Combinatorial Biosynthesis

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Multiplexing of Combinatorial Chemistry in Antimycin Biosynthesis: Expansion of Molecular Diversity and Utility**

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The control of biological phenomena by natural products, which have co-evolved with their macromolecular receptors, results in their critical role in medicinal chemistry and chemical biology.[1] The generation of libraries of natural-product-like compounds is a major area of current interest in diversity-oriented synthesis, however, synthetic accessibility and efficiency of these compounds may face challenges associated with their structural complexity.[1] Natural products that include polyketides, nonribosomal peptides, and their hybrids often share a similar biosynthetic logic,[2] featuring the preparation of building blocks, skeleton assembly catalyzed by polyketide synthase (PKS) or/and nonribosomal peptide synthetase (NRPS), and post-tailoring for molecule maturation. The formation of antimycins (ANTs), which display a remarkable variety of biological activities,[3] is not an exception (Figure 1).

ANTs have a nine-membered polyketide–peptide hybrid dilactone proven to be a privileged scaffold capable of binding multiple protein targets.[3] We and others have uncovered a uniform paradigm for their biosynthesis,[4] showing that a hybrid NRPS/PKS system programs the formation of the dilactone core in a linear way (Figure 1). The 44 natural ANTs differ in their alkylation at C7 and acylation at C8,[5] however, the effects of these functionalities on biological activities and associated modes of action remain unclear. Very recently, Zhang et al.[4d,e] established the biochemical basis for these two modifications (Figure 1): while alkylation at C7 occurs through PKS-catalyzed elongation that utilizes variable alkylmalonyl units as the extender unit, provided by a crotonyl-CoA reductase/carboxylase (CCR)-like protein AntE,[6] acylation at C8 is the result of post-NRPS/PKS modification of the dilactone core, mediated by a promiscuous acyltransferase AntB. The ANT NRPS/PKS system was also found to tolerate the change of the starter unit and to initiate assembly of a dilactone conjugated with a modified 3-formamidosalicyclic acid (FSA) moiety.[6b–d] Interesting questions thus arise regarding 1) how promiscuous the entire ANT biosynthetic machinery is and 2) whether multiple alterations can be achieved to create unnatural ANTs. By addressing these queries in the ANT-producing strain *Streptomyces sp.* NRRL 2288, we herein report a multiplex combinatorial biosynthesis approach, to expand the molecular diversity and utility upon applying an idea of combinatorial chemistry to different biosynthetic stages of ANTs.

We first deleted *antB*, aiming at the construction of an engineered biosynthetic apparatus for diversity-oriented production of dilactone scaffolds in vivo. The resulting mutant strain, AL2110, failed to produce mature ANTs but accumulated a series of C8-deacylated ANTs that vary in the alkylation at C7, including **DA-1**, **DA-2**, **DA-3**, **DA-4**, and **DA-5** (Figures 2 and S2). Three carboxylates, chloropentanoate (**1**), cyclohexanepropanoate (**2**), and 10-undecynoate (**3**), were then fed to AL2110, to examine whether naturally unavailable units can be incorporated into ANTs to increase the diversity at C7. These acids are known to be activated in cells by endogenous acyl-CoA ligase(s), giving chloropentanoyl-CoA (**4**), cyclohexanepropanoyl-CoA (**5**), and 10-undecynoyl-CoA (**6**), respectively, which go further through a partial β-oxidation pathway to generate α,β-unsaturated products, chloropentenoyl-CoA (**7**), cyclohexanepropenoyl-CoA (**8**), and (2*E*)-2-undecen-10-ynoyl-CoA (**9**), respectively (Figure 3a). As a result, all feedings produced new C8-deacylated ANTs, **DA-6**–**DA-9**, each of which became the major product in the chemical profile of AL2110 (Figure S2). The variation at C7 is well in line with the carboxylate precursors, as 3-chloropropyl of **DA-6** from **1**, cyclohexylmethyl of **DA-7** from **2**, and both 4-pentynyl of **DA-8** and 6-heptynyl of **DA-9** from **3** (Figure 2). Synthesis of 4-pentynyl in **DA-8** or 6-heptynyl in **DA-9** indicates that the CoA derivative **9** (eleven carbon atoms) was subjected to one or two complete round(s) of β-oxidation to produce the shortened derivatives (2*E*)-2-nonen-8-ynoyl CoA (**14**, −2C) or (2*E*)-2-hepten-6-ynoyl CoA (**15**, −4C; Figure 3a). For polyketide extension, in situ reductive carboxylation at C2 of **7**, **8**, **14**, and **15** requires the activity of the CCR-like protein AntE to generate the corresponding extender units **10**, **11**, **12**, and **13**, respectively, consistent with its surprising promiscuity

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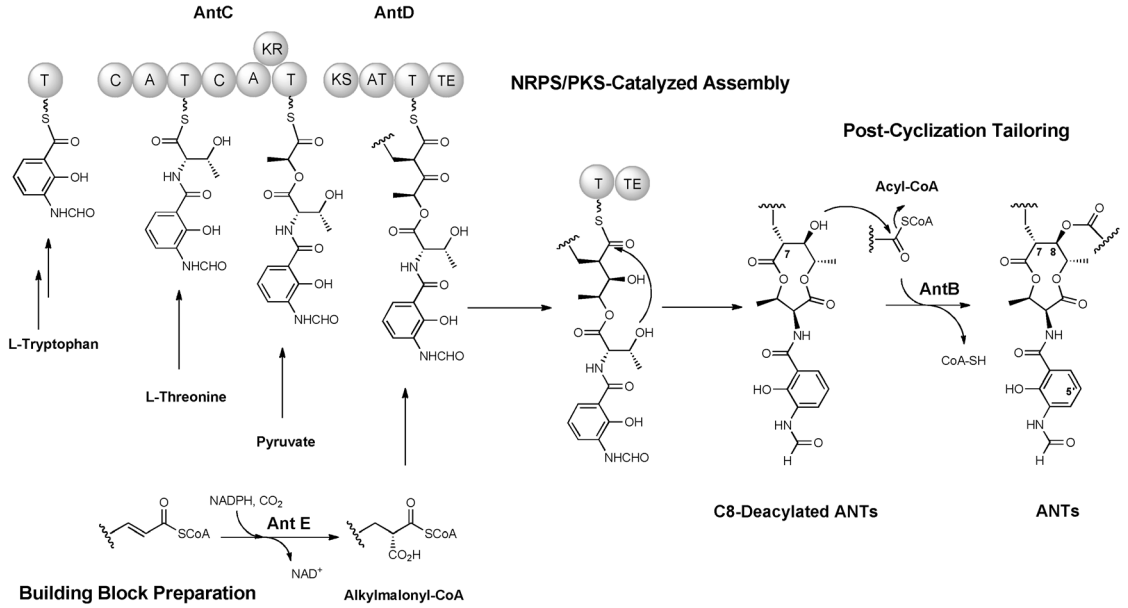

Figure 1. Proposed biosynthetic pathway toward ANT, showing the preparation of building blocks, PKS- or/and NRPS-catalyzed skeleton assembly, and post-tailoring. KS = ketosynthase; AT = acyl transacylase; TE = thioesterase; C = condensation; A = adenylation; T = thiolation domain; KR = ketoreductase. For details of the ANT members, see Figure S11.

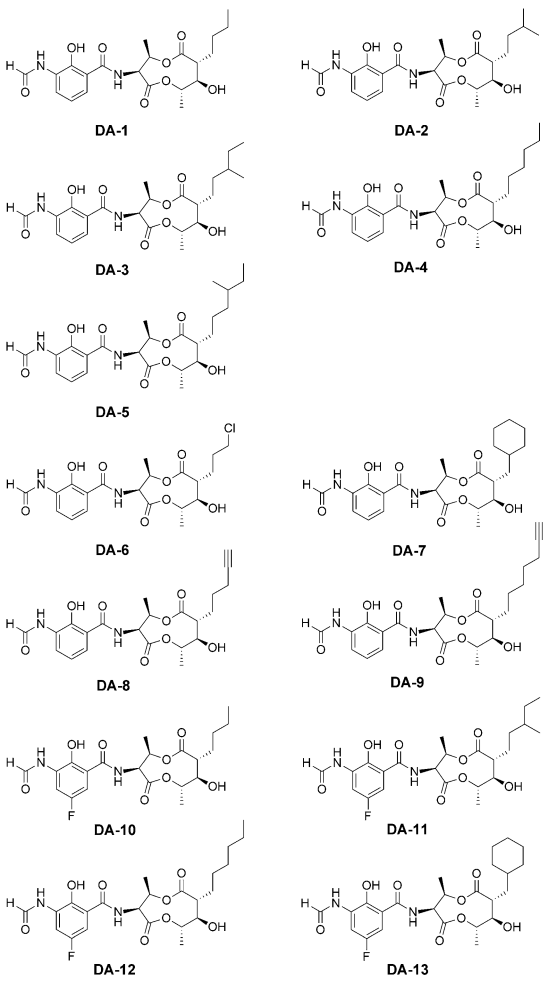

Figure 2. Structurally characterized C8-deacylated variants of ANTs.

characterized in vivo and in vitro (Supporting Information): inactivation of *antE* in AL2110 eliminated the diversity at C7 (Figure S2); in the presence of AntE, a number of α,β-unsaturated acyl-CoAs were efficiently converted into the alkylmalonylated CoAs (Figure S7). Remarkably, AntD, the most compatible PKS found to date, tolerates an extremely high variety of extender units, which can be linear, branching, cyclic, halogenated, or alkyne-containing, for polyketide diversity during the extension process. To our knowledge, this astonishing ability of PKS has not been reported to date.

In ANT biosynthesis, the assembly of the NRPS/PKS-programmed dilactone utilizes an L-tryptophan-derived FSA moiety as starter unit (Figure 1).[4c] By complementing 6-fluoro-L-tryptophan in the wild-type strain, we previously produced a set of FSA-fluorinated ANTs.[4c] Here, the same precursor was fed to the mutant strain AL2110, allowing the production of the 5′-fluoro-FSA-conjugated dilactones **DA-10**–**DA-12** (Figures 2 and S2). We thus considered whether FSA fluorination and diversification at C7 can simultaneously occur in vivo. As anticipated, combination of 6-fluoro-L-tryptophan and **2** in AL2110 resulted in the production of two major compounds, **DA-7** and a new product **DA-13. DA-13** was further characterized as a derivative of **DA-7**, featuring a 5′-fluoro-FSA moiety and a cyclohexylmethyl side chain at C7 (Figures 2 and S2). Therefore, the hybrid NRPS/PKS system appears amenable for variations in both the starter and extender units, leading to effective production of highly diverse dilactone scaffolds.

Next, we took advantage of the promiscuity of the acyltransferase AntB to attach acyl groups to C8 for diversity-oriented maturation of ANTs in vitro. We prepared a library of twenty-five CoA derivatives as the acyl donors (**16**–**38**, **43**, and **46**), among which twelve are commercially available and thirteen were synthesized in this study (Figure 3b). These derivatives contain acyl groups that vary in

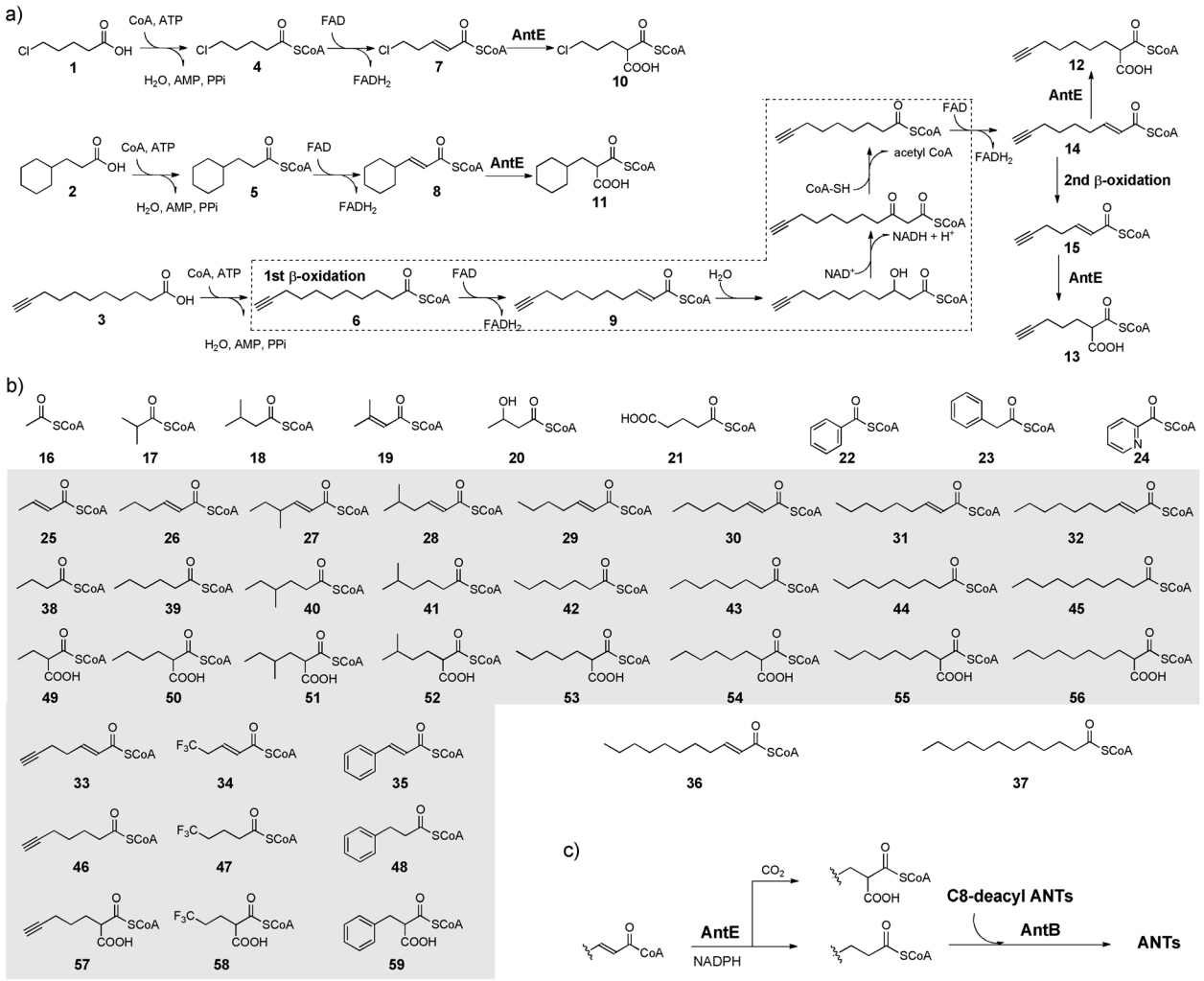

Figure 3. Building blocks for alkylation at C7 and acylation at C8 of ANTs. a) In vivo conversion of the carboxylates **1**, **2**, and **3**. For **3** in AL2110, the process includes two rounds of β-oxidation (first round in dashed rectangle). b) Library of acyl-CoAs, including the derivatives that are commercially available and chemically or enzymatically synthesized in this study. The α,β-unsaturated CoAs, along with their corresponding saturated and 2-carboxylated derivatives that were obtained by AntE-catalyzed shunt and complete reactions, are highlighted in grey. c) "Relay reaction" for acylation at C8 by cooperation of AntE-catalyzed shunt reduction with AntB-catalyzed acylation.

both length and structural features (e.g., they can be linear, branching, aromatic, saturated, unsaturated, halogenated, or heterocyclic). The C8-deacylated ANTs, **DA-1**–**DA-13**, then served as acyl acceptors. In the presence of AntB, permutation of **DA-1**–**DA-13** with acyl donors from the CoA-derivative library produced 285 mature ANT variants, 87.7% of the theoretical yield ($13 \times 25 = 325$; Figures S6 and S11). Each of the C8-deacylated ANTs completely accepted the acyl groups from the CoA derivatives **16**–**30, 33**–**35**, **43**, and **46**, ascertaining the effectiveness of AntB in catalyzing various acylations. The AntB-catalyzed reaction failed only when using the donor lauroyl-CoA (**37**) or 2-(2*E*)-undecenoyl-CoA (**36**), indicating that linear acyl transfer is limited by length (with a range from two to ten carbon atoms). Transfer of (2*E*)-2-nonenoyl (nine carbon atoms) from **31** or (2*E*)-2-decenoyl (ten carbon atoms) from **32** was optional and dependent on the acceptor, suggesting that the functionalities at C7 and perhaps C5' may in some cases modulate acylation at C8 by longer linear acyl groups.

To add new members to the CoA-derivative library, we employed the "shunt reaction" of the CCR-like protein AntE, which, without bicarbonate as the cosubstrate, only catalyzes the NADPH-dependent reduction of α,β-unsaturated acyl-CoAs (Figure 3c and Supporting Information).[6] Thus, eleven enoylated CoAs (**25**–**35**) were individually incubated with AntE to produce saturated acyl-CoAs (**38**–**48**) in vitro, which were then applied to AntB-catalyzed acyl transfer reactions to functionalize the C8-deacylated ANTs (**DA-1**–**DA-13**). This so-called "relay reaction" further expanded the diversity of ANTs at C8 and resulted in 121 variants, 84.6% of the theoretical yield ($13 \times 11 = 143$; Figures S6, S8, and S11).

Notably, most of the observed ANT variants, if not all, were produced effectively. As pilot experiments, we scaled up the AntB-mediated reactions to transfer octanoyl from **43** to **DA-1** and 6-heptynoyl from **46** to **DA-3** and **DA-13**. The resulting products were purified, analyzed by NMR spectroscopy, and ultimately confirmed to be identical to the anticipated variants **A1**–**43**, **A3**–**46** and **A13**–**46** (Figures 4a

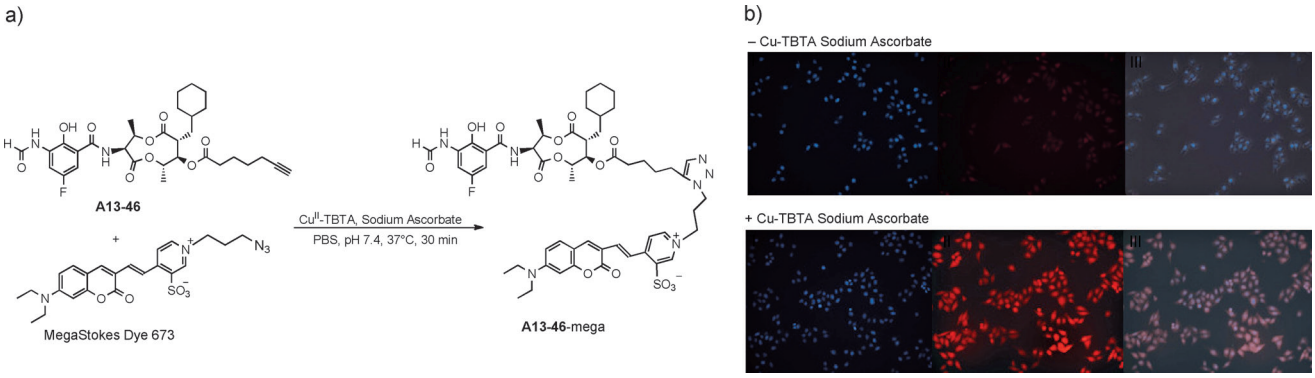

Figure 4. Reactivity of the unnatural ANT variant **A13–46**. a) Copper-catalyzed conjugation to give **A13–46**-mega. b) In situ labeling of **A13–46** within cells. HeLa cells were treated with **A13–46** and Azide MegaStokes dye 673 in the absence (top) or presence (below) of the catalytic reagents. Left: UV channel; center: red channel; right: UV and red channels merged. PBS = phosphate-buffered saline; TBTA = tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine.

and S11), therefore verifying the efficiency and scalability of the approach.

We selected 45 ANT derivatives for in vitro assays of cytotoxic activity against the mouse leukemia P388 cell line and antifungal activity against *Candida albicans*, using authentic natural **A3b** as the control (Table S14). In addition to 12 purified C8-deacylated ANTs, the 34 mature ANTs were generated in vitro by AntB-catalyzed acylations and quantified by HPLC. All of the mature ANTs were biologically active, whereas the C8-deacylated ANTs were nearly inactive. This finding clearly indicated that acylation at C8 is of importance to ANT actions. In comparison with **A3b**, 22 ($\approx$64.7%) of the mature ANTs exhibited improved cytotoxicity (up to 66.7-fold), and 7 ($\approx$20.6%) displayed enhanced antifungal activity (up to 2.0-fold). Notably, the tested ANT variants showed a wide range of ratios between cytotoxicity and antifungal activity (from 3.8×10^{-3} to 3.7×10^{5}). The fact that permutation of the three modifications at C5′, C7, and C8 can significantly modulate the relative bioactivity suggests the different modes of action of ANTs to reach different bioactivity.

We chose the alkyne-bearing variant **A13–46** to react with Azide MegaStokes dye 673, an azide-containing fluorescent molecule, through click chemistry (Figure 4a).[7] The reaction was first tested in vitro, leading to quantitative production of **A13–46**-mega, a conjugant with a triazole linkage. This copper-catalyzed conjugation was then carried out in HeLa cells. The cells that were efficiently labeled in situ were resistant to washing (Figure 4b), suggesting the binding of **A13–46** to the macromolecular targets. These findings confirmed the reactivity of the introduced alkyne group, which is naturally unavailable and could be a handle amenable to further chemical derivatization for investigating the in vivo actions of ANT derivatives.

In summary, on the basis of characterization of the compatibility in ANT biosynthesis, we developed a multiplex combinatorial biosynthesis approach for diversity-oriented biosynthesis of a library of ANT-like compounds. This approach combines the technologies of multiple mutasynthesis, pathway engineering, and chemoenzymatic transformation,[8] and depends on the core strategy using combinatorial chemistry at different biosynthetic stages for starter unit choice, extender unit preparation, and post-NRPS/PKS modification (Figure 5). Considering the convenience in qualification and quantification, we dissected the biosynthetic machinery of ANTs into two parts. The first part, constructed

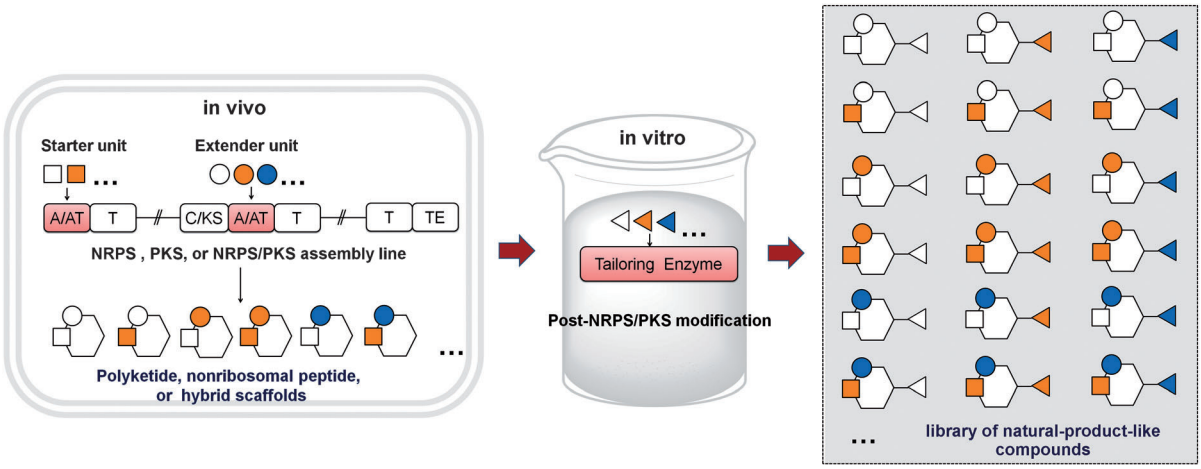

Figure 5. Strategy of multiplex combinatorial biosynthesis.

by inactivating the tailoring gene, focuses on the synthesis of the FSA-conjugated dilactone in vivo. The second part involves AntB, the highly promiscuous acyltransferase, for maturing dilactones in vitro. We generated a total of 380 distinct ANT-like products, of which 356 are new, and introduced a number of functional groups that are chemically active, pharmaceutically important, or naturally unavailable. Biosynthesis is promising to accomplish the formation of complex molecules; however, thus far there have been relatively few successes in achieving high diversification by this way, because of the limitation in the understanding, development, and utilization of compatible biosynthetic machineries. Given the increasing depth of knowledge in the field of biosynthesis and the significant progress in protein/pathway engineering,[9] the approach presented here provides a useful model for generating libraries of polyketides, nonribosomal peptides, and the hybrids that share a similar biosynthetic logic.

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