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In Vitro Precursor-Directed Synthesis of Polyketide Analogues with Coenzyme A Regeneration for the Development of Antiangiogenic Agents

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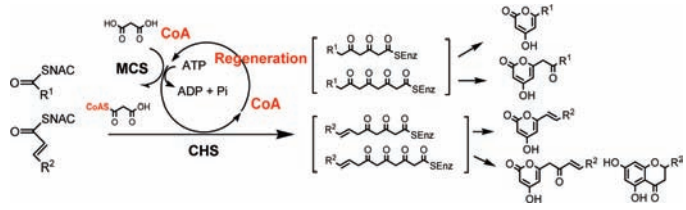
ABSTRACT



Polyketide analogues are produced via in vitro reconstruction of a precursor-directed polyketide biosynthetic pathway. Malonyl-CoA synthetase (MCS) was used in conjunction with chalcone synthase (CHS), thereby allowing efficient use of synthetic starter molecules and malonate as extender. Coenzyme-A was recycled up to 50 times. The use of a simple immobilization procedure resulted in up to a 30-fold higher yield of pyrone CHS products than that obtained with the free enzyme solutions.

Polyketides represent an exceptionally diverse class of natural products with applications as antibiotics, anticancer compounds, cholesterol-lowering drugs, and animal feed supplements.[1] Their often high biological activity and potential for broad structural diversity are attractive driving forces for exploration of polyketide analogues with tailored structures and biological function. Along these lines, chemical synthesis has been used to generate polyketide analogues;[2] however, the structural and chemical complexity of polyketides and their derivatives makes this route difficult.

Alternatively, an in vivo heterologous production of polyketides by metabolic engineering of biosynthetic pathways or combinatorial biosynthesis is playing an increasingly important role in natural product production.[3] For example, precursor-directed biosynthesis using *N*-acetylcysteamine thioester (SNAc) starter substrates has proven to be effective in yielding unnatural polyketide analogues.[3b] However, producing polyketides in vivo has numerous limitations, such as limited substrate permeability into the cell, the presence of competing pathways with complex regulatory controls, and cellular toxicity.[4]

As opposed to chemical- or cell-based polyketide synthesis, in vitro reconstruction of polyketide synthase (PKS) biosynthetic pathways is an emerging route to explore novel enzyme mechanisms, assess substrate specificity, and produce diverse polyketides.[5] However, the instability of PKS and the burden of expensive extender (malonyl- or methylmalonyl-coenzyme A (CoA)) substrates have made it difficult to achieve high-yield production of polyketide analogues and

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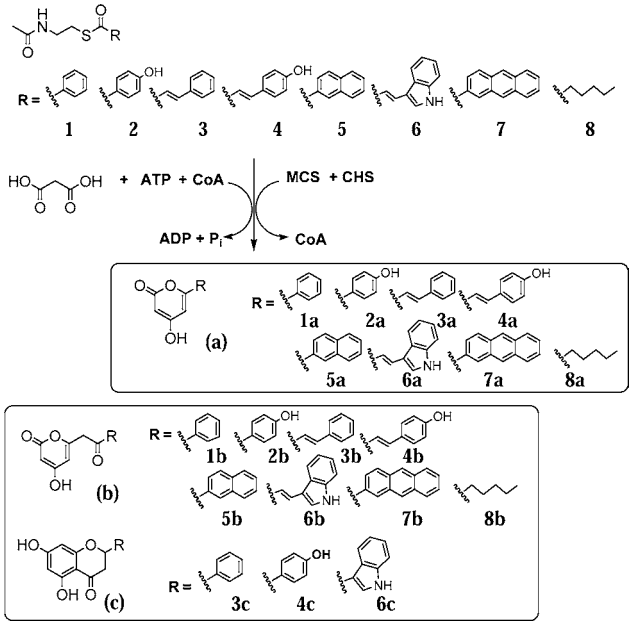
enable their efficient scale-up.[4a] Herein, we report an efficient in vitro, enzymatic synthesis of polyketide analogues by reconstructing a type III PKS biosynthetic pathway complete with a CoA regeneration system that mimics in vivo precursor-directed biosynthesis.

To demonstrate the in vitro function of a precursor-directed biosynthetic pathway, we used CHS[6a] with unnatural SNAc starter substrates (Scheme 1). A critical need for large-scale

Scheme 1. In Vitro Precursor-Directed Biosynthesis of Polyketide Analogues



synthesis of novel polyketides is high enzyme activity and stability. Unfortunately, ~40% of CHS activity was lost within 3 h, and after 2 days <10% activity remained for the reaction of benzoyl-SNAc with malonyl-CoA (Figure 1a and Figure 1S, Supporting Information).

To improve the in vitro stability of CHS, we evaluated several enzyme immobilization methods, including physical adsorption onto mesoporous silica (MPS) and hydrophobic methyltrimethoxysilane (MTMOS)-coated MPS,[7] covalent attachment onto aldehyde-functionalized MPS,[8] and cross-linked enzyme aggregate (CLEA).[9] Unfortunately, physical adsorption onto either hydrophilic or hydrophobic silica surfaces failed to improve CHS stability Figure 1S, (Supporting Information). Moreover, upon covalent attachment using the reactive amine residues of CHS, the activity and stability were substantially lower than that of free CHS. Similarly, CLEA formation using glutaraldehyde cross-linking of CHS amine residues resulted in poor stability, and this was not improved with a polymeric (dextran aldehyde) cross-linked residue.

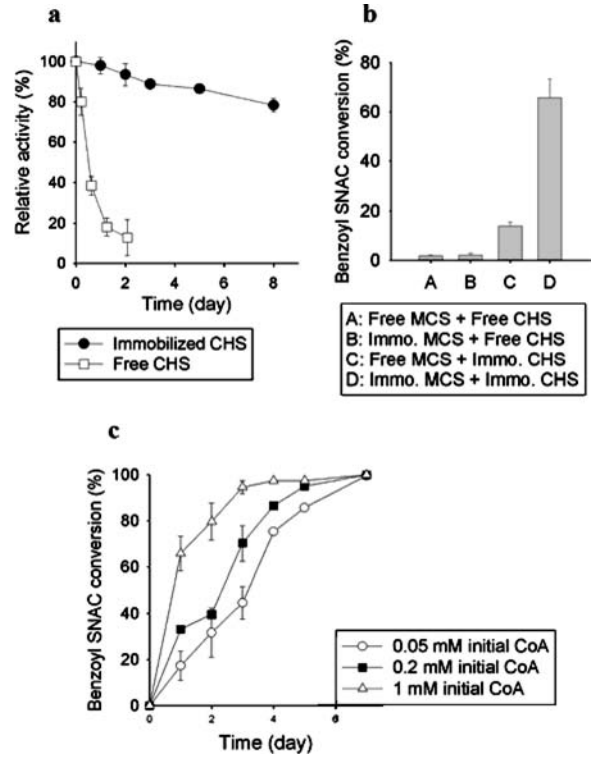


Figure 1. (a) Stability comparison between immobilized and free CHS (static condition, 22 °C). (b) Comparison of benzoyl SNAC conversion (%) among four combinations of MCS and CHS (1 day reaction). (c) Time course of benzoyl SNAC conversion (%) using both immobilized CHS and MCS.

These results can be rationalized by examining the CHS crystal structure. As depicted in Figure 2Sa (Supporting Information), CHS possesses numerous lysine and arginine residues (yellow and orange balls), which are located near the CoA binding site (green, substrate binding site). The covalent attachment on the silica beads or cross-linking with these reactive amine groups (lysine and arginine residues) may block the substrate entrance of active site, resulting in interference with starter CoA binding leading to decreased catalytic activity. As opposed to rather random adsorption or covalent attachment to a surface, the site-specific[10] coordinated covalent attachment using the N-terminal histidine (His)-tag of enzyme onto Ni^{2+}-nitrotriacetic acid (Ni-NTA) agarose beads enables attachment distant from the CHS active site (cyan, Figure 2Sa, Supporting Information). Immobilization onto Ni-NTA agarose beads resulted in ca. 50% retention of native solution activity, yet a highly stable

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enzyme. This stabilization was confirmed using spectroscopic analysis. Specifically, near-UV circular dichroism (CD) spectroscopy was used to evaluate the bulk tertiary structure of CHS free in solution at the initial time and after 15 h incubation in aqueous buffer (Figure 2Sb, Supporting Information) as well as immobilized onto Ni−NTA agarose. Secondary structural changes using far-UV CD could not be performed using the immobilized enzyme form due to interference by the support. A clear loss in tertiary structure was observed for the free enzyme after 15 h incubation; however, near-complete retention of the tertiary structure was evident with the immobilized CHS.

CHS immobilized onto Ni−NTA agarose was extremely stable under the operating conditions, with nearly 80% retention of initial enzyme activity following 1 week in aqueous buffer at room temperature (Figure 1a). This stability enabled recycling and reuse (via gravity centrifugation) of the immobilized CHS.

Following 20 cycles (2 h per cycle), ca. 50% of the initial CHS activity remained (Figure 2Sc, Supporting Information). Finally, the reactivity of Ni−NTA agarose immobilized CHS was tested using several acyl-CoA starter substrates and malonyl-CoA (Table 1). For benzoyl-CoA, the overall conversion was similar for the immobilized and free CHS, although a larger proportion of the product of the immobilized CHS was the tetrapyrone, indicative of a more extensive reaction. However, for the intrinsically less reactive butyryl-CoA and acetyl-CoA starter substrates, substantially higher conversion was achieved with the immobilized enzyme. Therefore, the combination of enzyme activity and stability, coupled with the ease of immobilization using Ni−NTA agarose beads (including combined protein purification and immobilization), led us to use this immobilization strategy for this study.

Table 1. Reactivity Comparison between Free and Immobilized CHS on Ni−NTA Agarose Using Several Acyl-CoA Substrates as Starter and Malonyl-CoA as Extender[a]

starter substrates	immobilized CHS		free CHS	
	tripyrone	tetrapyrone	tripyrone	tetrapyrone
benzoyl-CoA	**11**	60	**22**	39
butyryl-CoA	**47**	5	**33**	N.D.
acetyl-CoA	**37**	N.D.	**1**	N.D.

[a] 100 μL reaction volume, 40 μg enzyme used in Tris−HCl buffer. Three day reaction. Concentrations of starter substrate and malonyl CoA are 1 and 5 mM, respectively. Conversion (%) based on starter substrate as the limiting agent. N.D. indicates compounds were not detected.

CHS is capable of using SNAc thioesters as starter substrates[6b] as an alternative to natural CoA esters. Although the reactivity of CHS on SNAc substrates is lower than on their CoA counterparts (e.g., CHS was 2-fold less active on benzoyl-SNAc as compared to benzoyl-CoA), the ease of synthesis and the far lower cost of the SNAc esters sufficiently outweighs the reduced enzyme activity. CHS, however, is incapable of using malonyl-SNAc or methylmalonyl-SNAc as extender substrates and instead requires the respective CoA esters. For this reason, we coupled malonyl-CoA synthetase (MCS)[11] with CHS, which enabled malonyl-CoA to be used as the natural extender substrate and also allowed recycling of malonyl-CoA upon addition of malonate and ATP with a small amount of CoA. To that end, glutathione S-transferase (GST)-tagged MCS was used in either free solution or immobilized onto glutathione-sepharose (Figure 3S, Supporting Information).[11] A sequential two-step enzymatic reaction was performed with benzoyl-SNAc (1 mM) and excess malonate (10 mM) in the presence of CoA (1 mM) and ATP (5 mM) to produce benzoyl pyrones (tripyrone **1a** and tetrapyrone **1b**; detailed reaction conditions are described in the Supporting Information). The conversion of benzoyl-SNAc was ∼2% when free CHS and MCS were used, and the conversion was not increased upon immobilization of MCS. Modest improvement in starter unit conversion was achieved in the presence of immobilized CHS (Figure 1b). The largest conversion (>65%) was obtained in the two-step reaction using both immobilized CHS and MCS.

The efficient use of the CoA regeneration system enabled us to perform larger scale biotransformations, up to 50-mL reaction volumes, containing different concentrations of initial CoA (0.05 to 1.0 mM) with benzoyl SNAC (1 mM), 4 mM ATP, and 0.5 mM CoASH. The time course of benzoyl pyrone synthesis is shown in Figure 1c. As expected, with higher concentrations of initial CoA, the reaction proceeded faster. However, after 7 days, complete conversion of benzoyl SNAC was achieved even with 0.05 mM of initial CoA concentration. In the case of 0.05 mM initial CoA concentration, ca. 50 CoA regeneration cycles were achieved after 7 days for conversion of benzoyl-SNAc into tripyrone (two molecules of malonyl CoA used) and tetrapyrone (three molecules of malonyl CoA used) (Table 1S, Supporting Information). This immobilized in vitro system, therefore, did not require high concentrations of expensive CoA, increasing the potential for reaction scale-up.

The coupled CHS/MCS reaction system was then used to synthesize a wide range of type III polyketides. Eight starter SNAc compounds (**1**−**8**) were chemically synthesized and used in 50 mL reactions. After 3-day reactions, the expected tri- and tetrapyrones and flavonoids were detected and analyzed by LC−MS. Selected compounds were then purified by flash column chromatography and analyzed by ^{1}H NMR (Supporting Information). Overall reaction yields ranged from 27 to 81% (Table 2) based on conversion of the SNAc starter substrate. Several of the polyketide products have not been reported previously, including naphthoyl-primed pyrones (**5a** and **5b**), indole-primed pyrones and flavonoids (**6a**, **6b**, and **6c**), and anthracene-primed pyrones (**7a** and **7b**) synthesized from unnatural starter molecules (naphthoic, indolic, and anthracenoic-SNAc esters (**5**, **6**, and **7**, respectively). The starter SNAc esters having *trans*-cinnamoyl moieties (**3**, **4**, and **6**) were converted into flavonoids as well as pyrones. These results indicated that

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Table 2. Yields (% Based on Starter SNAc) of Polyketides Produced by in Vitro System[a]

	triketides		tetraketides		flavonoids	
starter	product	yield (%)	product	yield (%)	product	yield (%)
1	**1a**	23	**1b**	64		
2	**2a**	5	**2b**	47		
3	**3a**	36	**3b**	5	**3c**	10
4	**4a**	17	**4b**	1	**4c**	35
5	**5a**	10	**5b**	55		
6	**6a**	18	**6b**	3	**6c**	6
7	**7a**	40	**7b**	3		
8	**8a**	54	**8b**	24		

[a] Percent yields based on starter SNAc ester concentration; 10 mg of immobilized MCS and 5 mg of immobilized CHS were mixed in the reaction buffer (50 mL Tris−HCl, pH 7.5). 10 mM sodium malonate, 1 mM starter molecules, 10 mM $MgCl_2$, 4 mM ATP, 10 mM dithiothreitol, and 0.5 mM initial CoA were used for 3 day incubations. Coefficients of variation (CV) of the results were less than 7%.

CHS has extremely broad substrate specificity for aromatic starter SNAc esters. A total of 19 natural or unnatural polyketides were synthesized using combined immobilized CHS and MCS.

To test the bioactivity of isolated polyketide analogues, an antiangiogenesis assay was performed by incubating HER2 overexpressing breast cancer (BT-474) cells with the compounds and determining the expression level of vascular endothelial growth factor (VEGF). Because VEGF are crucial regulators of blood-vessel formation (angiogenesis) as well as vascular development (vasculogenesis), inhibition of VEGF gene expression and blocking of VEGF function are clinically used in cancer therapy.[12]

Previous reports indicate that known type III polyketides, including genistein and apigenin, inhibit VEGF expression and decrease tumor angiogenesis.[13] Therefore, we anticipated that unnatural polyketide analogues might also regulate VEGF secretion and synthesis. With this rationale in mind, we tested the polyketide analogues synthesized in vitro by the combined action of CHS and MCS catalysis for their ability to suppress the secretion of VEGF from BT-474 cells; activation or overexpression of HER-2 is associated with up-regulation of VEGF in human breast cancer cells.[13]

As a result, 10 μM each of the newly synthesized polyketides were modestly inhibitory against VEGF release from BT-474 cells. Among them, benzoyl tri- or tetrapyrones (**1a**, **1b**), cinnamoyl flavonoid (**3c**), and naphthoyl tri- or tetrapyrones (**5a**, **5b**) showed ~50% inhibition against VEGF secretion. To compare the effectiveness of these compounds with a known VEGF inhibitor, apigenin,[14] dose−response curves for **1b**, **3c**, and **5b** were obtained to calculate their IC_{50} (half maximal inhibitory concentration) (Figure 4S, Supporting Information) As summarized in Table 2S (Supporting Information), benzoyl tetrapyrone (**1b**) was the most potent of the compounds tested against VEGF secretion.

In summary, in vitro reconstruction of a PKS biosynthetic pathway was developed, resulting in high yields of polyketide analogues. Main bottlenecks of in vitro PKS catalysis, e.g., enzyme stability and cost of malonyl-CoA, were overcome by use of simple immobilization and CoA regeneration catalyzed by MCS, respectively. Regeneration of ATP can be considered as an additional approach to reduce consumption of expensive cofactors.[15]

Eight unnatural SNAc starter substrates were successfully used in this system, yielding the expected polyketides and then screened for antiangiogenesis activity. Because the in vitro reconstruction of PKS biosynthetic pathway comprised both high enzyme stability and continuous production of malonyl-CoA by CoA regeneration, this system may efficiently mimic in vivo PKS biosynthesis. This, in turn, results in convenient screening of novel unnatural polyketide analogues and may lead to new classes of compounds with antiangiogenesis activity.

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Supporting Information Available: Detailed experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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