

Rational Control of Polyketide Extender Units by Structure-Based Engineering of a Crotonyl-CoA Carboxylase/Reductase in Antimycin Biosynthesis

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Abstract: Bioengineering of natural product biosynthesis is a powerful approach to expand the structural diversity of bioactive molecules. However, in polyketide biosynthesis, the modification of polyketide extender units, which form the carbon skeletons, has remained challenging. Herein, we report the rational control of polyketide extender units by the structure-based engineering of a crotonyl-CoA carboxylase/reductase (CCR), in the biosynthesis of antimycin. Site-directed mutagenesis of the CCR enzyme AntE, guided by the crystal structure solved at 1.5 Å resolution, expanded its substrate scope to afford indolylmethylmalonyl-CoA by the V350G mutation. The mutant A182L selectively catalyzed carboxylation over the regular reduction. Furthermore, the combinatorial biosynthesis of heterocycle- and substituted arene-bearing antimycins was achieved by an engineered *Streptomyces* strain bearing AntE^{V350G}. These findings deepen our understanding of the molecular mechanisms of the CCRs, which will serve as versatile biocatalysts for the manipulation of building blocks, and set the stage for the rational design of polyketide biosynthesis.

Polyketides, one of the largest groups of natural products with remarkable structural diversity, are a valuable source of medicinally important compounds. Despite their structural complexity, all polyketides are biosynthesized from simple acyl or malonyl building blocks, in principle via decarboxylative Claisen condensation.^[1] During the condensation process, polyketide synthases (PKSs) catalyze either the partial or full reduction of the β -keto group, to yield differently modified structures.^[1–3] With the development of synthetic biology, considerable efforts have been devoted toward expanding the structural diversity of polyketides and improving their bioactivities. Extender units constitute the majority of polyketide scaffolds, thus engineering of extender units would dramatically increase the structural diversity of

polyketides. However, the engineering of extender units remains challenging, as compared to the starter unit engineering achieved by precursor-directed biosynthesis or mutasynthesis.^[4] Recent developments in extender unit engineering resulted in the introduction of terminal alkyne^[5] or fluorine^[6] substitutions to the polyketide products. However, the yields of the desired products were significantly lower when compared to those of the native products, and synthetic analogues are required as precursors. In this context, knowledge about the biogenesis of unnaturally substituted malonyl-CoAs and enzymology of PKSs is eagerly awaited for the effective bioengineering of extender units.

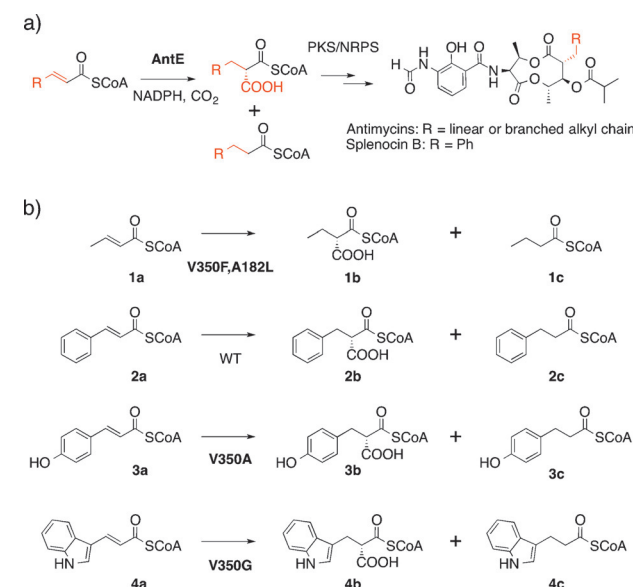
While most polyketides are derived from (methyl)malonyl-CoA extender units, some modular PKSs utilize various α -substituted malonyl-CoAs,^[7] which are mainly biosynthesized by crotonyl-CoA carboxylase/reductase (CCR) enzymes.^[8] CCRs catalyze the reductive carboxylation of α,β -unsaturated acyl-CoAs in the presence of CO₂ and NADPH, and yield the corresponding α -substituted malonyl-CoAs in a (2*S*)-stereoselective manner.^[9] These findings significantly enhanced the understanding of PKS building block biogenesis, and revealed the engineering potential of utilizing unique building blocks to generate “unnatural” natural products.^[8b,10]

Recently our group and others determined the biosynthetic pathway of antimycins (ANTs), the nine-membered antifungal polyketide-peptide hybrid dilactones in *Streptomyces* sp. NRRL2288,^[11] which includes the CCR enzyme AntE (Scheme 1). The native substrates of AntE range from crotonyl-CoA to octenoyl-CoA, as seen in the structures of naturally isolated ANT, and AntE not only catalyzes carboxylation but also simultaneously yields reduced byproducts of α,β -saturated-CoAs. Previous *in vivo* and *in vitro* analyses demonstrated that AntE carboxylates a variety of α,β -unsaturated CoAs, resulting in the production of various unnatural ANT derivatives bearing alkyl or even halogen or alkyne moieties. These findings indicated the high promiscuity of AntE, along with the downstream PKS enzymes in ANT biosynthesis.^[11b,12] Notably, AntE and its homologue SpnE catalyze the carboxylation of cinnamoyl-CoA, although the substrate scope for aromatic substrates seems to be limited only to cinnamoyl-CoA.^[12a,13]

To shed light on the molecular basis for the promiscuity of AntE, we first solved the structure of AntE in complex with NADP⁺ at 2.2 Å resolution. The overall structure showed high similarity to the 2-octenoyl-CoA carboxylase/reductase CinF from *Streptomyces* sp. JS360 (RMSD = 1.9 Å for the Ca

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Scheme 1. A) The reaction of AntE in antimycin biosynthesis. B) Substrates used in in vitro assays of AntE and the products generated.

atoms; PDB ID: 4A10).^[10d] The NADPH and CO₂ binding sites of AntE are well conserved among CinF and other CCRs, and form hydrogen-bond networks between NADPH, CO₂, and the surrounding residues (Figure 1; Supporting Information, Figure S1). An active-site comparison with CinF

revealed the unique structural features of AntE. First, a large-to-small substitution was observed at Ala182, where CinF and most CCRs have a conserved leucine forming a hydrophobic pocket (Figure 1B; Supporting Information, Figure S2), suggesting the significant expansion of the cavity around the NADPH and CO₂ binding sites in AntE. In contrast, Gly362 in CinF, which is proposed to be critical for accepting the bulky octenoyl-CoA as a substrate,^[10d] is replaced with the larger Val350 in AntE, suggesting the possibility of enzyme engineering.

To clarify the structural basis for substrate recognition, we performed structure-based mutagenesis targeting position Val350. In vitro analyses of the V350A, V350G, and V350F mutants were performed in the presence of NADPH and hydrogen carbonate, using crotonyl-CoA (**1a**), cinnamoyl-CoA (**2a**), and *p*-coumaroyl-CoA (**3a**) as substrates. As a result, the V350F mutant accepted only **1a**, whereas the V350A and V350G mutants accepted all three substrates, to produce both carboxylated and reduced products (Figure 1D). Furthermore, when 3-indolylacryloyl-CoA (**4a**) was tested, the V350G mutant even catalyzed the carboxylation of **4a** to afford indolylmethylmalonyl-CoA (**4b**), suggesting the crucial contribution of Val350 in determining the substrate specificity. Kinetics analyses revealed that V350F and V350G have enhanced catalytic efficiencies for **1a** and **2a**, respectively (Table 1). Importantly, the catalytic efficiency of V350G with **3a** was 3.0-fold higher than that of wild-type AntE with **2a**, indicating that the mutant can

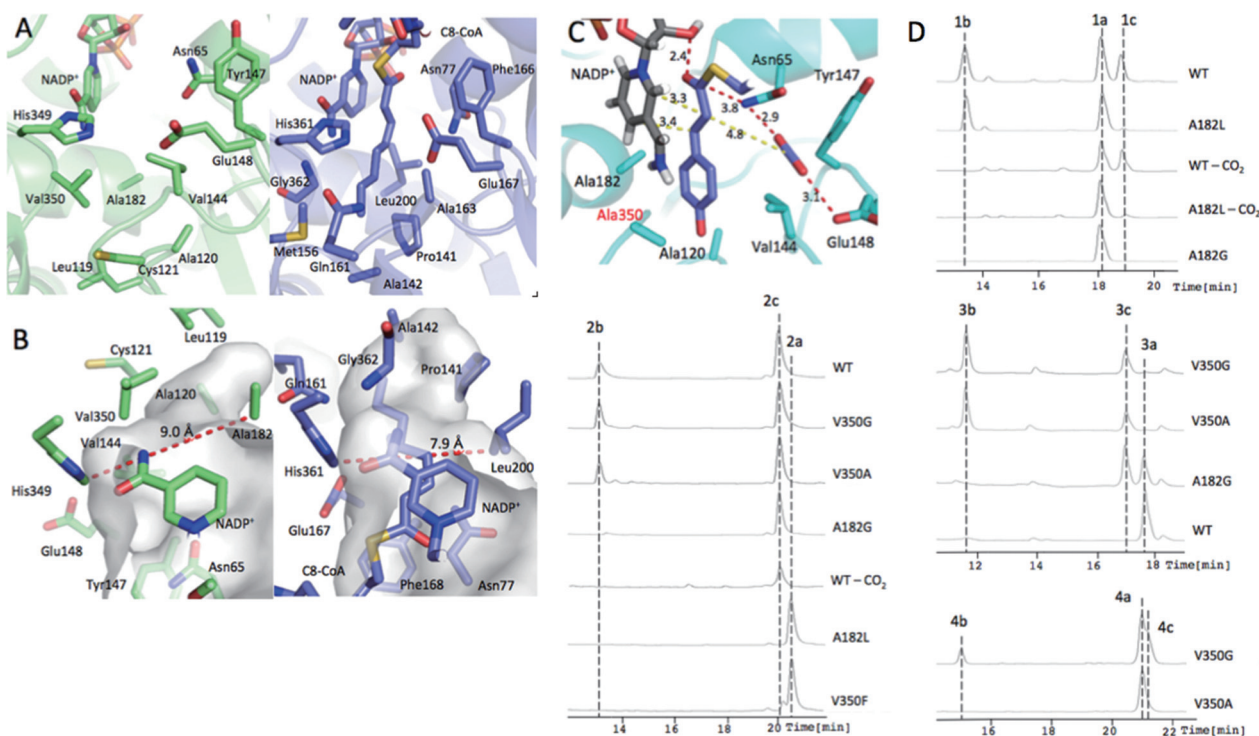


Figure 1. Structural analysis and in vitro assays of AntE. A) Structural comparison of the catalytic cavity of AntE (green, in complex with NADP⁺) with CinF (blue, in complex with NADP⁺ and octenoyl-CoA). B) Top view of the catalytic cavities of AntE and CinF, shown in gray. C) Docking model of *p*-coumaroyl-SNAC and carbon dioxide in the crystal structure of AntE^{V350A} with NADP⁺. Possible hydrogen bonds are shown in red, and the distances between the substrate and NADP⁺ or carbon dioxide are shown by yellow dotted lines. D) HPLC traces (UV: $\lambda = 260$ nm) of in vitro assays of AntE wt and mutants using crotonyl-CoA (**1a**), cinnamoyl-CoA (**2a**), *p*-coumaroyl-CoA (**3a**), and indolylacryloyl-CoA (**4a**). Reactions without hydrogen carbonate are labeled by $-\text{CO}_2$.

Table 1: Kinetics parameters of AntE and its variants.

Enzyme	Substrate	$k_{\text{cat}}^{[a]}$ [min ⁻¹]	$K_{\text{M}}^{[a]}$ [mM]	$k_{\text{cat}}/K_{\text{M}}$	Ratio ^[b]
AntE	1a	10.53 ± 1.5	1.58 ± 0.39	6.6	63:37
	2a	4.13 ± 0.47	0.38 ± 0.081	10.8	25:75
AntE ^{A182G}	2a	N/A	N/A	N/A	3:97
	3a	N/A	N/A	N/A	0:100
AntE ^{A182L}	1a	9.11 ± 1.1	1.31 ± 0.29	6.9	95:5
AntE ^{V350F}	1a	7.58 ± 0.51	0.49 ± 0.089	15.4	32:68
AntE ^{V350A}	1a	7.18 ± 0.75	1.21 ± 0.23	5.9	58:42
	3a	4.41 ± 0.24	0.14 ± 0.025	31.5	65:35
AntE ^{V350G}	1a	4.05 ± 0.66	1.17 ± 0.35	3.5	56:44
	2a	5.26 ± 0.44	0.089 ± 0.021	59.1	30:70
	3a	6.54 ± 0.35	0.20 ± 0.031	32.7	64:36
	4a	N/A	N/A	N/A	30:70

[a] Data represents mean value of three experiments ± S.D. [b] Product ratio of carboxylation: reduction in percentage.

accommodate the polar *p*-hydroxy group on the aromatic ring of **3a**. These results unequivocally revealed that, in accordance with the case of CinF,^[10d] Val350 in AntE is a key residue for determining the substrate specificity, and by rational mutagenesis, we successfully expanded the substrate scope of AntE to accept polar and fairly bulky unnatural substrates with increased catalytic efficiency.

We next focused on the Ala182 residue. We generated the A182L and A182G mutants and assessed the effects of the substitutions on the enzyme activities. Unexpectedly, A182G lost its carboxylation activity, but retained the reductase activities against substrates **2a** and **3a** (Figure 1D). In contrast, A182L no longer accepted the bulky **2a**, but exhibited dramatically increased carboxylation selectivity, and catalyzed the carboxylation of **1a** to afford **1b** with 95 % selectivity. Notably, CCRs catalyze ordinary reduction only in the absence of CO₂ by using the solvent water as a proton donor,^[9a] and are thought to have evolved from regular reductases by acquiring the carboxylation activity.^[14] In contrast, AntE catalyzes both reduction and carboxylation, even in the presence of CO₂. In this regard, it is remarkable that a single amino acid substitution drastically altered the reaction selectivity of AntE.

As for the increased carboxylation selectivity observed in the A182L mutant, we envision that the hydrophobic side chain of Leu182 prevents water molecules from entering the catalytic pocket, and simultaneously positions the substrate in a more preferable orientation for carboxylation in the narrowed cavity. This proposal is consistent with the selective reduction observed in the A182G mutant. In the wild-type enzyme, Ala182 would partially allow the access of a water molecule to the active center, resulting in the mixed reaction selectivity.

To gain further structural insights into the AntE-catalyzed enzyme reactions, we attempted the co-crystallization of AntE^{V350A} with *p*-coumaroyl-CoA. The crystal structure of AntE^{V350A} with NADP⁺ was obtained at 1.5 Å (RMSD = 0.4 Å for the Ca atoms, as compared with native AntE), although we could not observe clear electron density for the CoA-substrate. A ligand docking model of V350A with *p*-coumaroyl-SNAC as a substrate mimic, to clarify the active-

site binding, revealed that the substrate was located near Ala350 and fit well in the enlarged cavity (Figure 1C). Hydrogen bond networks could be drawn among the conserved Asn65, the OH-1 of NADPH, and the carbonyl oxygen of the substrate thioester, and thus the orientation of the substrate in the active-site cavity was fixed to afford (2*S*)-stereoselective carboxylation, as in the cases of other CCRs^[9a] (Figure 1C). We surmised that our structure-based AntE mutant could accept a large number of α,β-unsaturated acyl-CoAs with substitutions smaller than an indole group, ranging from polar phenols and halogens to non-polar alkynes and various branched and linear alkyl substitutions.^[12a]

The successful engineering of AntE led us to investigate its utility in polyketide biosynthesis *in vivo*, to achieve the selective production of ANTs by AntE^{A182L} or the production of novel ANTs by AntE^{V350G}. Thus, we expressed the AntE mutants A182L and V350G in the $\Delta antB\Delta antE$ strain AL2111,^[12a] and compared the metabolic profile of each strain with the control strain AL2110, a $\Delta antB$ strain producing C8-deacylated antimycins (DAs), and AL2111. As a result, the strain AL2112, in which the A182L mutant was introduced, selectively produced the deacylated antimycin DA-14 (**5**, 0.5 mg L⁻¹), but the mutation abolished the production of ANTs (**6–9**) with bulky side chains. The strain AL2113, in which the V350G mutant and a promiscuous CoA-ligase from *Lithospermum erythrorhizon*^[15] were introduced, altered the product ratio of ANTs (**5–9**) with a preference for longer and branched side chains, and afforded the novel derivative DA-15 (**10**, 0.7 mg L⁻¹). These results demonstrated the utility of an engineered CCR in ANT biosynthesis, and the expanded substrate scope of the V350G mutant afforded the production of a novel compound.

To examine the catalytic versatility of the AntE V350G mutant, we next performed feeding experiments with AL2113, with various aromatic substrates, including some that were not tested *in vitro*. By feeding 3-(2-thienyl)acrylic acid and *p*-methylcinnamic acid, two novel ANTs, DA-16 (**12**) and DA-17 (**13**), were produced (**12**: 4.7 mg L⁻¹, **13**: 1.1 mg L⁻¹, Figure 2). This is the first demonstration of the utilization of a heterocyclic or substituted arene extender unit by the PKS machinery, by the structure-based engineering of a CCR. Notably, another putative CCR, Clz4 from the chlorizidine polyketide pathway, reportedly generates a pyrrole-containing extender unit.^[16] On the other hand, the feeding of *p*-coumaric acid, 3-indoleacrylic acid, and urocanic acid did not afford the expected products, which is presumably caused by strict substrate specificity of PKS, or cellular catabolism. Nevertheless, our results indicated that AntE is a versatile biocatalyst for providing a variety of unnatural extender units.

In conclusion, we have demonstrated structure-based engineering of AntE, which can serve as a versatile catalyst to provide a wide range of α-substituted malonyl-CoAs. Utilizing more than a dozen acyl-CoA substrates (Supporting Information, Figure S3), the engineered AntE shows great potential not only as a biocatalyst for CO₂ fixation, which may inspire developments in biomimetic catalysis in organic chemistry, but also as a powerful tool in synthetic biology to provide novel building blocks for the creation of unnatural polyketide carbon skeletons. Further engineering of CCR

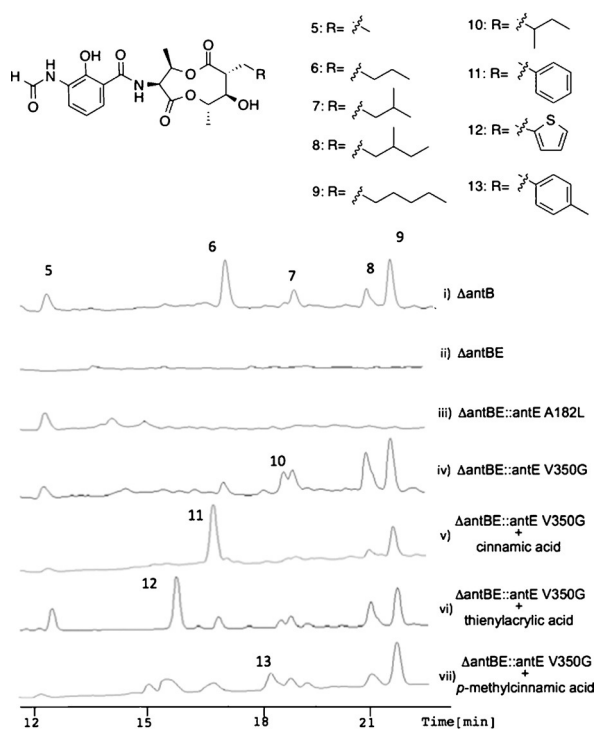


Figure 2. HPLC analysis of antimycin production. UV spectra were monitored at 230 nm. i) AL2110, ii) AL2111, iii) AL2112, iv) AL2113, v) AL2113 with 1 mM cinnamic acid supplementation, vi) AL2113 with 1 mM 3-(2-thienyl)acrylic acid, and vii) AL2113 with 1 mM *p*-methylcinnamic acid.

enzymes to improve their robustness and scalability, and clarification of the downstream PKS enzymology are awaited for the successful manipulation of natural product biosynthesis.

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