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Structural Basis of a Broadly Selective Acyltransferase from the Polyketide Synthase of Splenocin

Yuan Li[+], Wan Zhang[+], Hui Zhang, Wenya Tian, Lian Wu, Shuwen Wang, Mengmeng Zheng, Jinru Zhang, Chenghai Sun, Zixin Deng, Yuhui Sun, Xudong Qu, and Jiahai Zhou**

Abstract: *Polyketides are a large family of pharmaceutically important natural products, and the structural modification of their scaffolds is significant for drug development. Herein, we report high-resolution X-ray crystal structures of the broadly selective acyltransferase (AT) from the splenocin polyketide synthase (SpnD-AT) in the apo form and in complex with benzylmalonyl and pentynylmalonyl extender unit mimics. These structures revealed the molecular basis for the stereoselectivity and substrate specificity of SpnD-AT, and enabled the engineering of the industrially important Ery-AT6 to broaden its substrate scope to include three new types of extender units.*

Polyketides are a large class of pharmaceutically important natural products. Their natural forms and derivatives thereof have been widely utilized as medicines and agrochemicals.[1] To develop these compounds into drugs, polyketides typically need to be structurally modified to improve their pharmacological properties.[2] However, chemical efforts seeking to modify polyketide carbon scaffolds have frequently been impeded by their structural complexity and/or inert reactivity.[3] Biosynthetic engineering, particularly alterations of the specificity of acyltransferase enzymes (ATs), has now been established as an effective alternative to traditional chemical modification of polyketide skeletons.[4] Such approaches use domain swapping or direct AT engineering.[4,5]

Classical ATs in an extension module selectively transfer extender units (malonyl- or alkylmalonyl-CoA) onto polyketides.[4] Currently, there are only few reports on the successful engineering of canonical ATs to accept various extender units.[5] The lack of structural information on AT–substrate complexes has made these efforts challenging; previous attempts have relied on the screening of large numbers of mutations and have resulted in limited extensions of the substrate scope.[5] While it is known that typical ATs only accept a very limited class of substrates (malonyl- or methylmalonyl-CoA), we and others have recently identified several new, broadly selective AT domains from polyketide biosynthetic pathways.[6] The substrate scopes of these enzymes, and especially of the AT domain (SpnD-AT) of the splenocin (Figure 1 a) polyketide synthase (PKS), are highly variable and can therefore accept a number of diverse extender units, including very long aliphatic chains (up to C_7-malonyl-CoA) and even aromatic benzylmalonyl-CoA.[6a] Structural characterization of broadly selective ATs in complex with substrates or substrate analogues will provide key insight into the molecular basis of the substrate flexibility of these enzymes and will almost certainly guide the engineering of other canonical AT domains. Herein, we report high-resolution X-ray crystal structures of SpnD-AT in the *apo* form and in complex with benzylmalonyl and pentynylmalonyl extender unit mimics. Based on these structures, we can explain both the stereoselectivity and the broad substrate scope of SpnD-AT. Moreover, using the information from our

[*] Y. Li,[+] W. Zhang,[+] H. Zhang, W. Tian, S. Wang, M. Zheng, C. Sun, Prof. Z. Deng, Prof. Y. Sun, Prof. X. Qu
Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education
Wuhan University School of Pharmaceutical Sciences
185 Donghu Road., Wuhan 430071 (China)
E-mail: quxd@whu.edu.cn
L. Wu, J. Zhang, Prof. J. Zhou
State Key Laboratory of Bioorganic and Natural Products Chemistry
Center for Excellence in Molecular Synthesis
Shanghai Institute of Organic Chemistry
Chinese Academy of Sciences
345 Lingling Road, Shanghai 200032 (China)
and
Shaanxi Key Laboratory of Natural Products & Chemical Biology
College of Chemistry and Pharmacy
Northwest A&F University
3 Taicheng Road, Yangling 712100, Shaanxi (China)
E-mail: jiahai@mail.sioc.ac.cn
[+] These authors contributed equally to this work.
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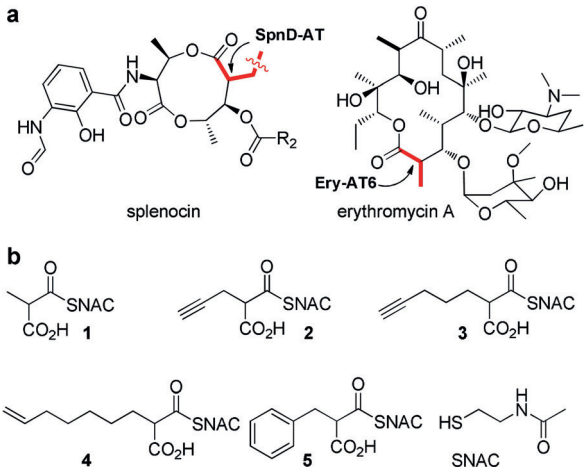

Figure 1. a) Structures of splenocin and erythromycin A, whose AT domains were studied in this work. The structural variations introduced by SpnD-AT and Ery-AT6 are highlighted by red. b) Extender unit mimics used in this study, including methylmalonyl-SNAC (**1**), propargylmalonyl-SNAC (**2**), pentynylmalonyl-SNAC (**3**), heptenylmalonyl-SNAC (**4**), and benzylmalonyl-SNAC (**5**).

structural study, we were able to mutate key residues of the canonical Ery-AT6 (Figure 1a) from the erythromycin PKS and thereby change its substrate scope to accept diverse bulky extender units.

A form of SpnD-AT comprising its N-terminal ketosynthase (KS) AT linker (residues 1–80) and its post-AT linker (396–402) was overexpressed in *Escherichia coli* BL21 (DE3) cells. The highly purified protein (43 KDa) was then crystallized in 1.5 M lithium sulfate monohydrate solution containing 0.1 M bis-tris propane (pH 7.0) at 18 °C. Its *apo* structure (PDB ID: 5YDA) was solved by molecular replacement with the atomic coordinates of Ery-AT3 (PDB ID: 2QO3)[7] as a searching model, and was refined to 2.35 Å. Similar to other canonical AT domains, such as Ery-AT3, Zma-AT (PDB ID: 4QBU),[8] and Ery-AT5 (PDB ID: 2HG4),[9] the core structure of SpnD-AT contains two subdomains, a small ferredoxin (residues 207–272) and a large α/β hydrolase fold (residues 81–206, 273–395; Figure 2a). To investigate the substrate binding mode of SpnD-AT, we used two SNAC substrate mimics for co-crystallization experiments, namely pentynylmalonyl-SNAC (**3**) and benzylmalonyl-SNAC (**5**; Figure 1b). The structures of the benzylmalonyl SpnD-AT (PDB ID: 5YDM) and pentynylmalonyl SpnD-AT (PDB ID: 5YDL) complexes were refined to 2.50 Å and 2.40 Å resolution, respectively (Figure 2b). In both structures, the substrate extender units were successfully tethered to S173 of SpnD-AT through ester bonds, with clear electron densities (see the Supporting Information, Figure S1). These structures are very similar to that of the *apo* form (with RMSDs of 0.225 Å for pentynylmalonyl SpnD-AT and 0.243 Å for benzylmalonyl SpnD-AT), suggesting that substrate binding does not cause any obvious conformational changes in the enzyme. Both complex structures show that the extender units are stabilized by 1) a bidentate salt bridge formed between the guanidyl group of R198 and its carboxylate group and by 2) two hydrogen bonds formed between the backbone nitrogen atoms of G92 and L174 and their respective carbonyl groups (Figure 2b). These multiple stabilizing interactions tightly fasten the malonyl plane and force the α-substituents into an *S* configuration pointing towards the hinge region, which is constituted by helix α9 and a loop with a ^{268}AAAH^{271} motif (Figure 2a). Although the *S* substrate preference of the AT domain had been biochemically confirmed in experiments using the DEBS system,[10] the possibility of *R* form extender units in the AT cavity remained under discussion.[5c,11] With the help of our complex structures, it is now clear that the rigidity of the malonyl plane makes the flexibility of the substituent much lower than previously assumed; we anticipated that the occurrence of *R* form substituents would cause a significant repulsion with the loop of ^{268}AAAH^{271}, and found this to be the case (data not shown).

The substituents of the extender units in the complex structures are surrounded by hydrophobic residues, including A268, A269, A270, A203, I202, I199, M266, and F145 (Figure 2c). I199, I202, and A203 form the bottom of the cavity, and together function to confine the maximum acceptable length of the substituents. Residues M266 and F145, as well as the ^{268}AAAH^{271} motif, encircle the substituents of the extender units and form the side walls of the cavity. Interestingly, F145 also forms a strong π-interaction with the phenyl and alkynyl groups of the extender units (Figure 2b). As the SNAC substrate mimics in the AT cavity are identical to native CoA substrates,[5c] and having shown that the specificity of the acylation of an extender unit can be predictive of the substrate scope of a given AT, we used a panel of malonyl-SNACs (Figure 1b), including propargylmalonyl-SNAC (**2**, C_3), pentynylmalonyl-SNAC (**3**, C_5), heptenylmalonyl-SNAC (**4**, C_7), and benzylmalonyl-SNAC (**5**) to evaluate AT specificity by monitoring the release of free thiols from SNAC substrate mimics after reaction with the mBBr reagent (Figure 3). Unlike the C_5 and benzylmalonyl-SNAC, the terminal unsaturated bonds of C_3 and C_7 point away from the phenyl plane of F145; thus, the π-interactions between these substrates and F145 should be

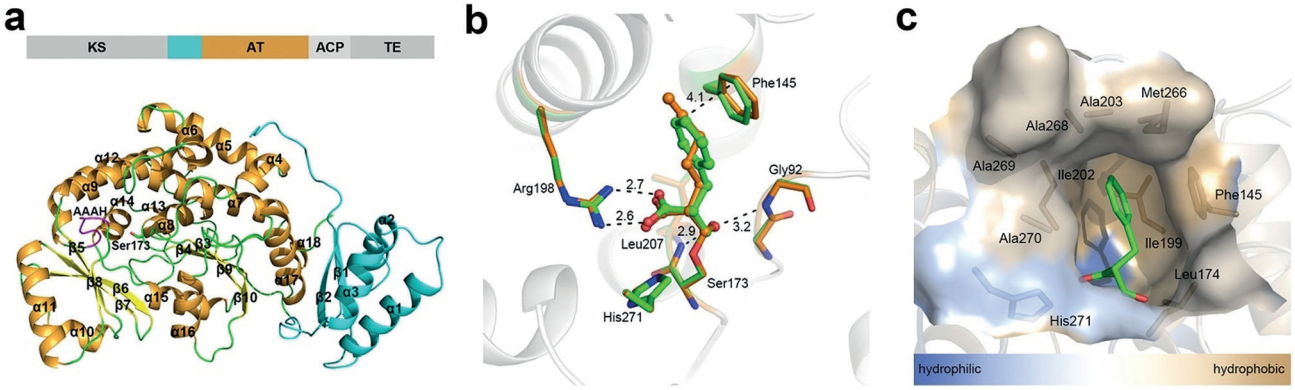

Figure 2. a) The overall structure of SpnD-AT. The *apo* structure of SpnD-AT is shown as a cartoon, and the secondary structures are numbered sequentially. The KS-AT linker is colored in cyan, and the α-helix, the β-sheet, and the loop of the AT domain are shown in bright orange, yellow, and green, respectively. The loop of ^{268}AAAH^{271} is colored in magenta, and the side chain of the catalytic residue Ser173 is shown in stick representation. b) Superposition of the active sites in the two complexes and the detailed interactions between SpnD-AT and the substrate. The extender unit forms a covalent bond to the hydroxy group of the catalytic residue Ser173. The atoms of the pentynylmalonyl and benzylmalonyl units with the corresponding active residues are shown in orange and green, respectively. c) The active pocket of SpnD-AT, which contains a number of hydrophobic residues, including A268, A269, A270, A203, I202, I199, M266, and F145. Hydrophobicity and hydrophilicity are indicated by brown and blue shading, respectively.

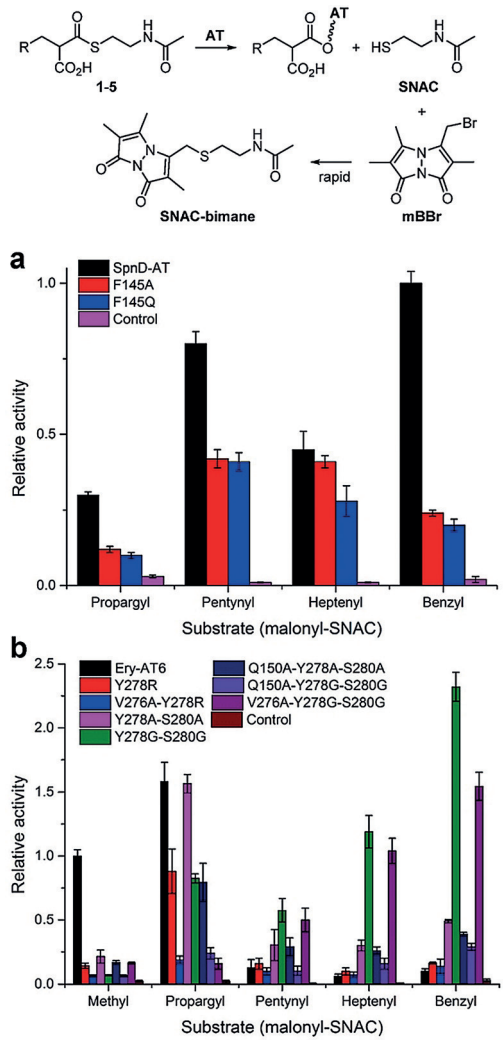

Figure 3. Characterization of the AT specificity in vitro. Malonyl-SNACs (**1**–**5**) were hydrolyzed by ATs; the catalytic efficiency was measured based on the generation of SNAC-bimanes (Figure S2), which were produced by the reaction of mBBr and released SNAC. Each experiment was performed in triplicate. a) Catalytic efficiency of SpnD-AT and its related mutants for substrates **2**–**4**. b) Catalytic efficiency of Ery-AT6 and its related mutants for substrates **1**–**5**. The conditions in the control reaction are the same to those of the AT reactions except for omission of the enzyme.

negligible. As expected, activity assay results (Figure 3a) revealed that the wild-type SpnD-AT indeed prefers the C_5 and benzyl substituents, with about twofold higher activity than for the C_3 and C_7 substituents. Moreover, mutation of F145 into either alanine or glutamine resulted in a significant reduction in the acylation activity for the C_5 and benzyl substrates (ca. 60% and 80% reduction for either mutant), yet only a small (8% by F145A) and medium reduction (33% by F145Q) reduction for the C_7 substrates. These results confirm the significance of the π-interactions to F145 in determining the substrate scope of SpnD-AT. Furthermore, F145A and F145Q mutations also induce a severe reduction (ca. 70%) in the acylation activity for the C_3-substituted substrate, suggesting that a large cavity does not promote this AT to accept substrates with small extender units efficiently, probably owing to the loss of hydrophobic stabilization from the binding cavity.

To gain further insight into the substrate specificity of ATs in a more general sense, we compared SpnD-AT with two other canonical AT domains, namely Ery-AT3 (PDB ID: 2QO3)[7] and Ery-AT5 (PDB ID: 2HG4;[9] Figures S3 and S4). Although SpnD-AT shares a low degree of sequence identity with Ery-AT3 (31%) and Ery-AT5 (30%), their overall architectures are highly similar. By mapping the substrate-surrounding residues onto the structures of SpnD-AT, Ery-AT3, and Ery-AT5, we found that conserved residues for substrate binding, including S173, R198, and H271, are all clustered in the interior of the active site (Figure S5). Interestingly, in Ery-AT3, we observed an unusual 56.3° rotation of the guanidyl group of R208 (Figure S5); this can significantly change the orientation of the malonyl plane, and forces the substituents to point towards a helix (D152–Y172). This conformation leaves only a very limited space for the accommodation of the extender units. Unlike in Ery-AT3, the arginine residues in other solved extender AT structures (PDB IDs: 3IM8, 5DZ6, 3RGI, 3TZW, 4RR5, 4MZ0, 4QBU, and 2HG4) are all identical to that of SpnD-AT, suggesting that the binding mode of extender units is highly conserved in most ATs.

The bottom of the Ery-AT5 binding pocket is formed by S204, L207, and R208 (Figure S4a). The observed most influential residues in SpnD-AT are A268 and A270 in the ^{268}AAAH^{271} motif and F145, which correspond to Y278, S280, and Q150 in Ery-AT5 (Figure S4a). Unlike the phenyl group of F145, which is parallel to the substituent of the extender units, the side chain of Q150 protrudes deeply into the pocket. It pushes the substrate outward to force it closer to the bulky Y278 and S280 residues. These two residues form direct repulsive interactions with large substrates and hence restrict Ery-AT5 to only accepting methylmalonyl-CoA as a substrate (Figure S4a and S6). Significantly, these constraints are common among canonical ATs; both Q150 and the bulky residues in the ^{278}YASH^{281} (Y278 and S280) or ^{278}HAFH^{281} (H278 and F280) motifs are highly conserved in methylmalonyl-CoA-specific or malonyl-CoA-specific ATs.[5d]

The constraint factors that we have identified provide insight that can inform the rational engineering of other canonical ATs to expand their substrate scopes. Ery-AT6 has been widely studied in the past; previous studies with site-directed mutagenesis forms of the Ery-AT6 enzyme have shown that it is possible to alter the ability of the enzyme to accommodate various C3 extender units (e.g., propargylmalonyl-CoA),[5a–c] and demonstrated the use of altered substrate scopes for the biosynthesis of macrolactones of erythromycin. The structure of Ery-AT6 was modeled and superimposed on the SpnD-AT complex structures (Figure S4b). As for Ery-AT5, its residues for the stabilization of the malonyl plane and for the pocket structure are identical (Figures S4b and S3), and Q150, Y278, and S280 appear to form the major repulsive interactions with bulky substrates (Figure S4b). Previously, Ery-AT6 and the two best mutant forms Y278R and V276A–Y278R have been evaluated with methylmalonyl (C_1) and propargylmalonyl (C_3) for their

substrate scope.[5b] To test their ability to accept bulkier substrates, these enzymes were prepared and assayed for acylation activity against the aforementioned synthetic malonyl-SNACs (**1**–**5**). In vitro assays (Figure 3b) revealed an identical result, namely that 1) wild-type Ery-AT6 is able to effectively accept both methylmalonyl and propargylmalonyl extender units, actually showing a slight preference for propargylmalonyl-SNAC (**2**); 2) the Y278R mutant form retains only weak activity for methylmalonyl-SNAC and other substrates but reacts well with propargylmalonyl-SNAC; and 3) the Y278R–V276A mutant has weak but balanced activity (10–20% activity of Ery-AT6 on **1**) to extender units. Mutation of Y278 into arginine is able to alter the substrate binding conformation to specifically accept C_3 extender units but this arginine residue apparently prevents Ery-AT6 from accommodating larger extender units. A previous study showed that the combination of three mutations (Y278G, S280G, and V276A) is able to increase the efficiency of Ery-AT6 to accommodate C_3 extender units,[5c] results that suggested that reducing the area occupied by residues 278 and 280 would be beneficial if the goal was to enable accommodation of larger extender units. It was assumed that the V276A mutation increased the flexibility of the YASH motif in a way that facilitated accommodation of propargylmalonyl-CoA by Ery-AT6.[5c] However, our findings in the present study indicate that the V276A mutation may also have a detrimental influence on the AT activity of Ery-AT6-Y278R (by sacrificing the activity to broaden its specificity). We therefore chose to keep the wild-type residue at position 276, and replaced two key Ery-AT6 residues (Y278 and S280) with either the corresponding two residues from SpnD-AT (both of which are alanine residues: "Y278A–S280A double mutant") or two more flexible glycine residues ("Y278G–S280G double mutant"). Biochemical assays indicated that these mutations dramatically broadened the substrate scope. Whereas the Y278A–S280A double mutant showed a strong preference for propargylmalonyl substrates, the Y278G–S2280G double mutant form of Ery-AT6 can effectively accommodate C_3, C_5, C_7, and benzyl substrates, but not the C_1 substrate (Figure 3b). Introducing additional V276A into the Y278G–S280G mutant decreased the activity towards the C_3, C_5, C_7, and benzyl substrates (Figure 3b), suggesting that Y278G–S280G is better for engineering erythromycin structures.

Our structural analysis indicated that Q150 also constrains the substrate scope of Ery-AT6. In the binding pocket, the side chain of Q150 points towards the middle, which pushes the substrate into the vicinity of Y278 and S280 (Figure S4b). To confirm the functional role of Q150, two triple mutants were generated and assayed (Q150A–Y278G–S280G and Q150A–Y278A–S280A). Compared to their double mutant forms, both of these triple mutants had significantly reduced abilities to accommodate extender unit substrates, and these reductions were more pronounced for larger substrates such as heptenylmalonyl- (**4**) and benzylmalonyl-SNACs (**5**). We suspect that mutation of Q150 into alanine could have a powerful impact in increasing the volume of the pocket; however, this mutation might also sacrifice the hydrophobic interaction to the substrate, which we had earlier confirmed to be very important for both the activity and substrate specificity of SpnD-AT (see above). Thus, to further broaden the substrate scope without decreasing the AT activity, an ideal mutation at this position would cause reduced steric hindrance while promoting increased hydrophobic interactions; we are currently exploring this hypothesis. Kinetic data revealed that Y278G–S280G is indeed as efficient as SpnD-AT in binding heptenylmalonyl-SNAC (**4**) (with k_{cat}/K_m values of 0.47 and 0.50×10–3×10^{-3} $min^{-1}\,\mu M^{-1}$, respectively) and benzylmalonyl-SNAC (**5**; with k_{cat}/K_m values of 1.48 and 1.42×10^{-3} $min^{-1}\,\mu M^{-1}$, respectively). More so, Y278A-S278A shows a comparative activity on propargylmalonyl-SNAC (**2**) ($k_{cat}/K_m = 0.54 \times 10^{-3}$ $min^{-1}\,\mu M^{-1}$; Figure S7). These results show that the artificially engineered canonic ATs are highly competent in accepting bulky substrates.

Understanding the physical interactions between AT enzymes and their substrates is crucial for AT engineering, but this structural knowledge has been previously restricted to a malonyl-tethered transacylase (FabD) of the *E. coli* type II fatty acid synthase (FAS) family.[12] This structure not only has low identity with PKS AT (e.g., 28% protein identity with SpnD-AT), but also lacks information for substituted groups. Recently, an acyl-complex structure of a transacylase (SAT) domain from a fungal iterative PKS was also solved;[13] however, this enzyme only selects an acyl substrate and shows a very different substrate binding mode from those of classical PKS extension modules; hence the utility of these complex structures for informing AT engineering has been limited. Our *apo* structure and two complex structures of the highly promiscuous AT domain of SpnD-AT guided our engineering of Ery-AT6, and our results suggest that future efforts to alter AT activity and substrate scope should be focused on the engineering of these three residues (Q150, Y278, S280 in methylmalonyl-CoA-specific AT and Q150, H278, F280 in malonyl-CoA-specific AT) to enable the accommodation of various extender unit substrates. To the best of our knowledge, this is the first successful example of the characterization of PKS extender AT–substrate complex structures and the first demonstration of an engineered AT domain that accepts substrates with very bulky extender units. These new structures open the door for introducing diverse structural modifications into many polyketide carbon scaffolds.

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Conflict of interest

The authors declare no conflict of interest.

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