Combinatorial Enzymatic Synthesis of Unnatural Long-Chain β-Branch Pyrones by a Highly Promiscuous Enzyme

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Supporting Information

ABSTRACT: In this study, we described in detail a combinatorial enzymatic synthesis approach to produce a series of unnatural long-chain β-branch pyrones. We attempted to investigate the catalytic potential of a highly promiscuous enzyme type III PKS to catalyze the non-decarboxylative condensation reaction by two molecules of fatty acyl diketide-N-acetylcysteines (diketide-NACs) units. Two non-natural long-chain (C16, C18) fatty acyl diketide-NACs were prepared successfully for testing the ability of non-decarboxylative condensation. In vitro, 12 novel naturally unavailable long-chain β-branch pyrones were generated by one-pot formation and characterized by ultraviolet−visible spectroscopy and high-resolution liquid chromatography−mass spectrometry. Interestingly, enzymatic kinetics result displays that this enzyme exhibits the remarkable compatibility to various non-natural long-chain substrates. These results would be useful to deeply understand the catalytic mechanism of this enzyme and further extend the application of enzymatic synthesis of non-natural products.

1. INTRODUCTION

Combinatorial biosynthesis helps to enrich the novelty and diversity of the natural product architectures, offer an environmentally friendly way to produce natural product analogs, and increase the titer of the compound, eventually resulting in less expensive drugs.1−3 Enzymes are used in biocatalytic processes for the efficient and sustainable production of pharmaceuticals, fragrances, fine chemicals, and other products.4 Combinatorial enzymatic synthesis is a green and efficient synthesis strategy compared with traditional chemical synthesis. Combined with the utilization of non-natural substrates and the nonspecificity of enzymes, enzymatic synthesis achieves further evolution and ascension in combinatorial chemistry.5,6

Polyketide synthases (PKSs) are an important class of biologically active and structurally diverse secondary metabolites in nature. Polyketides are synthesized through decarboxylative condensation of two-carbon acetate units by PKSs.7−11 According to their sequences, primary structures, and catalytic mechanisms, PKSs fall into three groups: type I PKS, type II PKS, and type III PKS.12−17 Type III PKS catalyzes sequential decarboxylative condensations of malonyl-CoA with an aromatic or aliphatic CoA-linked starter molecule without using the acyl carrier protein to produce a variety of biologically active secondary metabolites.18,19 Despite their structural simplicity, type III PKS produces a wide array of compounds such as chalcones, pyrones, acridones, phloroglucinols, stilbenes, and resorcinolic lipids.20,21

Recently, CsyB from Aspergillus oryzae is a unique type III PKS that is involved in the biosynthesis of short-chain product csypyrone B1. This is the first type III PKS that performs not only the polyketide chain elongation by decarboxylative condensation but also the condensation of two β-ketoacyl units by non-decarboxylative condensation (Figure 1). Interestingly, a fatty acyl CoA binding tunnel of a length of about 16 Å is located in its active center, so it is a very likely hypothesis that more diversified start units, in particular, a number of precursors of unnatural long-chain fatty acyl...
diketide-NAC, would be accepted to form the novel non-natural products by in vitro enzyme assays. Due to this, the I375F mutant of CsyB can accept the C4−C9 fatty acyl diketide-NAC substrates to synthesize the unnatural novel product, while the wild type only accepts the C4−C7 fatty acyl diketide-NAC substrates. Therefore, for testing this hypothesis, the catalytic capability and substrate diversity of the I375F mutant of CsyB were evaluated for synthesis of the non-natural long-chain β-branch pyrones in this paper.

2. RESULTS AND DISCUSSION

2.1. Expression and Purification of CsyB. The recombinant CsyB was expressed in Escherichia coli as a fusion soluble protein with a His6-tag at the C terminus and purified to homogeneity by a combination of Ni-affinity chromatography. The purified His6-tag-fused CsyB protein migrated as a single band with a molecular mass of 45 kDa on SDS-PAGE, which agrees well with the calculated value of 45.2 kDa (Figure S1).

2.2. Synthesis of Different Length Diketide SNACs. For examination of the substrate selectivity of CsyB for fatty acyl diketide-NAC substrates with various lengths, short-chain (C4 to C10) and long-chain (C16 to C18) diketide-NACs were synthesized according to the published method (Scheme S1). The synthetic C16 and C18 diketide-NAC compounds were isolated, purified, and identified on a small scale. Based on the high-resolution liquid chromatography−mass spectrometry (HRLC−MS) and nuclear magnetic resonance (NMR) data, C16 and C18 diketide-NACs had a molecular formula of C22H41NO3Sa/m/z 400.2819 [M + H]+ (calcd for C22H41NO3S, 400.2807) and 428.3102 [M + H]+ (calcd for C24H45NO3S, 428.3120), respectively, and data of 1H and 13C NMR also were in accordance with their structures (Figure 2) (Figures S2−S7).

2.3. Enzymatic Synthesis of Unnatural Long-Chain β-Branch Pyrones Using C16 Diketide-NAC. The CsyB-specific fatty acyl CoA binding tunnel is highly similar to long-chain pyrone enzymes such as ORAS in Neurospora crassa and PKS18 in Mycobacterium tuberculosis. It is suggested that this enzyme may also be able to accept a series of long-chain substrates such as ORAS and PKS18, although its native substrates are short-chain. To test this hypothesis, we performed the enzyme reactions using various lengths of diketide-NAC thioesters as substrates. The in vitro enzyme assay was carried out as previously described.

To check the substrate diversity and tolerance, C16 diketide-NAC and several short-chain diketide-NACs from C4 to C9 diketide-NACs were used to synthesize the non-natural long-chain β-branch pyrones by the purified CsyB mutant enzyme I375F by high-performance liquid chromatography (HPLC) in vitro. The CsyB I375F catalyzed the coupling reaction between one molecule of a short-chain (C4−C9) diketide-NAC and one molecule of long-chain C16 diketide-NAC to yield the six new products 1−6 with various lengths of acyl chains (Figure 3).

The HPLC result showed that six new peaks were detected at 310 nm, which stood for specific absorption of β-branch pyrones. Furthermore, for confirming the molecular weights
(MWs) of new compounds, these new peaks were measured by HRLC−MS. The result showed that compound 1 had a molecular formula of C24H40O4 at m/z 393.2941 [M + H]+ (calcd for C24H40O4 393.2927). Similarly, the molecular formulas and m/z of other five compounds 2−6 are summarized in Table 1.

Table 1. Molecular Weights of Csypyrone Derivatives

| no. | formula   | calc MW  | MW       |
|-----|-----------|----------|----------|
| 1   | C24H40O4  | 392.2927 | 392.2941 |
| 2   | C25H42O4  | 406.3083 | 406.3067 |
| 3   | C26H44O5  | 420.3240 | 420.3256 |
| 4   | C27H46O5  | 434.3396 | 434.3379 |
| 5   | C28H48O5  | 448.3553 | 448.3541 |
| 6   | C29H50O4  | 462.3709 | 462.3723 |
| 7   | C30H52O5  | 476.3866 | 476.3882 |
| 8   | C31H54O5  | 490.4022 | 490.4001 |

2.4. Enzymatic Synthesis of Unnatural β-Branch Pyrones Using C18 Diketide-NAC. For further testing of the substrate diversity and tolerance, C18 diketide-NAC and several short-chain diketide-NAC from C4 to C9 diketide-NACs were used to synthesize the non-natural long-chain β-branch pyrones by the purified CsyB mutant enzyme I375F in vitro. The CsyB I375F using one molecule of a short-chain (C4−C9) diketide-NAC catalyzes coupling reactions to yield the six products 7−12 with various lengths of acyl chains (Figure 4).

Figure 4. HPLC results of CsyB I375F with long-chain C18 diketide-NAC.

Furthermore, for confirming the MWs of new compounds, these new peaks were measured by HRLC−MS. The result showed that compound 7 had a molecular formula of C26H44O5 at m/z 420.3240 [M + H]+ (calcd for C26H44O5 420.3226). Similarly, molecular formulas and m/z of other five compounds 8−12 are summarized in Table 1.

2.5. Kinetics Parameter Comparison between Short-Chain and Long-Chain Diketide-NAC. The steady-state kinetics values of the CsyB I375F mutants for C4−C18 diketide-NAC substrates are summarized in Table 2 according to previous work. The kinetics parameters for the reactions of C18−C18 diketide-NAC substrates were also determined with the substrate C3 diketide-NAC. Steady-state kinetics parameters were determined from the Lineweaver−Burk plot method (Figures S8−S12).

The steady-state kinetics values of CsyB I375F for long-chain diketide-NACs were a little better than those of short-chain diketide-NACs. In addition, C18 diketide-NAC has a bit stronger affinity than C16 diketide-NAC to enzyme in the long-chain diketide-NACs. Furthermore, the kinetics parameters for different substrates reported in Table 2 show that kcat instead of Km is the major parameter to be affected, while one would expect that varying the carbon chain length of the substrate should mainly affect substrate binding instead of chemical catalysis. The reason is quite likely related to the catalytic mechanism of this KS class. Because CsyB and other type III PKSs such as ORAS and PKS18 accept the various lengths of fatty acyl-CoAs or SNACs, a kind of CoA analog) by binding the CoA or SNAC group as well as the fatty acyl group as shown in Figure 5. Therefore, these enzymes exhibit the extensive substrate specificity for fatty acyl diketide-NACs with various lengths, short chain (C4 to C16) and long chain (C16 to C18).

3. CONCLUSIONS

CsyB, a novel fungal type III polyketide synthase, can sequentially accept one molecular of short-chain fatty acyl CoA as a start unit, one molecular of malonyl-CoA, and one molecular of acetoacetyl-CoA as an extend unit to produce the short-chain csypyrone B1−B3 in natural conditions. Because a fatty acyl CoA binding tunnel of 16 Å length is located in its active center, more diversified start units are proposed to be accepted by CsyB. In order to attempt to synthesize the long-chain β-branch pyrone, an in vitro enzyme assay was done using a number of precursors of unnatural fatty acyl SNAC
using CsyB. The results of HPLC revealed that a series of long-chain cyppyrone derivatives were detected. These 12 new long-chain β-branch pyrone compounds were preliminarily analyzed by UV–visible spectroscopy and HRLC–MS. By comparing the enzymatic kinetics parameters of the long- and short-chain substrates, it was found that the enzyme could not only utilize the long-chain substrates but also show better affinity to the long-chain substrates than to the short-chain substrates. These results demonstrate that CsyB exhibits a remarkable ability to synthesize non-natural products and broad specificity for long-chain fatty acyl SNAC. The application of CsyB in combinatorial chemistry was realized, which laid a foundation for its industrial application.

4. EXPERIMENTAL SECTION

4.1. Materials. Coenzyme A (CoA), fatty acyl-CoA, acetoacetyl-CoA, malonyl-CoA, and SNAC were obtained from Sigma-Aldrich. Acyl diketide-NACs with various lengths were synthesized according to the published methods.22,23 Oligonucleotides were obtained from Sangon Biotech (Shanghai) Co., Ltd. The expression vector pET-CsyB was stored at a −80 °C freezer in our laboratory.

4.2. Synthesis of the Various Alkyl Diketide-NACs. The synthesis scheme of alkyl diketide-NAC is shown as follows. Various fatty acids were used to synthesize the alkyl diketide-NACs. There are two steps in this reaction. First, fatty acid reacted with Meldrum’s acid at room temperature for 4 h in CH2Cl2 along with dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP). After purifying the intermediate, the second step was performed at 80 °C for 5 h in toluene. Then the targeted compound was isolated from the final product using silica gel. The eight unnatural alkyl diketide-NAC substrates were synthesized using the above method.

4.3. Expression and Purification of CsyB. The pET-CsyB/E. coli BLR (DE3) strain was incubated to an OD600 of 0.6 at 37 °C in an LB medium containing 50 mg mL−1 kanamycin. The target protein expression was induced from the IPTG (1 mM) to a final concentration of 0.6 mM. The E. coli cells were harvested by centrifugation at 7000g for 15 min. The cells were suspended and disrupted by ultrasonication, and the lysate was centrifuged at 10000g for 30 min. After protein was purified by metal affinity chromatography, gel filtration chromatography was used as the last step on a HiLoad 16/60 Superdex 200 pg (GE Healthcare). All of the procedures were performed at 4 °C.

4.4. Enzyme Assay and Kinetics Parameters of CsyB and Mutants. The enzyme assay was performed, and the kinetics parameters were measured as previously reported.22 Different chain lengths of diketide-NACs and the purified enzyme were preincubated at 37 °C for 5 min, and then the reactions were initiated by adding the purified enzyme and then further incubated at 37 °C for 20 min. The decreased amount of substrate was quantified by HPLC analysis. Steady-state kinetics parameters were determined from the Lineweaver–Burk plots.

ASSOCIATED CONTENT

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02473.

SDS-PAGE of purified CsyB I375F, synthetic scheme of alkyl diketide-NACs, and 1H and 13C NMR spectra of C4 to C9 diketide-NACs (PDF)

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Notes
The authors declare no competing financial interest.

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