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Communication

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Chemoinformatic-guided engineering of polyketide synthases

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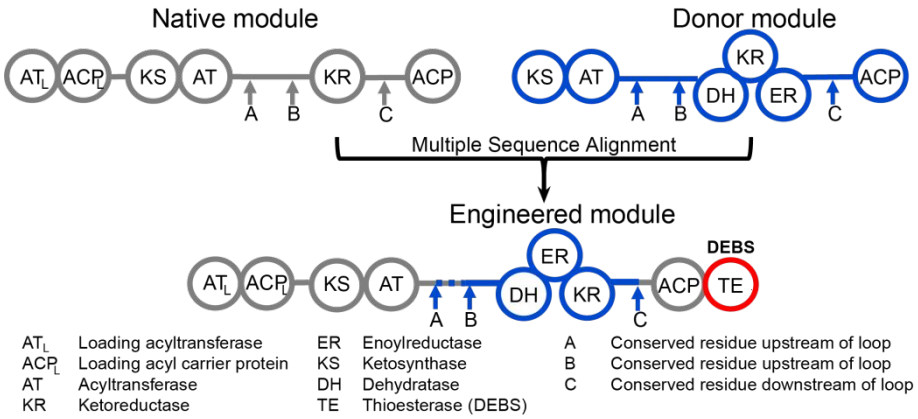
ABSTRACT: Polyketide synthase (PKS) engineering is an attractive method to generate new molecules such as commodity, fine and specialty chemicals. A significant challenge in PKS design is engineering a partially reductive module to produce a saturated β-carbon through a reductive loop exchange. In this work, we sought to establish that chemoinformatics, a field traditionally used in drug discovery, offers a viable strategy for reductive loop exchanges. We first introduced a set of donor reductive loops of diverse genetic origin and chemical substrate structures into the first extension module of the lipomycin PKS (LipPKS1). Product titers of these engineered unimodular PKSs correlated with chemical similarity between the substrate of the donor reductive loops and recipient LipPKS1, reaching a titer of 165 mg/L of short chain fatty acids produced by *Streptomyces albus* J1074 harboring these engineered PKSs. Expanding this method to larger intermediates requiring bimodular communication, we introduced reductive loops of divergent chemosimilarity into LipPKS2 and determined triketide lactone production. Collectively, we observed a statistically significant correlation between atom pair chemosimilarity and production, establishing a new chemoinformatic method that may aid in the engineering of PKSs to produce desired, unnatural products.

Rational reprogramming of PKS enzymes for the biosynthesis of new polyketides has been a major research thrust over the past three decades.[1–3] PKSs load a malonyl-CoA analog onto the acyl carrier protein (ACP) using the acyltansferase (AT) domain and extend the growing chain from the ketosynthase (KS) domain through a decarboxylative Claisen condensation reaction. After chain extension, the β-carbonyl reduction state is determined by the module's reductive domains, namely the ketoreductase (KR), dehydratase (DH), and enoylreductase (ER), which generate the β-hydroxyl, α-β alkene, or saturated β-carbons respectively, when progressively combined. Unlike fatty acid synthases, which faithfully produce saturated fatty acids, PKSs have this variability in β-carbonyl reduction. Consequently, multiple studies have reported PKS module engineering for various β-carbon oxidation states.[4–8] However, design strategies for introduction of reductive loop exchanges (*i.e.* KR-DH-ER domains) into partially reductive modules remain elusive. In this work, we compare bioinformatic and chemoinformatic approaches to guide reductive loop (RL) exchanges and develop a new method for RL exchanges based on the chemical similarity of the RL substrate. Chemoinformatics, an interdisciplinary field blending computational chemistry, molecular modeling and statistics to analyze structure-activity relationships, was first established for drug discovery.[9] Recently, we suggested that a chemoinformatic approach to PKS engineering could be valuable, particularly in RL exchanges where the KR and DH domains are substrate-dependent[1]: acyl chain length has critically affected dehydration in stand-alone DH[10] and full PKS module studies.[7,13]

Chemoinformatic methods such as atom pair (AP) similarity, which characterizes atom pairs (*e.g.* length of bond path, number of π electrons), and maximum common substructure



Scheme 1. Experimental design of RL swaps. Conserved residues are identified through multiple sequence alignment surrounding the reductive domains ("A", "B" and "C"). Donor RLs are inserted into the native lipomycin module 1, and the attached DEBS thioesterase hydrolyzes the product.

(MCS) similarity, which identifies the largest common substructure between two molecules,[14] could beneficially describe substrate profiles. While divergent in chemical characterization, both similarity methods translate to a Tanimoto coefficient with a range of 0 (least similar) to 1 (most similar).[14] We hypothesized that chemosimilarity between the substrates of donor and acceptor modules in RL exchanges may correlate with production levels, thereby leading to engineered modules that better control the reductive state of the β carbon.

Bioinformatic studies of PKS evolution have guided engineering efforts in closely related biosynthetic gene clusters (BGCs).[15,16] We therefore undertook a phylogenetic analysis of the reductive domain common to all RLs, the ketoreductase (KR). The KR not only reduces the β-keto group to a β-hydroxyl, but also sets the stereochemistry of the β-group and, if a branched extender is used, sets the α-carbon stereochemistry resulting in subtypes A1, A2, B1, B2 (**FigureS1A**). We generated a phylogenetic tree from all manually curated ketoreductases and ketosynthases in ClusterCAD, an online database and toolkit for Type I PKSs, totaling 72 biosynthetic gene clusters (BGCs) and 1077 modules.[17] As in previous investigations,[18,19] the KR domains clustered by subtype (**FigureS1B-S2**). In contrast, the RL type (e.g. KR, KR-DH, KR-DH-ER) did not phylogenetically cluster with its upstream or downstream KS domain (**FigureS3-S4**).[18] This suggests a link between KR evolution and product specificity, analogous to the evolution of KS domains of cis-AT[18] and trans-AT PKS modules[20,21] towards substrate specificity. As KRs from KR-DH-ER modules evolved distinctly from KR-only modules, we hypothesized that neither KR sequence identity nor phylogenetic distance, a pairwise comparison of phylogenetic tree members, between the donor loops and acceptor module were likely to correlate with RL exchange production levels.

To evaluate the importance of chemical similarity and phylogenetic distance in RL exchanges, we swapped diverse, full RLs into the first module of the lipomycin PKS (LipPKS1) using conserved residues as exchange sites (**Scheme1**).[7] In our previous work, we introduced a heterologous thioesterase from 6-deoxyerythronolide B synthase (DEBS) into the C-terminus of LipPKS1; the resulting truncated PKS produced a β-hydroxy acid.[22] In this work, we selected N-terminal junctions ("A" and "B") located immediately after the post-AT linker, which is important for KS-AT domain architecture,[23] and the C-terminal junction ("C") directly before the ACP domain (see **TableS1** for sequences) based on previous work with the first module of borreledin.[7]

We identified four donor RLs (IdmO, indanomycin, *S. antibioticus*; SpnB, spinosyn, *S. spinosa*; AurB, aureothin, *S. aureofaciens*; NanA2, nanchangamycin, *S. nanchangensis*; final products in **FigureS5**) to swap into LipPKS1. A pairwise comparison of phylogenetic distance and amino acid sequence identity determined that IdmO, AurB, and SpnB have the highest KR similarities to LipPKS1 (**Figure1A**). A similar trend holds in the analysis of these donor modules upstream and downstream KS domains (**FigureS6**). In contrast, the NanA2 substrate has the highest chemical similarity based on AP and MCS similarity to LipPKS1, followed by SpnB (**Figure1B**). With the introduction of RL swaps, the chimeric enzymes should produce 2,4-dimethyl pentanoic acid. As *in vitro* PKS studies have shown divergence from *in vivo* results[24,25] due to underestimation of factors including limiting substrate, crowding, and solubility,[26] we cloned eight chimeric modules and a control expressing red fluorescent protein (RFP), into an *E. coli* -*Streptomyces albus* shuttle vector and conjugated into *S. albus* J1074 (**TableS1**).[27] Following ten-day production runs in a rich medium in biological triplicate, cultures of *S. albus* harboring each of the constructs were harvested and analyzed for product (**Supplemental Methods**).

Consistent with our hypothesis, we found a perfect correlation between titers of the desired product and the AP/MCS chemosimilarities between donor and LipPKS1 module substrates (R_s=1.00 and p=0.00) (**Figure1C**). On the other hand, no significant correlation between product titer and phylogenetic distance or sequence similarity of the KR domain (R_s=0.04, p=0.60) was found. The lack of phylogenetic correlation was not surprising based on our bioinformatics analysis since the lipomycin KR is an A2-type, evolving separately from KRs with full RLs. This trend held in both junctions, though junction B chimeras generally resulted in higher product titers, consistent with a previous

study of RL exchanges as the extra residues in junction A are distal to the ACP docking interface and active site.[7] Substituting the donor loop most chemically similar to LipPKS1, NanA2, resulted in the highest titers of desired product, 2,4-dimethyl pentanoic acid, reaching 165 mg/L (**Supplemental Methods**). Low titers of the intermediate 2,4-dimethyl-3-hydroxypentanoic acid were produced,

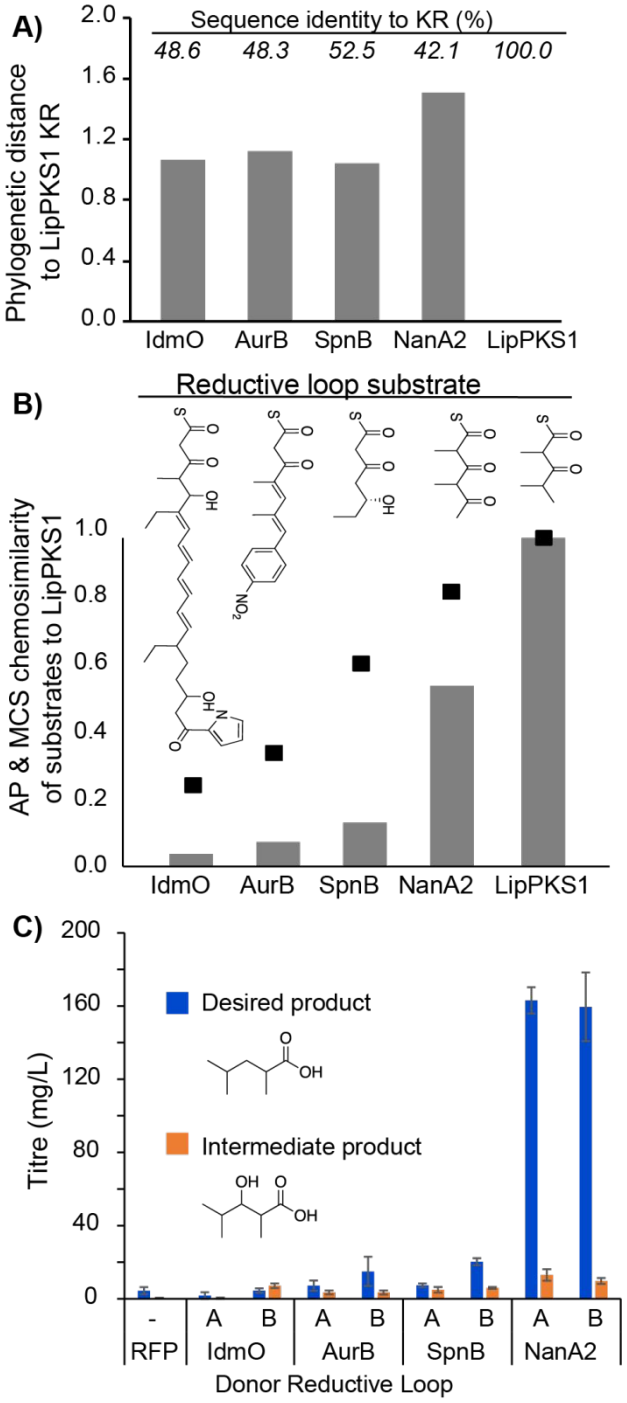


Figure 1. Phylogenetic and chemical similarity effects on reductive loop exchanges. **A)** Phylogenetic distance of the native LipPKS1 KR domain to each donor KR. The value above each bar denotes KR sequence identity comparison. **B)** AP (bar) and MCS (dots) chemical similarity between the native LipPKS1 KR domain and each donor KR. Chemical structures display native KR substrate in each module **C)** Polyketide production of engineered PKSs at both junction "A" and junction "B" in biological triplicate (error bars denote standard deviation).

which we hypothesize is due to a comparatively lower rate of turnover at the energetically intensive DH domain,[28] allowing for premature cleavage of the stalled product by non-enzymatic or TE-mediated hydrolysis. Like our previous study of

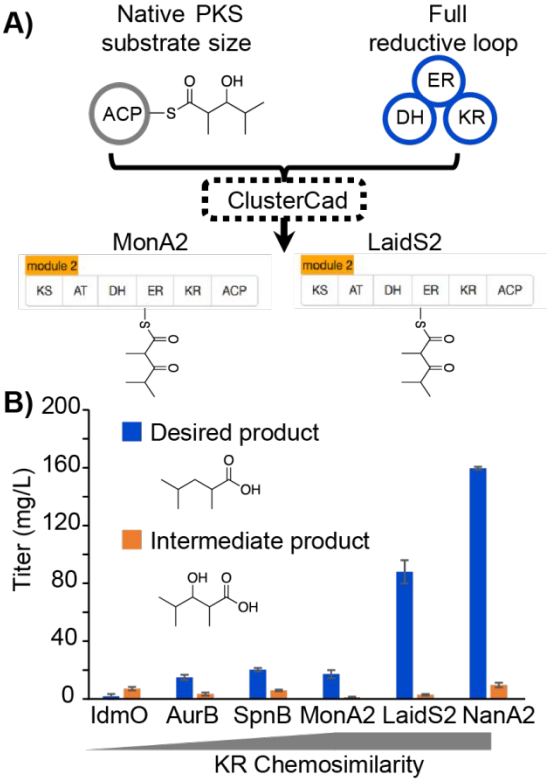


Figure 2. A chemoinformatic approach to reductive loop exchanges. **A)** ClusterCad search revealed the closest substrates to LipPKS1 containing full RLs **B)** Production levels of junction "B" RL exchanges ordered from highest KR substrate similarity with LipPKS1 (MonA2, LaidS2 and NanA2) to progressively less similarity (IdmO, AurB, SpnB) in biological triplicate (error bars denote standard deviation).

in vitro production of adipic acid, we did not detect alkene or keto acid stalled products [7]; non-functional KRs produce short chain β-keto acids that spontaneously decarboxylate to form ketones, which was also not observed, and ERs rapidly reduce *trans* double bonds.[28]

Based on these results, we took a chemoinformatic approach to further test our hypothesis that chemosimilarity of RL substrates is critical to PKS engineering. Using the ClusterCAD[17] database, we identified donor RLs from laidlomycin and monensin that use a KR substrate (identical to the NanA2 KR substrate) with the highest chemically similarity to LipPKS1 (**Figure2A**). As junction B resulted in superior levels of production, the RLs of LaidS2 and MonA2 were cloned into junction B of lipomycin. Like NanA2, LaidS2 loops produced high titers of desired product, while MonA2 performed similarly to SpnB and AurB (**Figure2B**). As protein levels may influence product titers, we determined the quantitative levels of all LipPKS1 constructs using targeted proteomics at the conclusion of the production run and observed no correlation between PKS protein levels and product titers (R_s = -0.15 and p=0.77) (**FigureS7**). Reduced

protein levels in the MonA2 swap could partially explain the lower levels of production in the MonA2 swap compared to LaidS2 and NanA2. However, targeted proteomics of three peptide peaks across the PKS does not eliminate the possibility of proteolytic degradation or

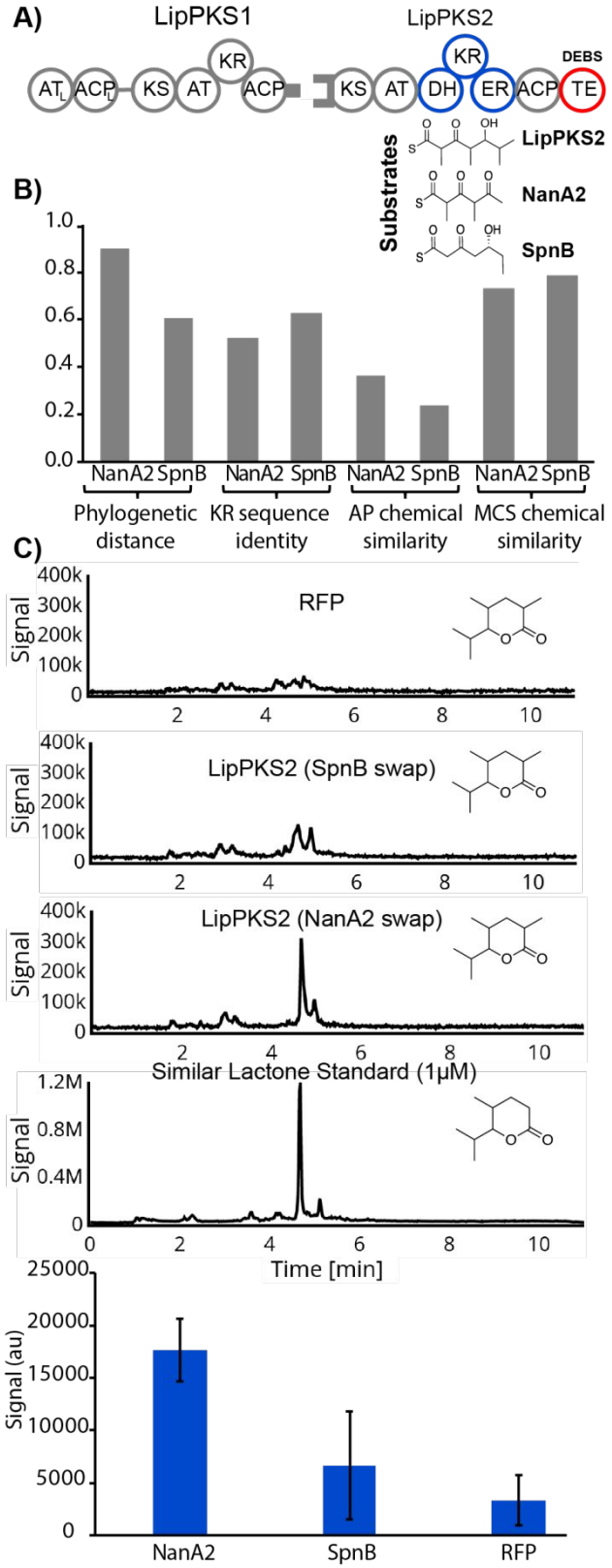


Figure 3. Bimodular reductive loop exchange **A)** Schematic of reductive loop exchanges in LipPKS2 with substrates **B)** Phylogenetic distance, KR sequence identity, AP and MCS similarity between reductive loop donors and LipPKS2 **C)** Chromatograms of RFP, LipPKS2 with donor loops SpnB and NanA2, and a structurally similar standard spiked into RFP cultures **D)** Production levels of desired lactone in biological triplicate (error bars denote standard deviation).

variability in protein quality. AP Tanimoto and MCS chemosimilarity had equivalent Spearman rank correlation to product titers (R_s of 0.82, p = 0.045).

To better demonstrate the utility of this approach, we further evaluated RL exchanges where AP and MCS chemosimilarity diverge and tested this method in modules located at the center of assembly lines, thus requiring docking domain interactions and larger substrates. We therefore performed RL swaps on the second module of lipomycin, LipPKS2 (**Figure3A**), to generate triketide lactones. Donor loops from SpnB and NanA2 were selected, as NanA2 has higher AP chemosimilarity while SpnB has higher MCS chemosimilarity (**Figure3B**). As in our single-module swaps, KR phylogenetic similarity and sequence identity did not correlate with product titers. We found higher correlation with AP chemosimilarity due to higher product levels with NanA2 (**Figure3C-D**). Proteomics on each PKS of these bimodular systems was not performed to rule out the effect of variable protein levels. AP chemosimilarity more heavily weights substructures, so NanA2 and LipPKS2 have higher similarity levels because both select methylmalonyl-CoA in the first two modules. In contrast, MCS chemosimilarity simply considers the largest common substructure, which ignores the influence of commonality at the growing chain by methyl groups. While extension of this phenomenon to account for variances in chemical similarity metrics (*e.g.* AP, MCS) requires further study, we hypothesize that chemosimilarity metrics that best match PKS enzymatic processing may prove most successful. Overall, in our reductive loop exchanges in both LipPKS1 and LipPKS2 we determined a Spearman correlation between AP Tanimoto chemosimilarity and product titer to have an R_s of 0.88 and a p-value of 0.004 (**Supplemental Methods**).

Based on previous literature regarding the importance of substrate size in reductive domains, in this study we hypothesized that the field of chemoinformatics, traditionally used in drug discovery, could be applied to PKS engineering. Using different RLs of varying phylogenetic and chemical similarity, we determined that chemosimilarity between donor KRs and recipient KRs correlated with production, in contrast to phylogenetic distance and sequence similarity. Extending our method into multi-modular systems that use larger substrates and communication domains, we performed RL swaps in LipPKS2 and found that AP chemosimilarity correlates with production. While our approach did not find a correlation between genetic similarity and production in these diverse RL swaps, it has been shown that within highly similar BGCs, the downstream KS groups with the upstream RL type (e.g. KR, KR-DH, KR-DH-ER)[18]. In this study, the donor modules do not share close homology with the lipomycin recipient module, but donor loops with high chemosimilarity located within a BGC may prove more compatible than chemosimilarity alone. Overall, our results determined statistical significance in the correlation between production and the chemosimilarity of the substrate between the donor and recipient modules. More generally, chemoinformatics may provide guideposts for other engineering goals (*e.g.* KR

domain subtype swaps to switch stereochemistry). With our incomplete understanding of PKS processing, design principles may accelerate the combinatorial approach currently used for *de novo* biosynthesis and help provide a framework to more rapidly produce valuable biochemicals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supplementary tables and figures, plasmids, strains, and detailed experimental procedures

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ABBREVIATIONS

PKS polyketide synthase; KS ketosynthase; AT acyltransferase; ACP acyl carrier protein; KR ketoreductase; DH dehydratase; ER enoylreductase; RL reductive loop; AP atom pair; MCS maxium common substructure; BGC biosynthetic gene cluster

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