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ORIGINAL ARTICLE

Engineered biosynthesis of the antiparasitic agent frenolicin B and rationally designed analogs in a heterologous host

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The polyketide antibiotic frenolicin B harbors a biosynthetically intriguing benzoisochromanequinone core, and has been shown to exhibit promising antiparasitic activity against *Eimeria tenella*. To facilitate further exploration of its chemistry and biology, we constructed a biosynthetic route to frenolicin B in the heterologous host *Streptomyces coelicolor* CH999, despite the absence of key enzymes in the identified frenolicin gene cluster. Together with our understanding of the underlying polyketide biosynthetic pathway, this heterologous production system was exploited to produce analogs modified at the C15 position. Both the natural product and these analogs inhibited the growth of *Toxoplasma gondii* in a manner that reveals sensitivity to the length of the C15 substituent. The ability to construct a functional biosynthetic pathway, despite a lack of genetic information, illustrates the feasibility of a modular approach to engineering medicinally relevant polyketide products.
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INTRODUCTION

Type II polyketide synthases (PKSs) catalyze the biosynthesis of the carbon skeletons of numerous polyfunctional aromatic natural products from the actinomycetes, including the clinically important antibiotic oxytetracycline and the anticancer agent doxorubicin.[1,2] Recent analysis has demonstrated considerable functional modularity within these multienzyme assemblies, which in turn can be exploited to alter priming units and change polyketide backbone length.[3,4] At the same time, the substrate flexibility of late-stage tailoring enzymes in these natural product pathways can also be exploited to create designer molecules.[4,5] Here, we have harnessed each of these modular elements to reconstruct a chimeric pathway to the antiparasitic antibiotic frenolicin B (**1b**), as well as targeted analogs in a heterologous host. Biological evaluation of our analogs against *Toxoplasma gondii* has revealed the strong sensitivity of this family of antibiotics to the identity of the C-15 substituent, thereby highlighting a new direction for future antiparasitic drug design.

The benzoisochromanequinone (BIQ) antibiotic frenolicin B, produced by the actinomycete *Streptomyces roseofulvus*, has been extensively investigated as an anticoccidial agent owing to its potent activity against *Eimeria tenella*.[6–9] Nonetheless, low fermentation titers[10] and challenging synthetic procedures[11,12] appear to have limited further development of this promising anti-infective lead substance. We sought to address both of these problems by reconstructing a biosynthetic pathway to frenolicin B in the heterologous host *Streptomyces coelicolor* CH999, from which the entire actinorhodin biosynthetic gene cluster has been deleted.[13]

MATERIALS AND METHODS

General

S. coelicolor CH999/pBOOST*,[14] which lacks the complete actinorhodin (*act*) gene cluster, was used as the host for production of polyketides. The plasmid pBOOST* has been shown to cointegrate with vectors containing the SCP2* origin of replication, thereby resulting in higher copy numbers and correspondingly improved antibiotic titers.[14] The transformation of shuttle vectors bearing BIQ pathway genes into *S. coelicolor* CH999/pBOOST* were performed following standard procedure.[15] Deuterated solvents for NMR experiments were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and all other solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA) at the highest available grade. ^{1}H and ^{13}C NMR spectra of purified polyketide products were recorded on Varian Inova 500 or 600 MHz instrument (Varian, Walnut Creek, CA, USA) in CD_3OD or DMSO-d_6. The ^{1}H NMR spectra were referenced to the solvent peak at 3.31 p.p.m. for CD_3OD or 2.50 p.p.m. for DMSO-d_6. The ^{13}C NMR spectra were referenced to solvent at 49.0 p.p.m. for CD_3OD or 39.52 for DMSO-d_6. Single-bond ^{1}H–^{13}C, multiple-bond ^{1}H–^{1}H and ^{1}H–^{13}C connectivity was determined by HSQC, COSY and HMBC, respectively on Varian Inova-600 NMR instrument. Mass spectra were obtained by ESI at the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University.

Construction of shuttle vectors

All cloning steps were performed in either *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) or *E. coli* DH5α (MCLab, South San Francisco, CA, USA). Plasmids

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used for cloning included pUC18 (New England Biolabs, Ipswich, MA, USA) and pET28b (Novagen, now EMD Bioscience, Madison, WI, USA). Expression vectors were constructed using standard molecular biology techniques. See Supplementary Information for a detailed description of the primers and construction.

Production, isolation and characterization of polyketide products

Each transformed strain was grown on R5 agar plates containing 50 mg l^{-1} thiostrepton and 100 mg l^{-1} of apramycin at 30 °C for 7 days,[15] after which metabolites were extracted with either 100% EtOAc or EtOAc/MeOH/acetic acid (89:10:1). This crude material was purified by a variety of methods including preparative HPLC and $NaHCO_3$ extraction. Structural determination was performed using a variety of 2D heteronuclear NMR experiments and LC/MS. See Supplementary Information for full details.

T. gondii growth inhibition

Tachyzoites of *T. gondii* strain RHΔ*hxgprt* YFP[16] were harvested from human foreskin fibroblast (HFF) monolayers grown in 25 cm^2 T-flasks. These parasites were then inoculated into a 96-well culture plate containing a confluent layer of HFF cells with ~8×10^3 tachyzoites/0.32-cm^2 well. Parasites were pre-incubated with HFF cells for 6 h at 37 °C, 5% CO_2. After pre-incubation, the media was exchanged for fresh DMEM complete ((Dulbecco's modified eagle medium) supplemented with 1% PS (penicillin–streptomycin) and 10% fetal bovine serum) containing a given concentration of antiparasitic agent in DMSO. Cultures were then incubated for 72 h at 37 °C, in 5% CO_2. Parasite growth inhibition was monitored in a SpectraMax Gemini EM fluorescent plate reader (Sunnyvale, CA, USA) at 510 nm excitation and 540 nm emission. ED_{50}s were determined using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Cytotoxicity assay

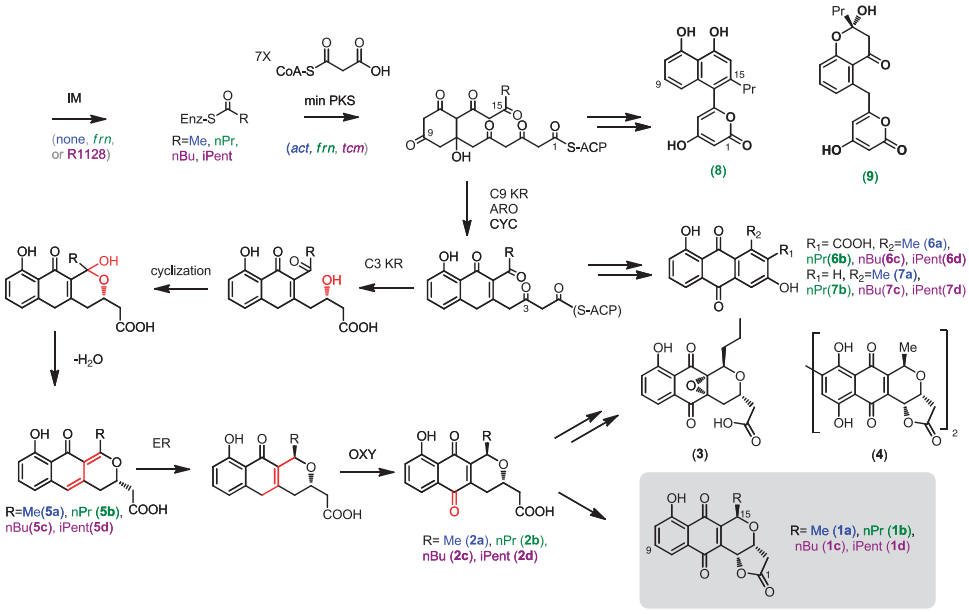
HFF cell cytotoxicity was determined using a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxylphenyl)-2H-tetrazolium) assay (CellTiter 96 Aqueous One Solution Assay; Promega, Madison, WI, USA). HFF cells (2×10^3 cells per well) were grown in a 96-well plate until confluence (~48 h) in phenol red-free DMEM complete at 37 °C, 5% CO_2. Varying concentrations of antiparasitic agent were then added to each well and incubated for 48 h at which point the medium was replaced with 100 µl phenol red-free DMEM and 20 µl MTS. After an additional 1.5 h of incubation at 37 °C, and the absorbance at 490 nm was calculated and the ED_{50} was determined using GraphPad Prism.

RESULTS AND DISCUSSION

Determining the tailoring enzymes necessary for BIQ core formation

Before embarking on the reconstruction of the frenolicin pathway, we first attempted to complete the biosynthesis of the simplest BIQ, deoxykalafungin **2a** (Scheme 1),[17] which does not require an initiation module and is an intermediate in the actinorhodin (**4**) pathway.[18] Previous studies have shown that introduction of the plasmid pRM5 (which encodes *act KS/CLF*, *ACP*, *C-9 KR*, *ARO* and *CYC* genes; for details, see Scheme 1) into CH999 leads to the production of 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (**6a**) and aloesaponarin II (**7a**), as summarized in Table 1.[13,14] The next step in BIQ production is stereoselective reduction of the C-3 ketone into an alcohol capable of cyclizing the third ring of the scaffold (Scheme 1 in red). To accomplish this, we cloned the C-3 ketoreductase gene, encoded by *act*VI-ORF1, onto pRM5, yielding the plasmid pJF6 (Table 1). As predicted, *S. coelicolor* CH999/pJF6/pBOOST* yielded **5a** after cyclization and loss of water (Supplementary Figure S16).[5,19]

The next step in the pathway is enoyl reduction of **5a** followed by oxygenation. Mutants of the actinorhodin-producing strain *S. coelicolor* A3(2), in which the enoyl reductase gene *act*VI-ORF2 has been inactivated, produce no actinorhodin, and mutants of the secondary enoyl reductase gene *act*VI-ORF4 produce only 18% of the actinorhodin of the native strain.[20] We therefore constructed two derivatives of pJF6, pCR66 and pCR67, harboring *act*VI-ORF2 and *act*VI-ORF2/*act*VI-ORF4, respectively (Table 1). The product profiles of *S. coelicolor*



Scheme 1 A biosynthetic pathway to the benzoisochromanequinones. The presumed biosynthetic route to **1a**–**d** is shown along with isolable intermediates and shunt products. The natural product frenolicin A (**3**) produced by *S. roseofulvus*, and the natural product actinorhodin (**4**) produced by *S. coelicolor*, are also shown as forming from their corresponding carboxylic acids, **2**. For details, see text. ARO, 1st ring aromatase encoded by *act*VII; CYC, 2nd ring cyclase encoded by *act*IV; C3 KR, C-3 ketoreductase encoded by *act*VI-ORF1; C9 KR, C-9 ketoreductase encoded by *act*III; ER, enoyl reductase encoded by *act*VI-ORF2; IM, initiation module (ketosynthase III, acyl carrier protein, acyl-ACP thioesterase); min PKS, minimal polyketide synthase (ketosynthase/chain length factor (KS/CLF), acyl carrier protein (ACP), malonyl-CoA:ACP transacylase (MAT)); OXY, oxygenase, encoded by an unidentified enzyme in the *S. coelicolor* genome.

Table 1 Plasmid constructs and resulting polyketide products

Plasmid[a]	*KS-CLF*	*ACP*	*Initiation module*	*Other relevant PKS genes*	*Tailoring*[b]	*Polyketide products*[c]
pRM5[d]	*act*	*act*		*act* KR, *act* ARO, *act* CYC	*act*VB	**6a, 7a**
pJF6	*act*	*act*		*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VB	**5a, 6a, 7a**
pCR66	*act*	*act*		*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VI-ORF2, *act*VB	**2a, 5a, 6a, 7a**
pCR67	*act*	*act*		*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VI-ORF2, *act*VI-ORF4, *act*VB	**2a, 5a, 6a, 7a**
pJF35	*frn*	*frn*	*frn*	*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VI-ORF2, *act*VB	**2b, 6b, 8, 9**
pYT127[d]	*tcm*	R1128	R1128	*act* KR, *act* ARO, *act* CYC	*act*VB	**6c, 6d, 7c, 7d**
pJF7	*tcm*	R1128	R1128	*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VB	**5c, 5d 6c, 6d, 7c, 7d**
pJF9	*tcm*	R1128	R1128	*act* KR, *act* ARO, *act* CYC	*act*VI-ORF1, *act*VI-ORF2, *act*VB	**2c, 2d, 5c, 5d, 6c, 6d, 7c, 7d**

[a]Each pRM5-derived shuttle vector was introduced into *S. coelicolor* CH999/pBOOST* via transformation.
[b]Function of gene products: *act*VB flavin reductase; *act*VI-ORF1 C-3 ketoreductase; *act*VI-ORF2 enoyl reductase; *act*VI-ORF4 secondary enoyl reductase.
[c]See Supplementary Information for compound characterization.
[d]Previously reported constructs (see text).

Table 2 Bioactivity of compounds 1a–d and pyrimethamine

Compound	*T. gondii* ED_{50} *(nM)*	*HFF1 cytotoxicity* ED_{50} *(nM)*	*Therapeutic index*
1a	230 ± 15	5700 ± 800	25
1b	260 ± 15	2400 ± 300	9
1c	420 ± 30	—	—
1d	1200 ± 60	8000 ± 2000	7
Pyrimethamine	820 ± 60	> 50 000	> 60

Each compound was tested at in least triplicate. The therapeutic index is calculated by dividing the cytotoxic ED_{50} by the EC_{50} against *T. gondii*. See Supplementary Figures S1 and S2 for growth inhibition curves.

harboring either plasmid was identical, and included **2a** as the next isolable intermediate (Supplementary Figure S16). This finding has two interesting implications for BIQ biosynthesis. First, overexpression of *act*VI-ORF2 in a pRM5-derived vector obviates the need for a supplementary enoyl reductase in a heterologous system. Second, and more interestingly, the inferred product of the enoyl reductase reaction (Scheme 1 bottom left) was not observed, nor was there a large buildup of **5a**. It therefore appears that, although the *act* gene cluster harbors dedicated oxygenase genes,[21,22] the *S. coelicolor* genome either has a nonspecific oxygenase capable of catalyzing the same reaction, or that quinone formation proceeds spontaneously in air.

Production of frenolicin B

Having identified the genes required to produce **2a** in *S. coelicolor* CH999/pBOOST*, we turned our attention to frenolicin B and analogs thereof. Whereas the minimal PKS responsible for frenolicin production has been previously defined,[23] the initiation module and downstream tailoring enzymes have not yet been characterized. Intriguingly, homologs of the *act*VI-ORF1 and *act*VI-ORF2 genes are not known to exist in the frenolicin (*frn*) gene cluster.[24] In fact, constructs harboring all putative biosynthetic genes from the identified *frn* gene cluster failed to produce frenolicin or any related BIQ antibiotic (data not shown). In the face of insufficient genetic data, we hypothesized that the tailoring enzymes identified in production of **2a** could be functionally repurposed for the biosynthesis of longer chain analogs. To accommodate the longer alkyl priming chain, we replaced the octaketide *act* KS-CLF genes with the mixed octaketide/nonaketide *frn* KS-CLF genes, and also included the genes thought to encode the initiation module of the *frn* PKS.

The *frn* gene cluster harbors homologs of the genes encoding the well-characterized initiation module of the R1128 cluster.[25] Specifically, *frn*I is a homolog of *zhu*H, *frn*K is a homolog of *zhu*C and *frn*J is a homolog of *zhu*N. These genes encode a homodimeric KS responsible for chain initiation, an acyl-ACP thioesterase and a dedicated ACP for the initiation module, respectively. Co-expression of these three *frn* genes with the genes encoding the *frn* KS-CLF, the *frn*N ACP the *act* C-9 KR, the *act* ARO and the *act* CYC, as well as the *act*VI-ORF1 and *act*VI-ORF2 genes (pJF35; Table 1) yielded a mixture of butyryl-primed nonaketide derivatives that arise from off-path cyclization reactions (Supplementary Figure S17). In addition, very small quantities of **2b** were also observed. Aside from **6b**, the two other major shunt products were propyl analogs of the previously identified compounds dehydromutactin and SEK34.[26–28] They form either due to the inability of the *act* ARO to aromatize the first ring or the inability of the *act* CYC to form the second six-membered ring (Scheme 1). Notwithstanding the relative abundance of these shunt products, the ability of *act*VI-ORF1 and *act*VI-ORF2 to replace the unidentified components of the frenolicin pathway underscores the potential for rational design of a biosynthetic scheme using a diverse PKS toolbox in the face of incomplete genetic information.

Analogs of frenolicin B

With a route in hand to the bioactive natural product frenolicin B, we turned our attention to the rational design of biosynthetic analogs. Earlier work with *E. tenella* had shown that extension of the C-15 methyl substituent of **1a** to a propyl substituent (**1b**) led to improved antiparasitic activity.[6] We therefore reasoned that analogs **1c** and **1d** could be useful to investigate the steric effect of the C-15 substituent on biological activity. As chemical synthesis of such analogs is not straightforward, we replaced the genes encoding the *frn* initiation module and ACP with homologs from the R1128 cluster, which incorporates bulkier primer units into the polyketide backbone.[29] At the same time, to compensate for the anticipated increase in overall chain length of the polyketide skeleton, we also replaced the *frn* KS-CLF genes with homologs from the decaketide tetracenomycin (*tcm*) PKS.[30] In combination with the *act* KR, *act* ARO and *act* CYC, this hybrid bimodular PKS gene cluster yielded a mixture of compounds, including **6c**, **6d**, **7c** and **7d** (pYT127; Table 1).[23]

Further inclusion of the *act*VI-ORF1 and *act*VI-ORF2 genes yielded *S. coelicolor* CH999/pJF9/pBOOST*, which produced a spectrum of products including the desired **2c** and **2d** (Table 1 and Supplementary Figure S18). Although this strain produced a wide range of unchar-

acterized polyketide products, the ratio of the desired products **2c** and **2d** to the shunt products **6c** and **6d** was considerably higher in this recombinant strain than in *S. coelicolor* CH999/pJF35/pBOOST*. The lack of large quantities of butyl- and isopropyl-primed analogs of **8** and **9** indicate that *act* ARO and CYC interface with the *tcm* min PKS better than with the *frn* PKS. In addition, this suggests that substrate incompatibility arising from increased alkyl priming unit length is unlikely to be the cause of the large amounts of **8** and **9** observed in *S. coelicolor* CH999/pJF35/pBOOST*. These rationally designed analogs highlight the versatility of our approach to polyketide construction, and enabled evaluation of their antiparasitic activities.

Biological evaluation against *T. gondii*

Previous studies with *E. tenella* showed that the lactone forms of the BIQs **1a** and **1b** were more potent than the free-acid forms (**2a** and **2b**).[6] We therefore converted our isolated analogs **2a–d** into their corresponding lactones **1a–d** by previously reported methods.[31] The structures of the novel compounds **1c** and **1d** were confirmed by NMR and mass spectrometric analysis (Supplementary Tables S3, S5 and S6). The compounds were assayed against *T. gondii*, the causative agent of toxoplasmosis, along with the known antiparasitic agent pyrimethamine as a reference.[32] A slight modification of a previously reported yellow fluorescence protein reporter assay was used for this purpose[16] (Supplementary Figures S1 and S2). As summarized in Table 2, within the C_1–C_4 range, the alkyl substituent has little effect on antiparasitic activity against *T. gondii*, but the longer branched substituent leads to a notable decrease in activity. Thus, the benefit of the longer substituent against *E. tenella* does not hold in our assays, and could be due to improved pharmacokinetics or differential species specificity. To assess their relative tolerability by mammalian cells, we also measured the cytotoxicity of **1a–d** against HFF-1 cells using a MTS-based assay (Supplementary Figure S3). The therapeutic indices of **1a–d**, reported in Table 2, appear to be comparable.

CONCLUSION

In summary, notwithstanding incomplete genetic data for the natural product biosynthetic pathway, we have successfully engineered a chimeric, heterologous system for the production of frenolicin B. Furthermore, we have exploited this heterologous system to produce novel frenolicin analogs that are difficult to access by alternative methods. Our results showcase the utility of a modular approach to the creation of medicinally relevant polyketide products of type II PKSs. Last but not least, we have shown that the new compounds have antiparasitic activity against *T. gondii in vitro*. Further modification of the biosynthetic system presented here could yield additional improvements in BIQ productivity as well as additional analogs of frenolicin B.

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