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Investigating the Reactivities of a Polyketide Synthase Module through Fluorescent Click Chemistry

Amanda Jane Hughes^a, Matthew R. Tibby^a, Drew T. Wagner^a, Johnathan N. Brantley^a, and Adrian T. Keatinge-Clay^a

^aDepartment of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station A5300, Austin, TX, USA, Tel.: (512) 471-2977; Fax: (512) 471-8696; adriankc@utexas.edu

Abstract

A method for monitoring *in vitro* polyketide synthesis has been developed whereby nonchromophoric polyketide products are made brightly fluorescent in a simple, rapid, inexpensive, and bioorthogonal manner through CuAAC with sulforhodamine B azide.

Modular polyketide synthases (PKSs) are the largest enzymes known to man and are responsible for synthesizing some of the most important human medicines.^{1,2} While PKSs perform similar chemistry to fatty acid synthases, they possess an assembly-line organization.³ Sets of enzymes, termed modules, cooperate to extend and process a growing polyketide chain. These enzymes include a ketosynthase (KS) that acquires and extends a growing polyketide chain through a decarboxylative condensation, an acyltransferase (AT) that selects the appropriate extender unit for the chain extension, an NADPH-dependent ketoreductase (KR) that stereoselectively reduces the KS-generated β -keto group, and an acyl carrier protein (ACP) that shuttles the growing polyketide between these enzymes through a thioester linkage to a post-translationally appended phosphopantetheinyl arm. The mature polyketide is typically released by a C-terminal thioesterase (TE) via cyclization or hydrolysis. The ability of these enzymes to construct stereochemically-rich carbon chains containing diverse substituents has inspired the biosynthetic community to engineer these factories to produce molecules of medicinal relevance; however, in order to reprogram PKSs, the reactivities of the enzymes within their modules must be better understood.

There are many challenges associated with investigating the operation of modules, let alone employing them to synthesize desired compounds: products generated are typically nonchromophoric, substrates such as NADPH and methylmalonyl-CoA are costly, and modules are often difficult to express and purify. Traditionally, nanogram to microgram quantities of products are generated from radiolabeled precursors visualized by radio-TLC.⁴ While GC/MS has been employed, derivitization is sometimes necessary and signal-to-noise can be low.⁵ Previously, we employed fluorine NMR to follow fluorinated substrates

through reactions catalyzed by overexpressed PKS enzymes in the commonly-employed biocatalytic setting of dialyzed cell lysate;⁶ however, this method requires both sophisticated instrumentation and knowledge of the chemical shifts of generated products. Recent advances in biocatalytic methods have aided in scaling up module-catalyzed reactions - a glucose-fueled NADPH-regeneration system can provide a constant supply of reduced nicotinamide coenzyme, and extender units like methylmalonyl-*S*-*N*-acetylcysteamine (mm-*S*-NAC) can be enzymatically generated by MatB for the price of ATP.^{7,8} We sought a more general bioorthogonal approach that would allow visualization of polyketide products by HPLC.

Since fluorescent alkyne probes and click chemistry have been used to locate azide-containing glycolipids in the background of a zebrafish,⁹ we reasoned that polyketide products containing a unique chemical functionality could be selectively labeled by a complementary fluorophore in the background of bacterial cell lysate. Here, we showcase a quantitative detection method that reports the fate of alkynyl priming units in module-catalyzed reactions within cell lysate (Fig. 1). Copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) with sulforhodamine B azide enables normally nonchromophoric polyketide products to be sensitively visualized and identified using fluorescence spectroscopy and mass spectrometry.^{10,11} Employing this method, the incorporation of ethanethiol-linked priming and extender units into reduced diketide compounds by the terminal module-TE of the erythromycin PKS (EryMod6TE) was investigated.

All experiments reported here investigated the reactivities of EryMod6TE within *E. coli* cell lysate. Performing reactions with polyketide substrates in dialyzed cell lysate saves the time and expense associated with protein purification and does not suffer from appreciable adventitious catalysis.¹² EryMod6TE lysate was incubated with synthetic priming and extender units before initiating CuAAC with sulforhodamine B azide. Resulting adducts were analyzed by HPLC and characterized by high-resolution mass spectrometry (supplemental information). In the experiments reported here, a fluorimeter was employed for optimal sensitivity, although UV/vis detection also provides an excellent signal.

To decrease costs associated with investigating the reactivities of modules and biocatalytically producing polyketides, the use of a simplified methylmalonyl-CoA analogue as an *in vitro* extender unit was investigated (Fig. 2). Previous *in vitro* reactions have employed enzymatically- or synthetically-generated **1** (mm-*S*-NAC);^{8,12} however, the one-step, multigram synthesis of methylmalonyl ethanethioester (mm-*S*-Et) **2** is much more economical. Thus, EryMod6TE was incubated with priming unit **3**, extender unit **1** or **2**, and the NADPH regeneration system to produce reduced diketide **5**. In all experiments, saturating quantities of nicotinamide coenzyme were employed to obtain optimal activity from EryKR6. After 16 hrs, reactions were subjected to CuAAC with sulforhodamine B azide and analyzed by HPLC.

The analysis revealed that approximately equal quantities of **5*** (asterisks denote sulforhodamine adducts) were generated in reactions employing **1** or **2**; ~35% of the priming unit **3** was accepted, extended, reduced, and hydrolyzed by EryMod6TE to afford reduced diketide **5**. Analysis also showed that ~50% of priming unit **3** was hydrolyzed to

carboxylic acid **4** (Figure 2C-D). TE-catalyzed hydrolysis of priming units has been previously implicated in the low yields (~10%) of EryMod6TE-catalyzed reactions.^{13,14} Remaining **3** may not have reacted due to the gradual loss of EryMod6TE activity - precipitate was noticeable in reactions after 6 hrs.

To demonstrate the use of extender unit **2** in the preparative, biocatalytic syntheses of polyketides, 17 mg of **3** and 13 mg of **2** were incubated with EryMod6TE lysate and the NADPH regeneration system. After 16 hrs, the reaction was acidified, extracted with ethyl acetate, and chromatographed to afford 3 mg of reduced diketide **5** (22% yield), which was characterized by ¹H NMR (supplemental information).

Since the NAC handle of extender unit **1** could be replaced by ethanethiol, we investigated replacing the NAC handle of priming unit **3** with ethanethiol (Fig. 3). Thus, EryMod6TE was incubated with the NADPH regeneration system, extender unit **2**, and either NAC-linked **3** or ethanethiol-linked **9**. After 16 hrs, reactions were subjected to CuAAC with sulforhodamine B azide and analyzed by HPLC. A comparison of the amount of **5*** formed from priming units **3** and **9** revealed that EryMod6TE did not significantly discriminate between NAC or ethanethiol priming unit handles. Building on these results, the promiscuity of EryMod6TE towards NAC- and ethanethiol-linked priming units of varying chain length was assayed. The analysis showed less than 5% difference in product formation between reduced diketide products **13*** and **14***. Thus EryMod6TE does not significantly discriminate between priming units based on chain length or handle.

Having analyzed module-catalyzed reactions employing priming units **3** and **6-10**, we were interested in probing the reactivity of EryMod6TE with a more advanced priming unit, such as β -ketoacyl ethanethioester **15** (Fig. 4). The chemical synthesis of **15** was performed by C-acylation of 5-hexynoic acid with **2** through a reaction developed by Masamune and coworkers.¹⁵ Briefly, **2** was reacted with magnesium ethoxide to form its magnesium salt, 5-hexynoic acid was reacted with carbonyldiimidazole to form its imidazolide, and these intermediates were reacted with one another. EryMod6TE was incubated for 16 hrs with priming unit **15**, extender unit **2**, and the NADPH regeneration system. The reaction was subjected to CuAAC with sulforhodamine B azide and analyzed by HPLC. Interestingly, **15** was reduced by EryKR6 to yield at least two stereoisomers (**17**). Extension of one stereoisomer followed by cyclization yielded ketolactone **19**, while extension followed by another round of EryKR6-mediated reduction prior to cyclization yielded hydroxylactone **20**.

Several insights into the reactivities of the enzymes contained in EryMod6TE were made from its incubation with the β -ketoacyl ethanethioester priming unit **15**. This priming unit was reduced to both *syn*- and *anti*- products by EryKR6 (this KR also exhibits little stereocontrol towards α -methyl, β -ketopentanoyl-S-NAC).¹⁶ At least one of the reduced stereoisomers of **17** was extended, as evidenced by the significant production of ketolactone **19** and hydroxylactone **20**. The relative quantities of **19** and **20** may indicate that EryKR6 kinetically outcompeted EryTE for the ACP-bound intermediate, as observed in preparative reactions with substrates that do not contain a terminal alkyne.¹⁴

As reported for DEBS1+TE (the first polypeptide of the erythromycin PKS fused to EryTE), phosphate had a beneficial effect on the activity of EryMod6TE.¹⁷ In the presence of 50 mM phosphate, priming units were completely turned over by EryMod6TE, apparently owing to enhanced enzyme stability (supplemental information, Fig. S1). Reaction conditions that include glycerol should be used with caution since glycerol was found to attack acyl-TE intermediates, effecting the glycerolysis of the priming unit (supplemental information, Figure S2). To our knowledge, this is the first time TE-mediated glycerolysis activity has been reported.

TE-catalyzed hydrolysis was responsible for the largest loss of the priming unit (>50%). Before PKS module-catalyzed synthesis of new compounds becomes routine, reaction yields must be increased. Ideally, priming units would not be substrates for TE hydrolysis. In recent biocatalytic studies employing modular constructs from the pikromycin PKS, greater than 60% yields were obtained in experiments employing thiophenol-linked priming units.¹⁸ We are currently investigating a panel of alkyne-containing priming units that display decreased TE-mediated hydrolysis while remaining substrates for KSs.

The described method above will help answer many fundamental questions of single module and bimodular systems *in vitro*. Owing to its bioorthogonal nature, it may also be useful in the quantitation of polyketide intermediates and products *in vivo*. The precursor-directed biosynthesis of 15-propargyl erythromycin A is accomplished using a priming unit containing a terminal alkyne.¹⁹ Applying our strategy to such a system would be very informative, especially for engineered versions of the erythromycin synthase that do not produce expected products. In general, we anticipate the described method to greatly accelerate the investigation and optimization of PKS-catalyzed biosynthesis of complex polyketide products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Lighting up polyketide products through fluorescent click chemistry. **A)** The sulforhodamine B azide fluorophore. **B)** Extender units and terminal alkyne priming units are incubated with PKS constructs. Reactions are subjected to click chemistry, and the resulting adducts are visualized by reverse-phase HPLC.

Figure 2.

A comparison of *in vitro* extender units. **A**) Synthetic extender units **1** and **2** employed in experiments. **B**) Reduced diketide **5** was produced by EryMod6TE from priming unit **3**, **1** or **2**, and the NADPH-regeneration system. EryTE-mediated hydrolysis of **3** yields **4**. **C**) Fluorimeter-coupled HPLC analysis of reactions. **D**) EryMod6TE employed extender units **1** and **2** interchangeably, as determined through a comparison of the peak areas of **3***-**5*** (asterisks indicate sulforhodamine adducts).

Figure 3.

EryKS6 promiscuity towards thiol handles and chain lengths. **A**) Terminal alkyne thioester priming unit substrates. **B**) Reduced diketides **5**, **13**, and **14** were generated from *in vitro* EryMod6TE reactions with substrates **3**, **6-10**. **C**) Equivalent product profiles were observed for NAC-linked priming unit **3** and ethanethiol-linked priming unit **9**. **D**) Equivalent product profiles were observed for substrates of varying chain length, regardless of thiol handle.

Figure 4.

EryMod6TE reactivity towards an advanced priming unit. **A**) β -ketoacyl ethanethioester **15** is reduced by EryKR6. At least one of the resulting stereoisomers (**17**) is extended and processed by EryMod6TE to afford ketolactone **19** and hydroxylactone **20**.

B) Reverse-phase HPLC analysis of reactions. Diastereomer(s) **18a** are *syn* and diastereomer(s) **18b** are *anti*, as the retention time of **18a*** matches that of **5***. **C**) A bar graph compares the peak areas of the products.