Combinatorial Biosynthesis

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Minimally Invasive Mutagenesis Gives Rise to a Biosynthetic Polyketide Library**

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Natural products are of utmost importance in medicine and serve as valuable compounds for fundamental studies in chemical biology. Polyketides constitute a large and diverse class of natural products with often intriguing structural complexity and important bioactivities. They present significant high challenges for synthesis, and both synthetic and biosynthetic techniques have been applied for their preparation and derivatization.[1]

In their biosynthesis, polyketides are typically formed by means of a cascade of decarboxylative Claisen condensations between malonyl extender units and carboxylic acid thioesters. The biosynthesis of the reduced polyketides of bacterial origin proceeds in a modular fashion; after each extension cycle the intermediate β-keto thioester is processed further in optional steps through various enzymatic reactions. A remarkable variety of structures are generated by an array of reductive enzyme domains, which catalyze the stepwise processing of the β-keto thioester to a secondary alcohol, an olefin, or a fully reduced aliphatic thioester. In this reaction cascade, the growing polyketide chain is passed on from one catalytic enzyme domain to the next in an assembly line-like process.[2]

The biosynthetic logic is reflected by the architecture of the involved polyketide synthases (PKSs), a family of enzymes that are typically several thousand amino acids in size and are structurally poorly characterized.[3,4] A representative example of these complex enzymes is found in the biosynthetic pathway toward the polyether monensin.[5,6] The monensin PKS genes span roughly 80000 base pairs with large open reading frames;[7–9] these conditions test the limits of modern enzyme engineering, especially when a significant number of enzyme variants must be generated.[3] The modular organization of the biosynthesis has sparked interest in engineering PKS to give rise to novel natural product analogues, leading to the field of combinatorial biosynthesis. In most cases, new polyketides are generated through the swapping of PKS fragments, thus transplanting catalytic domains or whole modules between different PKSs.[3,10,11]

However, the success rate in these experiments is often unsatisfying as the resulting hybrid PKSs are in many cases unproductive.[11–13] A major unresolved problem in this field of research is the limited insight into the structure and mode of action of PKS. Hybrid assembly lines can be unproductive because of the substrate selectivity of the catalytic domains positioned downstream of the swapping site or because of the rather drastic structural changes upon swapping. Current experimental setups often do not allow for the systematic exploration of this question, rendering PKS swapping a trial-and-error experiment. This sets significant obstacles to the rational design of modified polyketide biosynthetic pathways, possibly slowing down the whole field.

We herein present a novel approach to combinatorial biosynthesis to deliver a library of complex reduced polyketides. In contrast to previous experiments, only site-directed mutagenesis is applied to alter the innate catalytic potential of a PKS, thereby minimizing the deleterious side effects of the manipulations. The systematic experimentation gives clear insight into the innate substrate promiscuity of bacterial Type 1 PKS, the most frequently used enzymes of this class. We demonstrate that through this approach the engineered biosynthesis of polyketides might well become feasible with a high degree of predictability. The targeted mutagenesis gives rise to previously undescribed polyketide derivatives of high complexity, which are otherwise hard to access.

By using protein sequence alignments, we identified catalytically critical active site residues in every reductive domain[14–17] of the monensin PKS. We developed a mutagenesis scheme intended to result in a loss of activity of the targeted catalytic domain types, such as ketoreductases, dehydratases, and enoylreductases (for design of these null mutants see Figure 1 and the Supporting Information). These experiments were designed to lead to oxidized derivatives of the monensin shunt-product premonensin, which structurally resembles the marine polyketide discodermolide (see Figure 3).[18]

The principle behind the mutagenesis is to promote the transfer of a polyketide to the successive module at a higher

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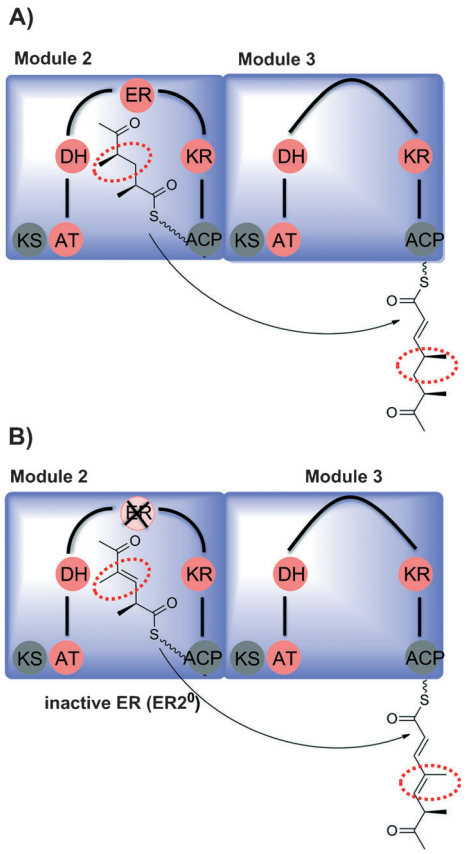

Figure 1. A) Organization of modules 2 and 3 in the monensin PKS (the complete monensin PKS is depicted in the Supporting Information). The nascent polyketide chain is tethered to a flexible ACP, which delivers it presumably in a stochastic fashion to the domains of the reductive loop. Following the reductive steps is a transthioesterification, relocating the polyketide chain to the subsequent module. Abbreviations: KS: ketosynthase, KR: ketoreductase, DH: dehydratase, ER: enoylreductase. B) The principle of the mutagenesis employed in this study is to slow down a reductive step through targeted mutagenesis of the respective domain type. This leads to a PKS variant in which the transthioesterification towards the subsequent module becomes faster than the reduction in the current module, leading to a less reduced nascent polyketide chain being passed on. Effectively, this is a deactivation of the targeted reductive domain.

oxidation state than in the wild-type enzyme through the blocking of the individual reductive domains. This should proceed with minimal concomitant alterations of the overall protein structure, thus allowing for a clear analysis of the substrate-specificity effects of downstream domains.

As initial targets for the mutagenesis experiments, the extension modules 2 to 8 of the monensin PKS were chosen. These modules comprise a total of 18 reductive domains. Mutagenesis of this PKS fragment may result in a maximum of 36 different polyketide compounds, each as a mixture of redox derivatives of premonensins A and B. The targeted modules of the monensin PKS comprise the majority of reductive domains, and mutagenesis was intended to leave the characteristic hydrophilic moiety of the compound intact.

The prerequisite experimental techniques for the targeted mutagenesis were developed in the course of this project (see Figure 2 and the Supporting Information) and comprise a high-fidelity PCR-based mutagenesis approach (PCR = polymerase chain reaction) suitable for the amplification of GC-rich sequences, combined with a sequence- and ligation-independent cloning (SLIC-MIX) of the resulting PKS mutant genes, as recently developed in our laboratory.[19] To minimize secondary effects on polyketide production through the genetic manipulation of *S. cinnamonensis*, the mutant PKS genes were introduced through in-place homologous recombination into the chromosome of the premonensin-producing strain *S. cinnamonensis* A495.[20] All mutations were confirmed by DNA sequencing of the resulting *Streptomyces* variants, except mutations in module 3, which repeatedly did not give the intended clones under any experimental condition tested.

Subsequently, the resulting 16 bacterial variants, corresponding to a theoretical library of 32 compounds, were cultivated in liquid media and their culture extracts were assayed by LC/ESI-MS (Figure 3B). The initial analysis was confirmed by HRMS and revealed the formation of 22 of the predicted premonensin redox derivatives (see Figure 3A,C).

The results indicate that the success rate of the mutagenesis in terms of new products being formed varies between the three different domain types. The acceptance of the nascent polyketide chain by downstream modules was found to depend on the functional group that was introduced through the mutagenesis. This is a trend also reported for the recently described trans-AT PKS family,[22,23] for which in silico analysis suggested that the KS domains discriminate between different redox patterns in the incoming substrate. However, the cis-AT PKS as studied here show much stronger substrate promiscuity.

The domain-skipping mutagenesis was fully reliable for all enoylreductase domains of the monensin cluster, resulting in four different polyketide products with newly formed alkene groups (Figure 3A; ER2^0, ER4^0, ER6^0, and ER8^0). The secondary alcohols resulting from the inactivation of dehydratase domains led to the formation of the predicted compounds in four out of six cases (DH2^0, DH4^0, DH5^0, and DH8^0). However, in the case of the mutagenesis of the ketoreductase domains, only three out of six domains tested led to the formation of the predicted ketones (KR2^0, KR4^0, and KR6^0). Additionally and in contrast to all other modules, none of the mutations introduced into module 7 led to any detectable premonensin analogue.

To characterize the structures of the predicted derivatives with methods besides ESI-MS, we investigated the structures of the polyketides produced by the wild-type and from the inactivated ER2 variant. After extraction and purification of the corresponding fermentation products, the structures of the obtained derivatives were analyzed by 1D and 2D NMR experiments and HRMS. The analysis confirmed the structures of premonensins A and B for the wild type[18] and the corresponding oxidized derivatives, ER2^0-A and ER2^0-B (Figure 3B and the Supporting Information), for the ER2^0 mutant. The structural difference between the wild-type premonensin and the reduced derivative of ER2 is evident from the NMR signals of the vinylic proton. In detail, the ER2^0 derivatives show a signal for an extra vinylic proton (H-5); in addition both the chemical shift and the multiplicity of

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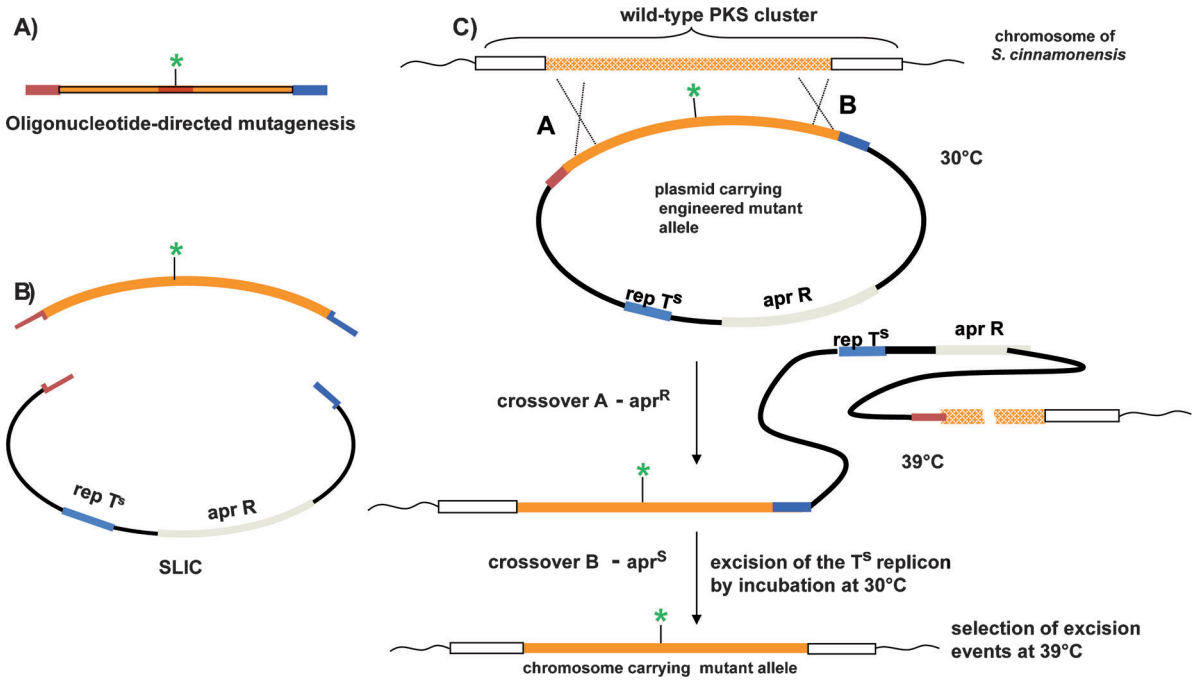

Figure 2. A) Oligonucleotide-directed mutagenesis is afforded by overlap-extension PCR. B) Sequence- and ligation-independent cloning (SLIC) with addition of single-stranded-DNA-binding protein (SSB) is used for the rapid cloning of mutated PKS fragments into a vector. Abbreviations: repT^s: temperature-sensitive origin of replication; apr^R: apramycin antibiotic resistance cassette; apr^S: apramycin-sensitive allele. C) Mutant genes are introduced into *S. cinnamonensis* by conjugation using the shuttle vector pKC1139 with a temperature-sensitive origin of replication (based on pSG5 by Muth et al.).[21] The use of this origin of replication was crucial to facilitate efficient double crossover for the scar-free introduction of the mutant PKS into the chromosome, which with other systems was too inefficient to enable the generation of a larger number of mutants.

the signal for proton H-4 were changed. After preparative HPLC, ER2^0-A and ER2^0-B were isolated in amounts of 0.7 mg L^{-1} and 2.2 mg L^{-1} from their respective fermentations, a reduction in yield by one order of magnitude in comparison to the wild-type preparations of premonensins A and B (8.2 mg L^{-1} and 15.2 mg L^{-1}).

More detailed inspection of the preparative-scale fermentation products of the ER2^0 variant led to unexpected findings which indicate a crosstalk between different segments of the PKS. Besides the expected products, two further premonensin derivatives, designated ER2^0-C and ER20-B*, were isolated and characterized by NMR spectroscopy and HRMS (see the Supporting Information). ER2^0-B* presumably is the consequence of the increased tautomerization tendency of the methyl ketone in ER2^0-B toward the corresponding enol as a result of conjugation with the adjacent C–C double bond, resulting in the epimer by-product.

ER2^0-C is, however, an unexpected desmethyl derivative, which can be isolated in milligram quantities from the *S. cinnamonensis* variant but whose premonensin analogue cannot be obtained from the wild type. The structure of this newly formed product corresponds to the incorporation of malonyl-CoA instead of the either methylmalonyl- or ethylmalonyl-CoA extender units in module 5 (Figure 4). Curiously, this might be the consequence of the increased substrate promiscuity of module 5 when it is presented with a nonnative substrate. Analogous behavior is described for single-step biocatalytic reactions but was unexpected for a canonical type I PKS, as it is not in accord with their typically modular behavior.[10,24] Alternatively, the effect might result from a yet to be identified proofreading mechanism within the monensin biosynthetic machinery, hydrolyzing some of the non-native products.[25–28] Regardless of the exact mechanism, this observation indicates an unusual crosstalk between modules 2 and 5 of the monensin PKS, whether through protein–protein or protein–substrate interactions remains to be elucidated (Figure 4).

Additional unpredicted crosstalk in module 5 was found upon introduction of the DH5^0 mutation. This mutation almost exclusively led to the formation of the methyl-branched premonensin B redox derivative with only negligible co-formation of the ethyl-branched analogue (see Figure 4). Mutagenesis of the adjacent KR5 domain led to an entirely unproductive variant.

Current understanding of PKS enzymology suggests that exclusively acyltransferase domains within the PKS architecture discriminate between different malonyl extender units. Our findings now indicate that, through an unprecedented and presumably subtle mechanism, the reductive loop acts not fully detached from the KS/AT core structure but contributes to the extender-unit selectivity.

In the course of our experiments the substrate specificity of domains located downstream from a mutagenesis site was evaluated in a PKS with 12 extension modules and all polyketide derivatives presented here were processed over between 10 and 33 successive reaction steps. Interestingly, 22 out of 32 theoretically predicted compounds were detected, corresponding to a success rate of 69 % in this experiment.

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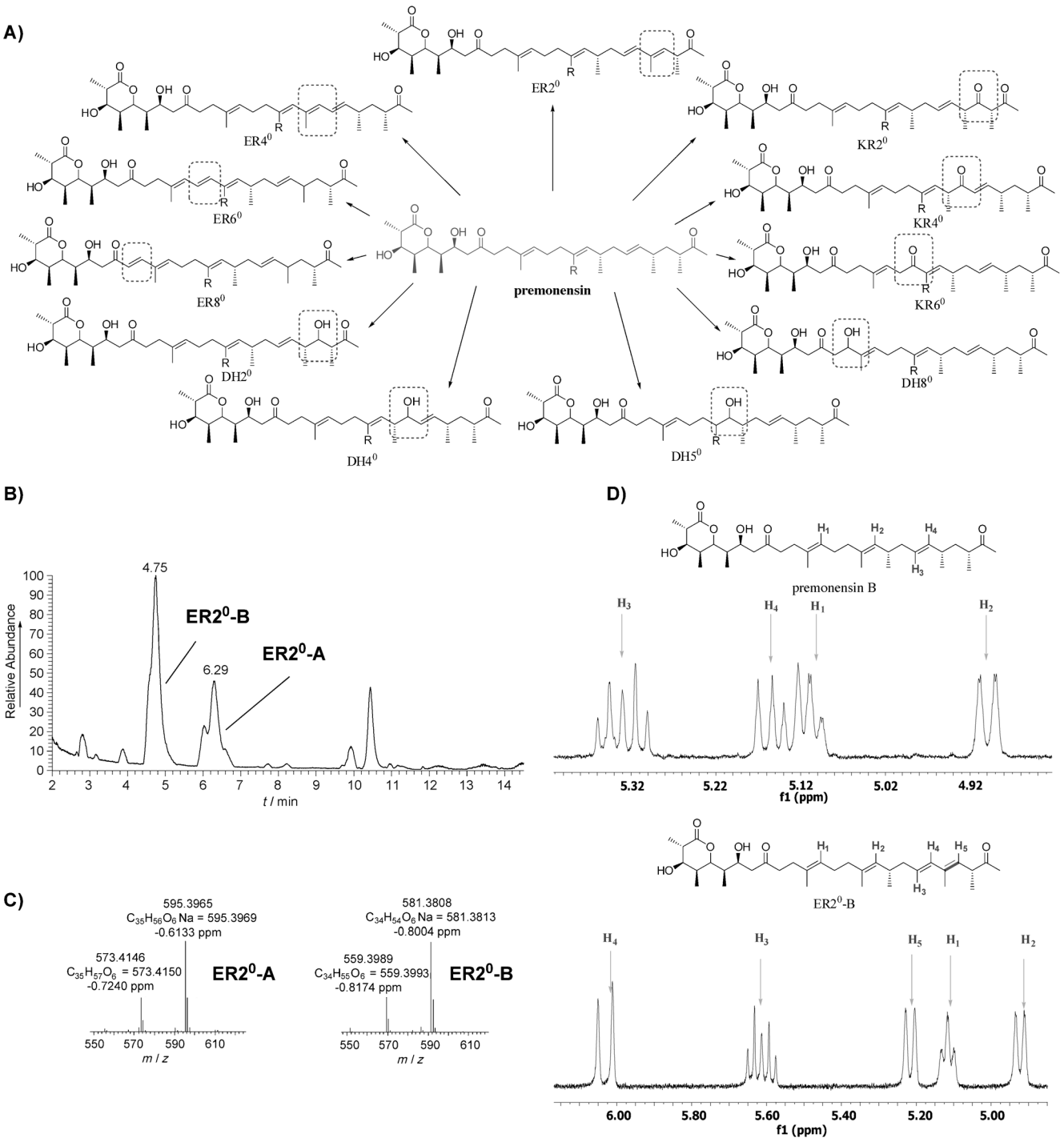

Figure 3. A) Predicted structures of the premonensin redox derivatives obtained in this study. R = CH_3 (premonensin B), R = CH_2CH_3 (premonensin A). The structural change induced through mutagenesis of the reductive domain is framed with dotted lines. The compounds are named after the domain type (KR, DH, ER) and the module number targeted in each case. The sum formula of each compound shown was found by HRMS analysis to match the predictions. B) LC/MS trace (base peak) from the initial screening of monensin PKS variants. This example comes from the $ER2^0$ variant. C) HRMS was later used to confirm the predicted product masses. D) As an example the $ER2^0$ variant was cultivated in preparative amounts and characterized by NMR spectroscopy; the NMR signals of the vinylic protons of premonensin B and its redox derivative $ER2^0$-B are shown. A new vinylic proton (H-5) is evident, and the chemical shift and multiplicity of the adjacent H-4 have changed from dd to d.

This is especially significant, as these experiments do not rely on a simplified model system but on a representative full-length PKS.

It is important to note that our site-directed-mutagenesis approach is solely mechanism-based and does not rely on longer homologous stretches in the protein sequence. That

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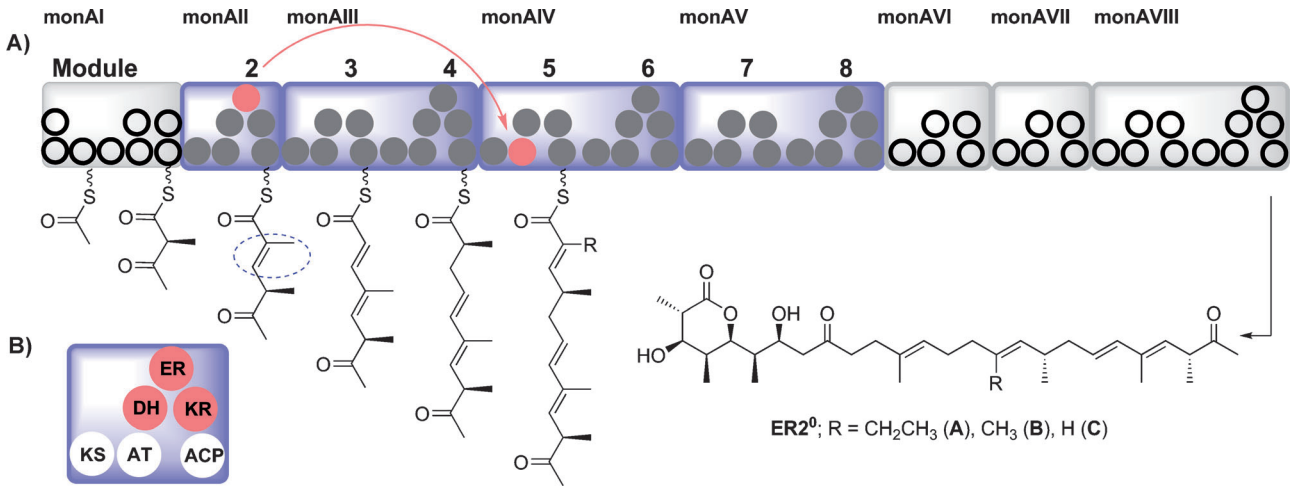

Figure 4. A) Schematic view on the unexpected crosstalk between modules 2 and 5 upon mutagenesis of domain ER2. The monensin PKS modules mutated in this study are highlighted as blue rectangles; ER2 and AT5 are shown in red, the redox derivatization is circled with a dotted blue line. Catalytic steps subsequent to module 5 are omitted for clarity. In the DH5^0 variant another case of crosstalk was observed. The ratio of A to B changed from approximately 2:3 in premonensin fermentations to less than 1:100 in this variant. For all other monensin PKS variants in this study the ratio between the two products was the same as for the wild-type. monAI to monAIII denote the individual enzymes in the monensin PKS, whereas arabic numbers are used to enumerate the individual modules. The labeling of single catalytic domains is omitted for clarity. B) Enlargement of a single module with a complete reductive loop (such as modules 2, 4, 6, and 8). All modules are organized in the same way, yet in several cases with an incomplete set of the reductive domains (KR, DH, ER). All modules contain the essential set of domains required for chain extension (KS, AT, ACP).

makes it intrinsically transferable to all related PKSs and even fatty acid synthases without time-consuming experiments to identify or optimize mutagenesis sites; this is typically the case even in the today's leading domain-swapping experiments employed to generate polyketide libraries.[29–31] However, it becomes apparent that the exclusion of nonnative nascent polyketide substrates by downstream domains is a feature not only of trans-AT-PKS but can also be found to a less critical extent in the more frequently studied cis-AT-PKS. In future experiments, this will have to be taken in consideration as it can significantly reduce the fermentation yield of the desired polyketide derivatives to a currently unpredictable degree. Presently the PKS substrate specificity cannot be addressed by single-residue exchanges alone.

Based on their similarity to known polyketides it can be assumed that the nonnative premonensin and its redox derivatives possess privileged structures[32] and they were tested for antimicrobial activity against a panel of bacterial indicator strains. Premonensins A and B show low bacteriostatic activity against the Gram-negative pathogen *Pseudomonas aeruginosa* DSM1117 (MIC 228 $\mu g\,mL^{-1}$). Interestingly, the purified derivatives ER2^0-A, -B, and -C all show an increase of antibacterial activity by two orders of magnitude against *P. aeruginosa* (MIC 1.8 $\mu g\,mL^{-1}$ for ER2^0-A) and even activity against the Gram-positive *B. subtilis* DSM10 (MIC 7.2 $\mu g\,mL^{-1}$ for ER2^0-A), against which premonensin shows negligible inhibitory activity (see the Supporting Information for full results with further bacterial strains).

In conclusion, we have presented a new strategy to generate polyketide compound libraries. To the best of our knowledge this is the first study using site-directed mutagenesis alone to systematically generate an array of different polyketide structures. In contrast to previous studies, this minimally invasive strategy avoids the swapping of domains or modules of a PKS, thereby minimizing deleterious effects of the manipulation. Through straightforward and intrinsically transferable experimental design the apparent useful substrate flexibility of bacterial cis-AT PKS can be exploited for the engineered biosynthesis of complex compounds, resulting in changes of their bioactivity.

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