Polyketide Biosynthesis

International Edition: DOI: 10.1002/anie.201510402
German Edition: DOI: 10.1002/ange.201510402

Extending Polyketide Structural Diversity by Using Engineered Carboxylase/Reductase Enzymes

*Jana Kundert and Tobias A. M. Gulder**

crotonyl-CoA carboxylase/reductases · enzymes · metabolic engineering · polyketide biosynthesis

Polyketides are among the most important sources of essential drugs, including the antibiotic erythromycin, the cholesterol-lowering drug lovastatin, and the antiparasitic avermectin. The biosynthesis of these natural products is catalyzed by multifunctional enzyme complexes, the polyketide synthases (PKSs).[1] Despite their highly diverse, often very complex structures, polyketides are assembled from simple acyl- and malonyl building blocks, bound either to coenzyme A (CoA) or to dedicated acyl carrier proteins (ACPs). Similar to fatty acid biosynthesis, the nascent polyketide gets successively elongated by decarboxylative Claisen condensation reactions with malonate-derived extender units. In the case of polyketides, however, reductive enzymes modify the resulting β-keto functions by partial or full reduction, thereby leading to impressive architectural diversity. Further structural variability is introduced through the incorporation of a large variety of different starter units, i.e., the first building block of the polyketide backbone.[2] By contrast, the chemistry of the extender units seems to be much more limited.[3] Indeed, most PKSs solely incorporate malonyl- or methylmalonyl-CoA, with a smaller number of examples utilizing ethylmalonyl-CoA, leading to H, Me, or Et substituents. In a few examples, ACP-bound hydroxy-, methoxy-, or aminomalonates are used as building blocks. However, some polyketides contain other unusual side-chains that cannot be explained by the incorporation of any of these extender units. Prominent examples include the proteasome inhibitors salinosporamide A (**1a**) and cinnabaramide A (**1b**), which bear a chloroethyl group and a long-chain alkyl group, respectively, the immunosuppressive drug FK-506 (**2**), which is equipped with an allyl group, and antimycin A_{7b} (**3**), which bears a branched alkyl side-chain (Figure 1).

The generation of the respective 2-substituted malonates used as PKS extender units is facilitated by reductive carboxylation of α,β-unsaturated acyl-CoA units at C_α (Figure 2A). This remarkable reaction is catalyzed by crotonyl-CoA carboxylase/reductase homologues [CCRs, broadly defined as enoyl-CoA carboxylase/reductases (ECRs)].[3] The carboxylating activity of such enzymes was only recently discovered by Fuchs, Alber, and coworkers in *Rhodobacter sphaeroides*, in which the prototypical CCR transforms crotonyl-CoA into (2*S*)-ethylmalonyl-CoA by efficient CO_2 fixation in primary metabolism.[4] Shortly thereafter, Moore and co-workers established this reaction as an important element in polyketide structural diversification, as demonstrated in the biosynthesis of the chloroethylmalonyl-CoA extender unit in **1a**.[5] Since then, many more examples for the allocation of 2-substituted malonyl-CoAs to PKSs by ECR-mediated processes have emerged.[3] In addition, the surprisingly relaxed substrate scope of natural ECRs and the involved downstream PKS domains in such biosynthetic pathways has been utilized to engineer novel natural product structures in vivo.[5b,6] However, a deep understanding of the ECR catalytic mechanism at the structural level has remained elusive.

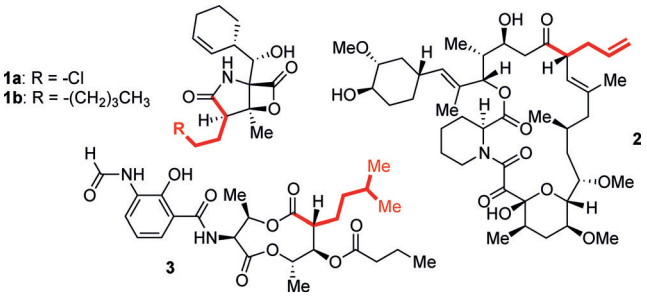

Figure 1. A selection of polyketides with unusual extender units (shown in red): salinosporamide A (**1a**), cinnabaramide A (**1b**), FK-506 (**2**), and antimycin A_{7b} (**3**).

In 2012, Heinz and Müller et al. beautifully illuminated the ECR catalytic process by thoroughly analyzing the first crystal structure of such an enzyme, CinF from **1b** biosynthesis, in complex with its cofactor $NADP^+$ and its substrate octenoyl-CoA.[7] By in silico docking and mutational analysis, they identified E167 and N77 as essential residues for CO_2 binding and activation. Furthermore, an extended hydrophobic pocket capable of accommodating the large octenoyl substrate was identified in CinF. While in reference CCRs thought to catalyze the carboxylation of crotonyl-CoA this pocket is blocked by F370 and I171, CinF harbors the smaller G362 and A163 residues at these positions. Single mutations

[*] M. Sc. J. Kundert, Prof. Dr. T. A. M. Gulder
Biosystems Chemistry, Center for Integrated Protein Science Munich (CIPSM) at the Department of Chemistry
Technische Universität München
Lichtenbergstraße 4, 85748 Garching (Germany)
E-mail: tobias.gulder@ch.tum.de
Homepage: http://www.biosystemchemie.ch.tum.de

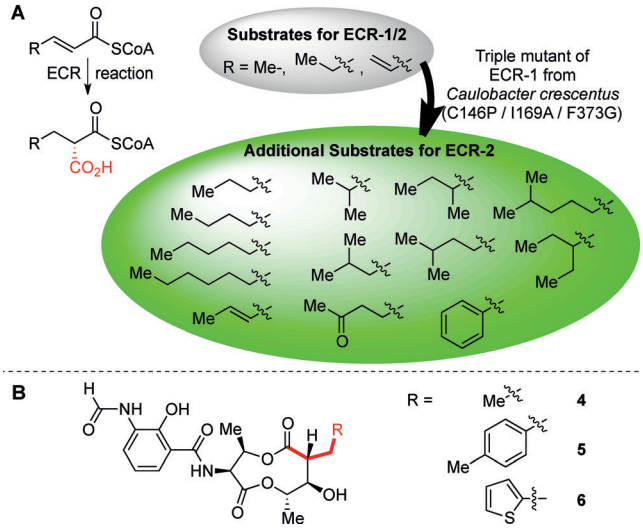

Figure 2. A) Exploring and expanding ECR-1/-2 substrate scope.[8] B) Production of novel antimycins **5** and **6** by ECR engineering.[10]

at either of these sites to larger residues led to complete abolishment of octenoyl-CoA carboxylating activity, while retaining some activity towards crotonyl-CoA. These studies firmly defined for the first time two ECR residues crucial for substrate recognition and binding.[7]

In a comprehensive study aiming at understanding and modulating the substrate scope of ECRs from primary (ECR-1) and secondary (ECR-2) metabolism, Erb et al. recently identified three active-site residues distinguishing these two groups of ECRs:[8] I169, F373, and C146 in ECR-1, which are replaced by A169, G373, and P146 in ECR-2.[9] By screening the activity of four homologues each from the ECR-1 and ECR-2 classes (including SalG/CinF from the biosynthesis of **1a/b**) against a large set of enoyl-CoA thioesters in vitro, the substrate scope of ECR-1 enzymes was demonstrated to be limited to linear C-4 and C-5 enoyl-CoAs. By contrast, members of the ECR-2 group additionally accept a broad variety of long-chain, branched, and further substituted substrates (Figure 2A). The only exception was SalG, the substrate scope of which rather resembled that of the ECR-1 group, as would be expected from the short natural substrates of SalG.

To verify the role of the above-mentioned amino acids in substrate recognition, Erb et al. generated single, double, and triple mutants by introducing the ECR-2 residues into the ECR-1 CcrCc. Indeed, a gradual increase in substrate tolerance was observed from the individual single mutants to the triple mutant. The latter showed a broad substrate scope comparable to that of a genuine member of the ECR-2 class. While its catalytic efficiency for crotonyl-CoA dropped approximately 2000-fold, the turn-over of larger substrates was in the range of the reference ECR-2 EcrSh. When assayed in vivo using a bacterial model system that requires ECR-1-mediated fixation of C_1 substrates for survival, the fitness of the double and triple mutants was significantly reduced. This nicely illustrates the fine tuning of catalytic activities for substrates from primary vs. secondary metabolism and might create the evolutionary pressure that maintains the restricted active site pocket in ECR-1 systems.

In a paper published back-to-back with the above work, Abe and co-workers report the structure-based engineering of the ECR homologue AntE, which is involved in antimycin biosynthesis.[10] Comparison of the AntE crystal structure with that of CinF[7] revealed a smaller A182 residue at the cavity close to the NADPH and CO_2 binding sites (L in CinF). In in vitro assays, the A182G mutant retained α,β-reductase activity while losing its crucial function as a carboxylase. A182L mutation restricted the substrate scope to crotonyl-CoA while significantly increasing carboxylation selectivity. These findings point to the importance of A182 for adjusting reductase vs. carboxylase activity, likely by preventing solvent water from entering the active site and serving as a proton donor in the reduction process.[4b] Abe et al. likewise identified V350 (equivalent to G362 in CinF) as a site for modifying the substrate scope of AntE. Intriguingly, V350G mutation led to an enzyme that even accepted the bulky 3-indolylacryloyl-CoA in vitro.

To test the significance of these findings for polyketide structural diversification, both the A182L and V350G mutants were independently used to replace wild-type AntE in an antimycin production strain. While A182L mutation led to the selective production of ethyl-substituted antimycin **4** (resulting from the incorporation of crotonyl-CoA-derived ethylmalonyl-CoA), V350G mutation shifted the product spectrum towards longer and branched side chains. Most significantly, feeding of *p*-methylcinnamic or 3-(2-thienyl)-acrylic acid to the mutant strain led to the production of mg quantities of novel antimycin derivatives **5** and **6**, respectively. This demonstrates for the first time that engineered ECRs can be used to significantly broaden PKS extender unit utilization, in this case leading to the first heterocyclic and substituted arene antimycins.

The above work provides detailed insights into how minimal ECR active-site manipulations can lead to enormously broadened substrate scope. This methodology has huge potential for the targeted engineering of polyketides. The signature motif of promiscuous ECR-2s identified by Erb[8] will help to identify PKS systems permissive for the incorporation of structurally diverse β-substituted malonates, as shown by Abe for the antimycins.[10] However, many challenges remain to be solved to make this approach a general tool for PKS engineering. These include potential incompatibilities of unusual building blocks with downstream PKS processes or proofreading mechanisms,[8,10] designing ECR selectivity for a desired substrate rather than achieving substrate promiscuity, or the incorporation of ECR biosynthetic logic into classical PKS machineries that do not naturally incorporate β-substituted malonate building blocks. It will thus be interesting to see how this methodology will expand in the future.

Acknowledgements

Research in our laboratory is generously supported by the DFG (Emmy Noether Program, CISPM). J.K. thanks the Jürgen Manchot Foundation for her PhD scholarship.

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 858–860
Angew. Chem. **2016**, *128*, 868–870

[1] C. Hertweck, *Angew. Chem. Int. Ed.* **2009**, *48*, 4688–4716; *Angew. Chem.* **2009**, *121*, 4782–4811.
[2] B. S. Moore, C. Hertweck, *Nat. Prod. Rep.* **2002**, *19*, 70–99.
[3] a) Y. A. Chan, A. M. Podevels, B. M. Kevany, M. G. Thomas, *Nat. Prod. Rep.* **2009**, *26*, 90–114; b) M. C. Wilson, B. S. Moore, *Nat. Prod. Rep.* **2012**, *29*, 72–86.
[4] a) T. J. Erb, I. A. Berg, V. Brecht, M. Müller, G. Fuchs, B. E. Alber, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 10631–10636; b) T. J. Erb, V. Brecht, G. Fuchs, M. Müller, B. E. Alber, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8871–8876.
[5] a) A. S. Eustáquio, R. P. McGlinchey, Y. Liu, C. Hazzard, L. L. Beer, G. Florova, M. M. Alhamadsheh, A. Lechner, A. J. Kale, Y. Kobayashi, K. A. Reynolds, B. S. Moore, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12295–12300; b) Y. Liu, C. Hazzard, A. S. Eustáquio, K. A. Reynolds, B. S. Moore, *J. Am. Chem. Soc.* **2009**, *131*, 10376–10377.
[6] Selected examples: a) A. S. Eustáquio, B. S. Moore, *Angew. Chem. Int. Ed.* **2008**, *47*, 3936–3938; *Angew. Chem.* **2008**, *120*, 4000–4002 (salinosporamides); b) S. Rachid, L. Huo, J. Herrmann, M. Stadler, B. Köpcke, J. Bitzer, R. Müller, *ChemBioChem* **2011**, *12*, 922–931 (cinnabaramides); c) Y. Yan, J. Chen, L. Zhang, Q. Zheng, Y. Han, H. Zhang, D. Zhang, T. Awakawa, I. Abe, W. Liu, *Angew. Chem. Int. Ed.* **2013**, *52*, 12308–12312; *Angew. Chem.* **2013**, *125*, 12534–12538 (antimycin).
[7] N. Quade, L. Huo, S. Rachid, D. W. Heinz, R. Müller, *Nat. Chem. Biol.* **2012**, *8*, 117–124.
[8] D. M. Peter, L. S. von Borzyskowski, P. Kiefer, P. Christen, J. A. Vorholt, T. J. Erb, *Angew. Chem. Int. Ed.* **2015**, *54*, 13457–13461; *Angew. Chem.* **2015**, *127*, 13659–13663.
[9] The numbering herein according to the CcrCc of *Caulobacter crescentus*; I169/F373 correspond to I171/F370 in CinF.
[10] L. Zhang, T. Mori, Q. Zheng, T. Awakawa, Y. Yan, W. Liu, I. Abe, *Angew. Chem. Int. Ed.* **2015**, *54*, 13462–13465; *Angew. Chem.* **2015**, *127*, 13664–13667.

Received: November 9, 2015
Published online: December 11, 2015

