Published in final edited form as:

Biotechnol Bioeng. 2018 November ; 115(11): 2771–2777. doi:10.1002/bit.26735.

Broadened Glycosylation Patterning of Heterologously Produced Erythromycin

Lei Fang^{1,†}, Guojian Zhang^{1,2,3,*,†}, Omar El-Halfawy^{4,5}, Max Simon¹, Eric D. Brown⁴, and Blaine A. Pfeifer^{1,2,3,*}

¹Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, New York, 14260, USA

²Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, People's Republic of China

³Laboratory for Marine Drugs and Bioproducts of Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, People's Republic of China

⁴Michael G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, L8N 3ZS, Canada

⁵Microbiology and Immunology Department, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt

Abstract

The biosynthetic flexibility associated with the antibiotic natural product erythromycin is both remarkable and utilitarian. Product formation is marked by a modular nature where directing compound variation increasingly spans both the secondary metabolite core scaffold and adorning functionalization patterns. The resulting molecular diversity allows for chemical expansion of the native compound structural space. Accordingly, associated antibiotic bioactivity is expected to expand infectious disease treatment applications. In the enclosed work, new glycosylation patterns spanning the deoxysugars D-forosamine, D-allose, L-noviose, and D-vicenisamine were engineered within the erythromycin biosynthetic system established through an *Escherichia coli* heterologous production platform. The resulting analogs highlight the expanded flexibility of the erythromycin biosynthetic process. In addition, the new compounds demonstrated bioactivity against multiple Gram-positive tester strains, including erythromycin-resistant *Bacillus subtilis*, and limited activity against a Gram-negative bacterial target.

Keywords

erythromycin; analog; antibiotic; *E. coli*; glycosylation; polyketide

*Correspondence: Department of Chemical and Biological Engineering, The State University of New York at Buffalo, Buffalo, New York 14260, USA; Tel.: 716-645-1198; blainepf@buffalo.edu (B.A. Pfeifer). Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, People's Republic of China: Tel: 0086-532-82031619; zhangguojian@ouc.edu.cn (G. Zhang).

[†]These authors contributed equally

1 INTRODUCTION

The erythromycin biosynthetic system features a modular polyketide synthase (PKS) with remarkable interchangeability of the enzymatic functional units (i.e., modules) responsible for polyketide formation (McDaniel et al., 1999; Pfeifer, 2001). Such systems thus gained broad scientific and engineering attention for the potential to rationally design new products through module exchange (Cane & Walsh, 1999; Cane, Walsh, & Khosla, 1998; Khosla & Harbury, 2001). In the case of erythromycin, six modules are divided across a three enzyme deoxyerythronolide B synthase (DEBS) PKS which converts one propionyl-CoA and six (2*S*)-methylmalonyl-CoA substrates to the polyketide product 6-deoxyerythronolide B (6dEB) (Figure 1).

Many polyketide and other complex natural products also feature adorning functionalities added by tailoring reactions (Thorson, Hosted, Jiang, Biggins, & Ahlert 2001). Here, erythromycin includes two deoxysugar moieties (L-mycarose and D-desosamine). Similar to the upstream core polyketide formation process, an emerging theme in engineered biosynthesis is the interchangeability or “modularity” of the downstream deoxysugar tailoring reactions (Salas & Mendez, 2007). Importantly, in the case of erythromycin (and likely other complex natural products), final antibiotic activity is only observed upon glycosylation of 6dEB. Hence, engineering new glycosylation tailoring reactions holds much promise in structural-based bioactivity variation.

Biosynthetic manipulation efforts require a functional recombinant system to enable engineering. Our group has systematically established erythromycin biosynthesis through *Escherichia coli* (Pfeifer, 2001; H. Zhang, Wang, Wu, Skalina, & Pfeifer, 2010). More specifically, metabolic engineering was applied to supply required intracellular biosynthetic precursors prior to the isolation and transfer of the pathway enzymes for polyketide formation and tailoring.

The heterologous *E. coli* erythromycin system allows for the assessment of deoxysugar pathway modularity, aided by the growing number of deoxysugar pathways revealed through genome sequence data. As such, we present in this work four new deoxysugar pathways assembled to produce D-allose, D-forosamine, L-noviose, and D-vicenisamine (Figure 2), representing a broad range of deoxysugars and associated complex natural product pathways to test broadened utility of the *E. coli* heterologous erythromycin platform (Anzai et al., 2003; Hong, Zhao, Melancon, Zhang, & Liu, 2008; Shindo, Kamishohara, Odagawa, Matsuoka, & Kawai, 1993; Thibodeaux, Melancon, & Liu, 2008; Thuy, Lee, Kim, Heide, & Sohng, 2005). The pathway construction approach relied upon DNA synthesis of the prerequisite genetic material to optimize codon usage for *E. coli* prior to the construction of plasmid-based operons, which were then tested for successful stand-alone heterologous compound formation.

In Figure 3, each of the target deoxysugars were identified by LC-MS assessment of production cultures. In confirming compound formation, support was provided for incorporation into the full erythromycin formation process, as lack of deoxysugar production would preclude successful incorporation into subsequent erythromycin analogs. Thus, the

new deoxysugar plasmids were individually introduced into an *E. coli* cell also containing the remaining tailoring reactions required for the conversion of the exogenously fed polyketide 6dEB compound (extracted from separate production cultures devoted to producing this precursor) to a fully active final product (Figure 2).

Establishment of the deoxysugar substrates within the *E. coli* heterologous system was a promising step towards new erythromycin analogs. However, a separate challenge is sufficient flexibility in the biosynthetic process to accommodate new elements. Specifically, the glycosyltransferase enzyme EryBV, natively responsible for attaching the original L-mycarose to the polyketide core of the erythromycin compound, would have to incorporate the new deoxysugars. Though, as we illustrate below, this and previous attempts on our part continue to highlight the flexibility of this enzyme to accept new pathway intermediates.

As demonstrated in Figure 3, production of new erythromycin analogs resulted for each deoxysugar pathway introduced. This result confirms the flexibility of the EryBV glycosyltransferase which, when added to the results of our previous studies, increases the number of deoxysugars incorporated by the EryBV enzyme to 20 (Jiang, Zhang, Park, Li, & Pfeifer, 2013; G. Zhang, Li, Fang, & Pfeifer, 2015). However, as we have observed previously (G. Zhang et al., 2015), the EryK C12 hydroxylase did not show activity towards the new erythromycin analogs and the EryG C3'-O-methyltransferase only showed activity towards the allose analog.

Expanded EryBV enzymatic capability to incorporate numerous deoxysugars may reflect a general flexibility in erythromycin biosynthesis (in line with upstream and downstream themes of biosynthetic modularity) as a cellular advantage relative to microbial competition for environmental resources. For example, extended flexibility in both the polyketide and tailoring portions of erythromycin biosynthesis would endow the native production host numerous resulting analogs to apply against microbial competitors capable of developing resistance to a certain version of erythromycin. The result would then be a biosynthetic system with great potential in molecular engineering for bioactivity augmentation, with particular promise within a platform as technically-supportive as the *E. coli* heterologous host. Given the requirement of glycosylation for molecular function, the results also support continued variation in the downstream steps of modularity for erythromycin and other complex natural product systems towards expanded bioactivity for the resulting analogs.

Bioactivity of the new analogs was then assessed across results presented in Figure 4. A solid medium assessment of analog antibiotic activity was first tested using streptomycin-resistant and erythromycin-resistant *B. subtilis* tester strains. For both initial analyses, results were compared to a series of negative controls (6dEB alone, background methanol solvent, and extract from cells containing the background pCOLADuet plasmid) and positive controls that included erythromycin A and an olivose analog, active against erythromycin-resistant *B. subtilis*, generated previously by our group (Jiang, Zhang, et al., 2013; G. Zhang et al., 2015). The qualitative results indicated that the new analog extracts retained activity against the streptomycin-resistant *B. subtilis* strain and that the forosamine and noviose analogs demonstrated activity against the erythromycin-resistant *B. subtilis* strain (Figure 4A).

To further probe and quantify bioactivity, liquid phase MIC assessment was completed for a range of Gram-positive and -negative tester strains (Figure 4B). With the streptomycin-resistant *B. subtilis* strain, the vicensamine analog matched the original erythromycin compound in MIC potency while the remaining analogs were within 2–4× the MIC level of erythromycin A. Importantly, when assessment was completed against the erythromycin-resistant *B. subtilis* tester strain, the noviose and forosamine analogs demonstrated bioactivity beyond that observed for olivose. This result, in particular, supports the premise of designer analogs as a response to the mutational events that produce drug-resistant bacterial strains. The noviose and vicensamine (and to a more limited extent forosamine) analogs demonstrated activity against Gram-positive *Staphylococcus aureus*, though 8× less effective than the original erythromycin compound. Though no activity was observed for the Gram-negative *Pseudomonas aeruginosa* tester strain (data not shown), limited activity against *E. coli* was observed over the first 18 hours of the assay (with the forosamine analog most potent), which diminished by 24 hours, possibly as a result of a resistant sub-population of cells dominating the culture over time.

The olivose erythromycin analog was included in the bioactivity analysis because it had previously demonstrated activity against erythromycin-resistant *B. subtilis* (Jiang, Zhang, et al. 2013; G. Zhang et al., 2015) and thus provided a good comparison to the new analogs tested in the current study. Beyond the erythromycin-resistant *B. subtilis* strain, the noviose and forosamine analogs demonstrated improved activity relative to the olivose analog against the newly tested Gram-positive and -negative tester strains (as did the vicensamine analog). Through any clear structure-activity relationship cannot be concluded at this stage without more dedicated structural biology information, the results indicate that chemical variety at this deoxysugar location in the erythromycin compound architecture promotes variation in, and in some cases improves, associated bioactivity.

In summary, bioactivity for four new erythromycin analogs was observed as a result of the successful incorporation of engineered deoxysugar pathways within the *E. coli* heterologous biosynthetic system. The results further support the approach of downstream deoxysugar pathway modularity in conjunction with enzymatic flexibility exhibited by the erythromycin EryBV glycosyltransferase. Bioactivity was particularly notable for the noviose, forosamine, and vicensamine erythromycin analogs due to activity against erythromycin-resistant and additional Gram-positive and -negative bacterial targets. These results extend the concept of complex natural product modularity as a designed approach and response to the antibiotic mutational evasion capabilities of pathogenic bacteria.

2 MATERIALS AND METHODS

2.1 Chemicals, reagents, plasmids, and strains

Primers were purchased from Eurofins Genomics (Huntsville, AL). Ampicillin, spectinomycin, kanamycin, erythromycin, chloramphenicol, lysogeny broth (LB), isopropyl β-D-1-thiogalactopyranoside (IPTG), arabinose, ethyl acetate, and buffer components were purchased from Fisher Chemical (Pittsburgh, PA). The chaperonin plasmid pGro7 was obtained from Takara (Madison, WI), and restriction endonucleases, T4 DNA ligase,

Phusion High-Fidelity PCR Master Mix, and Gibson Assembly Master Mix were purchased from NEB (Ipswich, MA).

Table S1 provides a summary of the plasmids and strains in this work. *E. coli* strains BAP1 and BL21(DE3) were used to generate 6dEB and erythromycin analogs, respectively, and formation of 6dEB required the plasmids pBP130 and pBP144, which when combined encode for the DEBS PKS(Pfeifer, 2001). Plasmid pGJZ10 contains *mtmD* (encoding TTP thymidylate transferase) and *mtmE* (encoding TDP-D-glucose 4',6'-dehydratase) to improve the accumulation of TDP-4-keto-6-D-glucose as the basis for subsequent deoxysugar biosynthesis(G. Zhang et al., 2015). The EryBV and ErmE enzymes are also encoded in this plasmid to facilitate transfer of deoxysugar moieties to the 6dEB polyketide macrolide core and to provide resistance to erythromycin products, respectively. Plasmid pJM3 (D-desosamine biosynthesis pathway and additional tailoring genes) enables the complete biosynthesis of erythromycin analogs(Jiang, Fang, & Pfeifer, 2013).

The deoxysugar gene clusters presented in Figure 2 were constructed as operons, with each preceded by a single T7 promoter and ribosome binding site. Separate Gibson assembly reactions were utilized to combine PCR-amplified operon fragments (primers indicated in Figure 2B; templates were synthetic DNA reading frames [optimized for *E. coli* codon usage] for deoxysugar pathway genes provided by Dr. George Church's group at Harvard Medical School) with pCOLADuet cut with the following restriction enzymes: *NcoI/HindIII* (D-forosamine, L-noviose), *NdeI/XhoI* (D-allose), or *NcoI/BamHI* (D-vicenisamine). An equal volume of digested pCOLADuet and PCR fragments were mixed with 10 μ L of the master mix solution at 50°C for 75 minutes prior to electroporating 5 μ L of the resulting reaction into TOP10 *E. coli* competent cells. Resulting transformant colonies were processed (using Qiagen miniprep kits [Frederik, MD]) for plasmid DNA, which was verified by restriction enzyme digestion.

2.2 Deoxysugar heterologous biosynthesis and analysis

An overnight LB culture of each deoxysugar pathway strain was used to inoculate a 25 mL culture (1% v/v) containing production medium at 37°C for 48 hours; production medium contained the following components: 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 15 g/L glycerol, 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L sodium chloride. Harvested cells were pelleted by centrifugation and stored at -20°C overnight prior to resuspending in 2 mL phosphate buffered saline (PBS) solution and sonicating for 2 minutes. Cell lysates were centrifuge at 4,000 $\times$ g for 10 minutes and supernatants were analyzed by LC-MS using a Shimadzu Prominence LC system coupled to an AP I3000 Triple Quad MS with a Turbo Ion Spray source (PE Sciex). Chromatography was performed on a Waters Xbridge amide 3.5 μ m column (2.1 $\times$ 150 mm). All MS analyses (scanning for parent/daughter fragment m/z ratios) were conducted in negative ion mode. A linear gradient of 10% buffer A (water and 5 mM ammonium acetate [pH adjusted to 9]) to 0% buffer B (10% water/90% acetonitrile/5mM ammonium acetate) was used at a flow rate of 0.2 mL/min for the LC.

2.3 Erythromycin analog biosynthesis and analysis

E. coli strain BAP1/pBP130/pBP144 was used to generate 6dEB with a 250 mL production medium culture inoculated (1% v/v) with an overnight starter culture and incubated at 22°C with shaking for five days once induced with IPTG (100 µM) at an OD₆₀₀ of 0.6 and supplemented with 20 mM sodium propionate. An equal volume of ethyl acetate was used to extract 6dEB from the culture, followed by rotary evaporation of the extract to dryness. The extracted 6dEB was then dissolved in 250 µL methanol.

Cultures (50 mL) of BL21(DE3)/pGJZ10/pJM3/pGro7/pSugars (individual deoxysugar plasmids outlined in Figure 2) were incubated at 37°C with shaking in production medium, induced with IPTG (100 µM) and arabinose (2 mg/mL) at an OD₆₀₀ of 0.6, fed 80 µL of the 6dEB extract, and incubated post-induction at 22°C with shaking for five days. The cultures were extracted with an equal volume of ethyl acetate twice, and the extract was concentrated to dryness using a rotary evaporator for subsequent resuspension in 100 µL methanol. For erythromycin analog quantification, roxithromycin (0.25 µg/mL) was added to a portion of the sample extract to serve as an internal standard during analysis. Titers were quantified by comparing to a standard curve generated from sequential control strain (BL21(DE3)/pGJZ10/pJM3/pGro7/pCOLADuet; this strain also served as a negative control [Figure S1 provides a representative LC-MS analysis result for the methylated allose-erythromycin analog]) cultures containing increasing levels of commercial erythromycin A that were then extracted, dried, resuspended in methanol (with roxithromycin) as indicated above. Experimental and calibration samples were analyzed using a Shimadzu Prominence LC system coupled to an AP I3000 Triple Quad MS with a Turbo Ion Spray source (PE Sciex). Chromatography was performed on a Waters X Terra C18 column 3.5 µm column (2.1 × 250 mm). All MS analyses were conducted in positive ion mode. A linear gradient of 70% buffer A (95% water/5% acetonitrile) to 100% buffer B (5% water/95% acetonitrile) was used at a flow rate of 0.2 mL/min for the LC; each buffer also contained 0.1% formic acid. Once quantified, all erythromycin analog extracts (without roxithromycin) were adjusted to the same concentration (5 mg/mL) prior to bioactivity assessment.

2.4 Antibiotic bioactivity assays

For antibiotic bioactivity analysis by filter paper disk, the assay began with overnight cultures of *Bacillus subtilis* in LB at 37°C with shaking; the cultures were then used to inoculate liquid LB agar (1% v/v) maintained at 40°C. After the liquid agar solidified within standard petri dishes, 0.8 cm diameter filter paper disks were placed onto the solid medium surface. The filter disks were then loaded with 5 µL of a given erythromycin deoxysugar analog, and the plates were incubated at 30°C overnight. Liquid-based minimum inhibitory concentration assays were conducted according to the protocol outlined by the Clinical and Laboratory Standards (Dalyan Cilo et al., 2018) for 24 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. Marc Guell and George Church (Harvard Medical School) for providing the genetic material for the deoxysugars pathways. This work was supported by the NIH (AI126367).

References

- Anzai Y, Saito N, Tanaka M, Kinoshita K, Koyama Y, Kato F. Organization of the biosynthetic gene cluster for the polyketide macrolide mycinamicin in *Micromonospora griseorubida*. FEMS Microbiol Lett. 2003; 218(1):135–141. [PubMed: 12583909]
- Cane DE, Walsh CT. The parallel and convergent universe of polyketide synthases and nonribosomal peptide synthetases. Chemistry and Biology. 1999; 6:R319–R325. [PubMed: 10631508]
- Cane DE, Walsh CT, Khosla C. Harnessing the biosynthetic code: combinations, permutations, and mutations. Science. 1998; 282(5386):63–68. [PubMed: 9756477]
- Dalyan Cilo B, Topac T, Agca H, Saglam S, Efe K, Ener B. Comparison of Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution methods for determining the susceptibilities of *Candida* isolates. Mikrobiyol Bul. 2018; 52(1):35–48. DOI: 10.5578/mb.63991 [PubMed: 29642828]
- Hong L, Zhao Z, Melancon CE 3rd, Zhang H, Liu HW. In vitro characterization of the enzymes involved in TDP-D-forosamine biosynthesis in the spinosyn pathway of *Saccharopolyspora spinosa*. J Am Chem Soc. 2008; 130(14):4954–4967. DOI: 10.1021/ja0771383 [PubMed: 18345667]
- Jiang M, Fang L, Pfeifer BA. Improved heterologous erythromycin a production through expression plasmid re-design. Biotechnol Prog. 2013; 29(4):862–869. DOI: 10.1021/btpr.1759 [PubMed: 23804312]
- Jiang M, Zhang H, Park SH, Li Y, Pfeifer BA. Deoxysugar pathway interchange for erythromycin analogues heterologously produced through *Escherichia coli*. Metab Eng. 2013; 20:92–100. DOI: 10.1016/j.ymben.2013.09.005 [PubMed: 24060454]
- Khosla C, Harbury PB. Modular enzymes. Nature. 2001; 409(6817):247–252. [PubMed: 11196653]
- McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, Ashley G. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel “unnatural” natural products. Proc Natl Acad Sci U S A. 1999; 96(5):1846–1851. [PubMed: 10051557]
- Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. Science. 2001; 291(5509):1790–1792. [PubMed: 11230695]
- Salas JA, Mendez C. Engineering the glycosylation of natural products in actinomycetes. Trends Microbiol. 2007; 15(5):219–232. DOI: 10.1016/j.tim.2007.03.004 [PubMed: 17412593]
- Shindo K, Kamishohara M, Odagawa A, Matsuoka M, Kawai H. Vicenistatin, a novel 20-membered macrocyclic lactam antitumor antibiotic. J Antibiot (Tokyo). 1993; 46(7):1076–1081. [PubMed: 8360102]
- Thibodeaux CJ, Melancon CE 3rd, Liu HW. Natural-product sugar biosynthesis and enzymatic glycodiversification. Angew Chem Int Ed Engl. 2008; 47(51):9814–9859. DOI: 10.1002/anie.200801204 [PubMed: 19058170]
- Thorson JS, Hosted J, Jiang TJ, Biggins J, Ahlert JBJ. Natures carbohydrate chemists the enzymatic glycosylation of bioactive bacterial metabolites. Current Organic Chemistry. 2001; 5(2):139–167.
- Thuy TT, Lee HC, Kim CG, Heide L, Sohng JK. Functional characterizations of novWUS involved in novobiocin biosynthesis from *Streptomyces spheroides*. Arch Biochem Biophys. 2005; 436(1):161–167. DOI: 10.1016/j.abb.2005.01.012 [PubMed: 15752721]
- Zhang G, Li Y, Fang L, Pfeifer BA. Tailoring pathway modularity in the biosynthesis of erythromycin analogs heterologously engineered in *E. coli*. Sci Adv. 2015; 1(4):e1500077.doi: 10.1126/sciadv.1500077 [PubMed: 26601183]
- Zhang H, Wang Y, Wu J, Skalina K, Pfeifer BA. Complete biosynthesis of erythromycin A and designed analogs using *E. coli* as a heterologous host. Chem Biol. 2010; 17(11):1232–1240. DOI: 10.1016/j.chembiol.2010.09.013 [PubMed: 21095573]

Figure 1.

The erythromycin biosynthetic process. The modular deoxyerythronolide B synthase (DEBS) generates the 6dEB polyketide precursor prior to tailoring reactions that include the addition of two deoxysugars. Abbreviations: AT=acyl transferase; ACP=acyl carrier protein; KS=β-keto-acyl synthase; KR=β-keto reductase; DH=dehydratase; ER=enoyl reductase; TE=thioesterase.

Figure 2.

Deoxysugar pathway design and incorporation into the erythromycin analog production scheme. (A) The genetic pathways indicated were designed to convert 6dEB to erythromycin analogs. R=OH with functional EryK activity. Plasmids pGJZ10, pJM3, and pGro7 support general deoxysugar biosynthesis, provide the pathway for D-desosamine biosynthesis, and express protein folding chaperonins, respectively (more details are provided in the Materials and Methods section) and when combined with the individual pSugar plasmids (outlined in part B), allow for the biosynthesis of new erythromycin analogs. (B) Sugar genetic pathways including primer pairs for individual gene amplification prior to pathway assembly and introduction into the erythromycin analog production platform.

Figure 3. Heterologous biosynthesis of deoxysugars and new erythromycin analogs as analyzed by LC-MS. LC traces provided for deoxysugars together with parent/daughter m/z fragments; LC traces (inset) and MS spectra provided for erythromycin analogs.

Figure 4. Bioactivity assessment of erythromycin analogs using filter disk (A) and MIC (B; $\mu\text{g/mL}$) assays.