ability of triclosan and these known FabI inhibitors to mimic the effects of ARC2 is consistent with a shared mechanism involving the inhibition of FabI.

To test this hypothesis genetically, we created a *S. coelicolor* strain that expressed the *fabV* gene from *Pseudomonas aeruginosa*, which encodes a triclosan-resistant FabI paralog (Zhu et al., 2010). The expression of this gene increased the MIC of triclosan against *S. coelicolor* from 3.13 to 100 μM but had no effect on the MICs of antibiotics that target translation or DNA replication (see Table S2). We compared the effects of ARC2, ARC3, ARC4, and ARC5 and compounds 16, 17, 33, 34, and

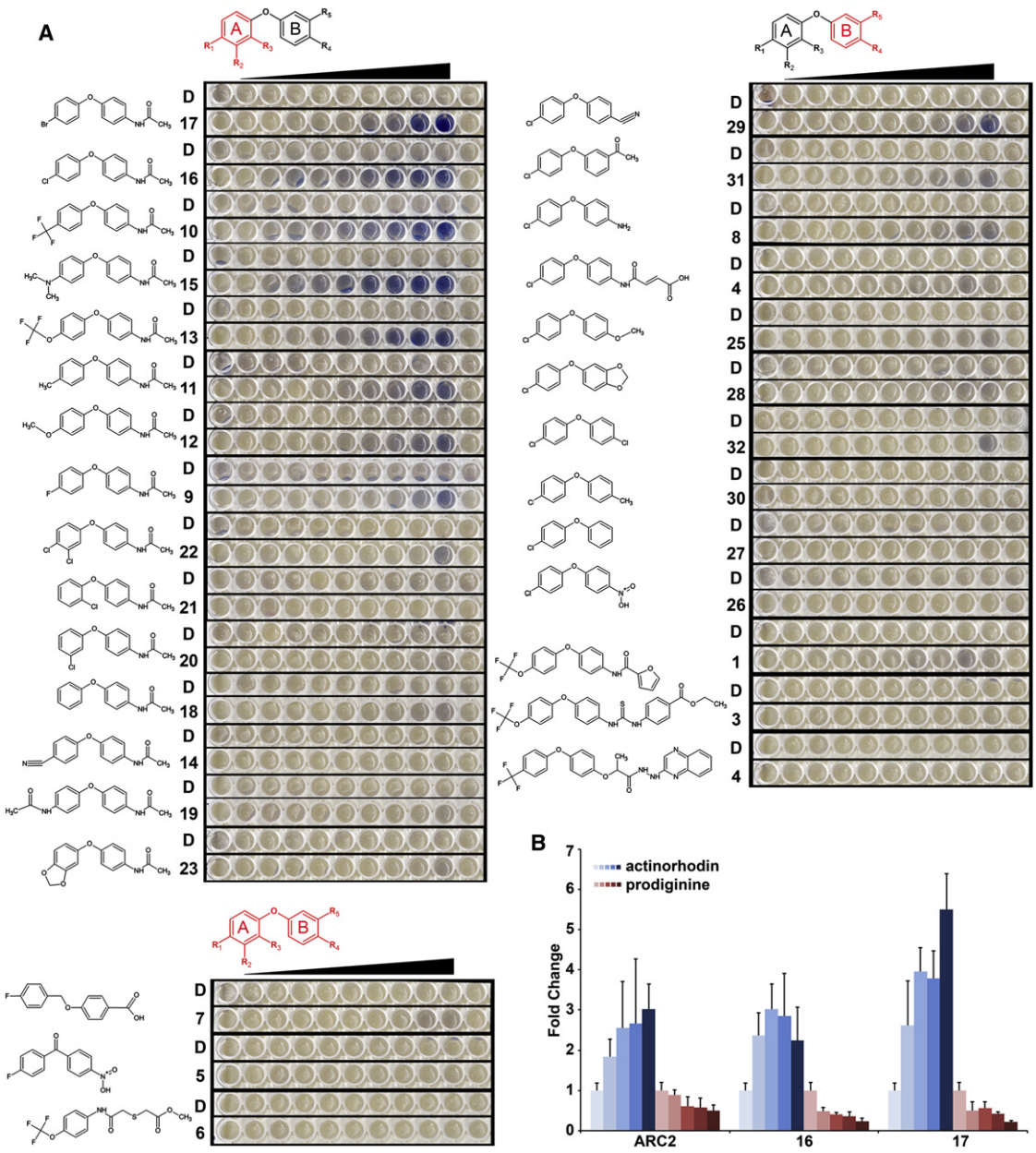

Figure 3. Effects of ARC2 and ARC2 Analogs on Actinorhodin and Prodiginine Biosynthesis

(A) Compounds 1–32 based on the ARC2 series were plated as a titration (0.39, 0.78, 1.56, 3.13, 6.25 12.5, 25, 50, and 100 μM) on solid MS agar in the presence of *S. coelicolor* M145 spores. The DMSO solvent controls are indicated (D) and outer wells (1 and 11) of compound rows. Photographs were taken after 3 days of growth with actinorhodin production denoted by the presence of a blue pigment.

(B) Titrations of increasing concentration of ARC2 and compounds 16 and 17 (1.56, 6.25, 12.5, 25 μM) were assessed for effects on actinorhodin and prodiginine production and compared to a DMSO control after 3 days of growth in liquid R5M ± SD. Actinorhodin production is shown in blue and prodiginine in red.

35 on actinorhodin yields in the resistant strain (V) and an isogenic control (E) (Figure 4C). Consistent with the targeting of fatty acid biosynthesis by these molecules, we found that the expression of FabV impaired the effects of all of the molecules on blue pigmentation. We observed residual actinorhodin-stimulating activity at the highest concentrations of some compounds (50 and 100 μM); however, there was a clear bypass effect of *fabV* at lower concentrations. We then tested two other unrelated actinorhodin-inducing molecules from our primary screen: ARC12 and 19. Neither of these is structurally related to ARC2. The capacity of these molecules to stimulate actinorhodin yields was not influenced by the *fabV* gene (Figure 4C).

We also explored the effect of *fabV* on the reduction of prodiginine yields by the ARC2 series (see Figure S3) and found that, indeed, this triclosan-resistant enoyl reductase restored undecylprodiginine (394.287 $[M+H]^+$) yields in the presence of

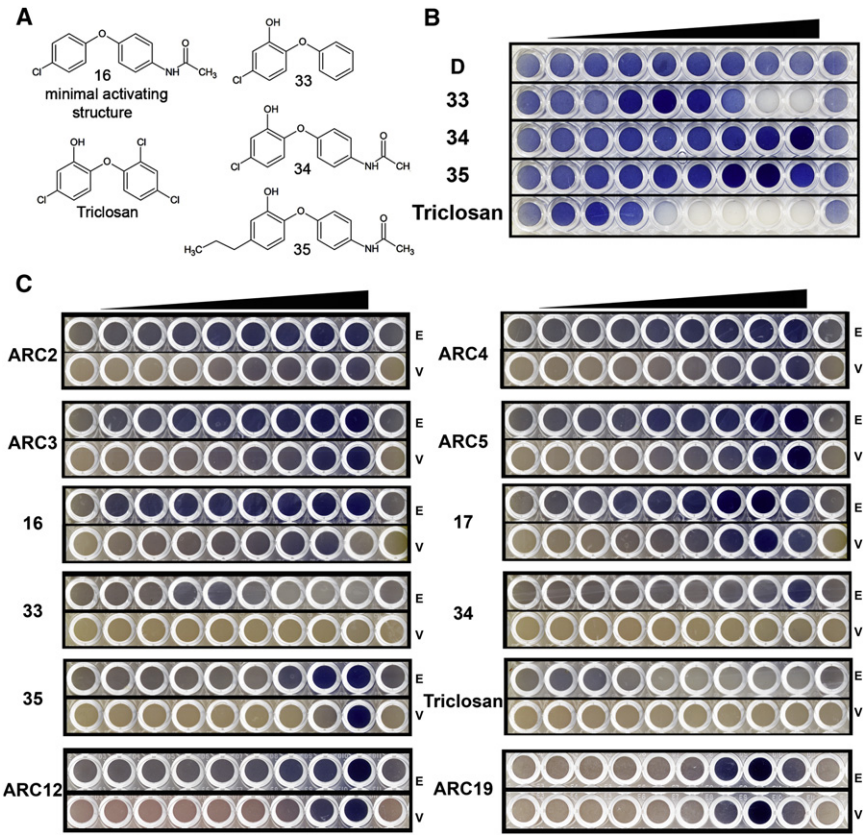

Figure 4. The ARC2 Series Acts through Partial Inhibition of Fatty Acid Biosynthesis

(A) Comparison of the minimally activating structure (compound 16) with triclosan and three triclosan/ARC2 series chimeras (compounds 33–35).

(B) The effects of these compounds on actinorhodin yields at 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 μM. Solvent controls are indicated (D) and in outer wells (1 and 10) of compound rows. Actinorhodin yield was assessed by blue pigmentation after 7 days of growth on solid MS agar.

(C) ARC2–ARC5, compounds 16 and 17, the triclosan/ARC2 chimeras (compounds 33, 34, and 35), and triclosan were added at 0.78, 1.56, 3.13, 6.25 12.5, 25, 50, and 100 μM to cultures of *S. coelicolor* expressing the triclosan-resistance allele *fabV* (rows denoted V) or a control vector (E). The DMSO solvent controls are in the outer wells (wells one and ten). Similar analysis was applied to ARC12 and ARC19, which are unrelated to the putative or known FabI inhibitors.

See also Figure S3.

ARC2. These data further support a mechanism of action for the ARC2 series that involves the inhibition of FabI.

Finally, we assessed the ability of the ARC2 series to interfere with the biochemical activity of purified FabI in vitro. We established an enzyme assay using *E. coli* FabI where the consumption of NADH during the reduction of the substrate crotonyl-CoA was measured (Liu et al., 2011). As expected, we observed 100% inhibition of FabI with 0.01 μM triclosan. Compound 33 inhibited FabI activity to completion at 1 μM; compounds 34 and 35 inhibited ~80% of the activity at 10 μM, consistent with their less-potent antimicrobial activities. Confirming a capacity to impair FabI activity, 50 μM ARC2 and compound 17 were able to inhibit FabI activity by 55% and 56%, respectively (Table 1). This work confirmed that the ARC2 series inhibit the FabI enoyl reductase and that inhibition of this step is important for their effects on secondary metabolism.

DISCUSSION

We have conducted a chemical screen against the life cycle of *S. coelicolor* and identified 19 chemical probes of secondary metabolism. We show that the ARC2 series is related to the antibiotic triclosan structurally and through their capacity to interfere with FabI activity in vitro and in vivo. The expression of a triclosan-resistant enoyl reductase, FabV, confers triclosan resistance and interferes with the effect of these molecules on actinorhodin and prodiginine biosynthesis, demonstrating that impairing fatty acid biosynthesis remodels secondary metabolite yields. The reduction in *S. coelicolor*'s two lipid-containing secondary metabolites, prodiginine and CDA, also correlates well with inhibition of fatty acid biosynthesis because there would be less lipids available for incorporation into these two metabolites.

The relationship between polyketide and fatty acid biosynthesis appears to be a very intimate one. For example most polyketide biosynthetic clusters, including those of actinorhodin, doxorubicin, baumycin, and germicidin, do not encode designated malonyl-CoA transferases for loading a starter unit onto the polyketide acyl carrier protein. Instead, they recruit the malonyl-CoA:ACP transacylase enzyme from the fatty acid biosynthetic machinery for this purpose (Koppisch and Khosla, 2003). The simplest interpretation of our data, therefore, is that there is a balance between fatty acid and polyketide biosynthesis in cells that helps set the upper limit on polyketide yields. This balance could be manifested at the level of precursor supply, malonyl-CoA:ACP transacylase availability, or both. Inhibiting this primary metabolic pathway therefore favors the polyketides and certain other metabolites also sharing these precursors (e.g., desferrioxamine). Polyketide biosynthesis is related to fatty acid biosynthesis biochemically; however, most polyketide biosynthetic pathways do not possess or require a designated enoyl reductase. This includes the pathways for the ARC2-stimulated polyketides that we have identified: actinorhodin, doxorubicin, baumycin, and germicidin.

Partial inhibition, rather than complete inhibition, of fatty acid biosynthesis is a critical feature of the most useful molecules we have identified. Fatty acid biosynthesis is essential for viability as demonstrated by the antimicrobial effect of triclosan and at least two of the ARC2 analogs. Indeed, the most potent inhibitors where characterized by a narrow concentration range,

Table 1. FabI Inhibition

Compound	Concentration (μM)	Inhibition (%)
Triclosan	0.005	86
Triclosan	0.01	100
33	1	100
34	10	75
35	10	81
ARC2	50	55
17	50	56

just below their MIC for growth (e.g., 0.78–3.13 μM for triclosan, and 3.13–12.5 μM for compound 33), at which they influenced secondary metabolism. In contrast the weaker inhibitors exhibited a broader concentration range at which they could influence secondary metabolism, and this range was not limited by a strong antimicrobial effect at higher concentrations. For example, for 16 and 17 we observed a stimulation of actinorhodin biosynthesis over the two orders of magnitude between 0.78 and 100 μM.

Many strategies have been applied to the improvement in yields of polyketides and other secondary metabolites, including the overexpression of pathway-specific or pleiotropic regulators (Martín and Liras, 2010). There have also been efforts to genetically enhance the availability of precursors (Olano et al., 2008; Rigali et al., 2008). Although all of these approaches have met with success, the majority requires genetic engineering of the starting strains. One exception is ribosomal engineering, which only requires the development of resistance to translation inhibitors (Hosaka et al., 2009). These strategies, however, are more difficult to accomplish in high throughput and can be blocked by, for example, the existence of restriction barriers to genetic engineering. In contrast a strategy for secondary metabolite screening that depends on small molecule perturbation is, in principle, readily scalable to large numbers of strains using existing high-throughput technologies. We note that whereas we observed relatively modest 2- to 5-fold enhancement in the yields of actinorhodin, which is normally produced abundantly during routine laboratory culture, the effect of ARC2 on some other metabolites was more dramatic. Indeed, we observed the induction of two unknown compounds in *S. peucetius* (417.103 and 433.097 m/z) that could not be detected in the absence of ARC2. This suggests that this strategy can be applied to strains in the absence of genome sequence information and that this could facilitate the discovery of new metabolites.

The fact that perturbation of fatty acid biosynthesis can enhance yields of some secondary metabolites suggests that it would be worthwhile to investigate other pathways in primary metabolism. For example the nature and importance of the links between primary metabolism and the biosynthesis of aminoglycoside, nonribosomal peptides, and other valuable secondary metabolites are poorly understood. It is clear that the manipulation of these pathways could have a significant impact on our ability to access the full spectrum of secondary metabolites in the many actinomycetes in academic and private sector collections.

SIGNIFICANCE

Secondary metabolites are vital to medicine, and strategies to facilitate the identification and production of novel metabolites are needed. Altering secondary metabolism through small molecule addition has significant advantages because it can be easily applied to any bacterial culture to alter metabolite production. Because this approach does not require genetic manipulation, it is readily scalable to high throughput. We have identified 19 compounds that alter the spectrum of metabolites produced by diverse streptomycetes and show that one set of these molecules works through the inhibition of fatty acid biosynthesis. In addition to providing an approach to secondary metabolite identification, this demonstrates an intimate link between a primary metabolic pathway, fatty acid synthesis, and many secondary metabolic pathways. Presumably, the mechanism by which this works involves shunting precursor molecules from one pathway to the other.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions

Plasmid propagation and cloning were carried out in the *E. coli* strain DH5α. *E. coli* strains were grown at 37°C in Luria broth media. Antibiotic concentrations were 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, 34 μg/ml of chloramphenicol, 25 μg/ml nalidixic acid, and 50 μg/ml of apramycin. All antibiotics were purchased from Sigma-Aldrich. For introduction of plasmids into *S. coelicolor*, plasmids were transformed into *E. coli* strain ET12567 containing the pUZ8002 plasmid allowing for conjugal transfer of the plasmid (Flett et al., 1997). *S. coelicolor* M145 was used for the high-throughput screen (HTS) and subsequent follow-up, unless stated elsewhere. All actinomycete strains were grown on solid R5M (100 g maltose, 10.12 g $MgCl_2 \times 6H_20$, 0.5 g K_2SO_4, 0.2 g Difco Casamino acids, 10 g yeast extract, 11.46 g TES, 4 ml trace elements, 10 ml [0.5%] K_2PO_4, 4 ml [5 M] $CaCl_2 \times 2H_20$, 15 ml [20%] L-proline, 7 ml 1 M NaOH/l) or MS agar at 30°C (Kieser et al., 2000). *Kutzneria* sp. 744, *S. pristinaespiralis* ATCC 25486, and *S. peucetius* 27952 were used for LC-MS analysis.

HTS for Small Molecules Altering *S. coelicolor* Growth

S. coelicolor M145 spores were dispensed at 20 cfu/well into 96-well screening plates containing 200 μl R5M agar using a Biomex FX liquid handler (Beckman Coulter). Two microliters of compounds was dispensed in duplicate from 1 mM master plates into the screening plate to give a final screening concentration of 10 μM of the Canadian Compound Collection of small molecules (McMaster HTS facility). Outer columns were reserved for control molecules with DMSO used as the normal growth control and thiostrepton (10 μM) used as a growth inhibition control. Plates were shaken to distribute the compounds evenly and then dried in a laminar flow hood for 2 hr. Phenotypes were recorded over 10 days of growth at 30°C.

Actinorhodin and Prodiginine Production

Liquid R5M cultures containing 2×10^7 cfu/ml of *S. coelicolor* were grown for 16 hr at 30°C and subcultured into 5 ml aliquots containing either DMSO or compounds of interest. DMSO concentration was kept constant at 0.1% for liquid analysis. Actinorhodin and prodiginine production was assessed after 72 hr in the presence of 25 μM compounds or DMSO, unless otherwise stated. Both metabolites were measured spectrophotometrically as previously described by Kieser et al. (2000). Briefly, 1 ml aliquots were centrifuged, and supernatant was extracted with 1:1 chloroform:acidic methanol. The chloroform phase was read at 542 nm corresponding to γ-actinorhodin. The pellet was weighed and 1 ml of acidic methanol added; the supernatant was then read at 530 nm, corresponding to prodiginines. Analysis of actinorhodin production was also assessed visually on MS agar with DMSO concentrations kept constant at 1%.

LC-MS Analysis

R5M was inoculated with *S. coelicolor*, *Kutzneria* sp. 744, and *S. pristinaespiralis* ATCC 25486, and MS agar was inoculated with *S. peucetius* 27952 in the presence of either DMSO or 10 μM ARC2 and grown for 7 days at 30°C. Strains were extracted in an equal volume of butanol, sonicated, and left overnight. Extracts were filtered through Whatman paper and evaporated. Extracts were resuspended in 1:1 CH_3CN:H_2O and subjected to LC-MS analysis. CDA was extracted after 7 days of growth on R5M as previously described by Powell et al. (2007). LC-MS analysis was performed on an Agilent 1200 "RR" series LC system coupled to a Bruker micrOTOF II with an ESI ionization source. LC was carried out using a Phenomenex Kinetex C18 column (50 × 2.1 mm, 2.6 μm, 100 Å), H_2O plus 0.1% formic acid (A), and CH_3CN plus 0.1% formic acid (B) as solvents, 40°C column block, and the following gradient: flow 0.2 ml/min, 0.5 min 5% B, 5–9 min 95% B, 10–15 min 5% B. The MS conditions were set to a capillary voltage of 4.5kV for positive mode, nebulizing gas pressure (N2) of 3 barr, dry gas flow rate (N2) of six l/min, temperature at 200°C, and a scan rate of 1 Hz.

Structure-Activity Library Construction and Analysis

Compounds 1–8 were purchased from Maybridge. Compounds 9–35 were synthesized as described in the Supplemental Experimental Procedures. Activity was assessed visually by the production of actinorhodin when spotted as a titration (0.78, 1.56, 3.13, 6.25 12.5, 25, 50, 100 μM) on MS agar. Outer columns contain DMSO only, and DMSO was kept constant at 1%.

FabV Complementation

FabV was PCR amplified from *Pseudomonas aeruginosa* PAO1 chromosomal DNA and introduced into pSET152 containing the ErmE* promoter through the use of the EcoRV and BamHI restriction sites. pSET152*ermE**p and pSET152 *ermE**p*fabV* were introduced into *S. coelicolor* by conjugal transfer. The ability of FabV to compliment the antimicrobial activity of triclosan and other antibiotics was assessed as a titration of compounds (0.39, 0.78, 1.56, 3.13, 6.25 12.5, 25, 50, 100 μM) on solid MS agar with outer wells containing DMSO only. Prodiginine complementation was assessed through LC-MS analysis. *S. coelicolor* M145 containing empty vector or FabV was grown for 7 days on solid MS agar in the presence of DMSO or ARC2 (1, 2.5, 5, 10 μM), extracted with butanol, dried, and resuspended in 1:1 CH_3CN:H_2O. Samples were analyzed using the aforementioned LC-MS conditions.

FabI Enzyme Assay

The assay was conducted as previously described by Liu et al. (2011). Briefly, 10 μM of *E. coli* FabI (Sino Biological) in the presence of PIPES (30 mM), NaCl (150 mM), NADH (250 μM), and crotonyl-CoA (200 μM) was assessed for its ability to convert the crotonyl-CoA substrate. The rate of reaction was determined by the decrease in absorbance at 340 nm corresponding to NADH consumption during crotonyl-CoA conversion by FabI. Crotonyl-CoA was added last to initiate the reaction.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.06.013.

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