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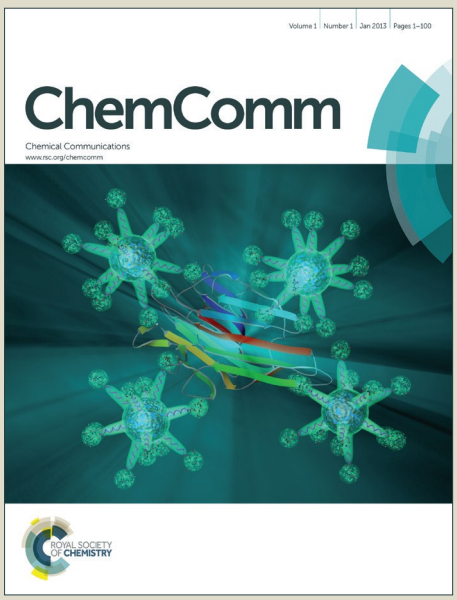

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Substrate Structure-Activity Relationships Guide Rational Engineering of Modular Polyketide Synthase Ketoreductases

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Modular polyketide synthase ketoreductases can set two chiral centers through a single reduction. To probe the basis of stereocontrol, a structure-activity relationship study was performed with three α-methyl, β-ketothioester substrates and four ketoreductases. Since interactions with the β-ketoacyl moiety were found to be most critical, residues implicated in contacting this moiety were mutated. Two mutations were sufficient to completely reverse the stereoselectivity of the model ketoreductase EryKR1, converting it from an enzyme that generates (2*S*,3*R*)-products into one that yields (2*S*,3*S*)-products.

Complex polyketides comprise a broad class of natural products whose stereochemical density have rendered them attractive targets for the development and application of asymmetric synthetic methodologies.[1] The complexity of these polyketides is generated from simple precursors through the activities of megasynthases known as modular polyketide synthases (PKSs). These assembly line enzymes catalyze polyketide extension primarily with malonyl and methylmalonyl building blocks via cycles of carbon-carbon bond formation and processing (e.g., reduction and dehydration).[2] Of the processing enzymes, ketoreductases (KRs) set most of the chiral centers within polyketides. In a process equivalent to dynamic kinetic resolution, KRs can stereospecifically set α-substituent orientations while stereoselectively reducing ß-ketoacyl intermediates. KRs that yield products with D-ß-hydroxyls are referred to as "A-type", while KRs that yield products with L-ß-hydroxyls are referred to as "B-type"; KRs that reduce D-α-substituted intermediates are denoted with a "1", whereas KRs that reduce L-α-substituted intermediates are denoted with a "2" (Scheme 1).[3,4]

KRs naturally reduce ß-keto intermediates linked to an 18-Å phosphopantheinyl prosthetic group (the "phosphopantetheinyl arm") of an acyl carrier protein (ACP) domain. The isolated enzymatic domains frequently retain their stereochemical fidelity *in vitro* when reducing diketides linked to a truncated mimic of the phosphopantetheinyl arm, the *N*-acetylcysteamine (NAC) handle (e.g. **1**; Scheme 1).[5] The relationship between the handle region of the substrate and the stereochemical outcome of KR reductions is largely unexplored, as the majority of investigations of stereocontrol have been performed with NAC-linked substrates.[5-9] To our knowledge, only one study has been reported in which stereocontrol towards a pantetheine-linked substrate was investigated, and in this study 2-methyl-3-oxopentanoyl-*S*-pantetheine and 2-methyl-3-oxopentanoyl-*S*-NAC (**1**) were reduced by the 1st KR of the erythromycin PKS (EryKR1) with equivalent kinetic parameters and stereochemical product distributions.[7] In contrast, studies in which the 1st KR from the tylosin PKS (TylKR1) was incubated with 2-methyl-3-oxopentanoyl diketides linked to handles through an oxo-ester showed that reversals in stereocontrol can result from unnatural handles.[10] To further dissect the interactions that mediate KR stereocontrol, we varied the chemistry and size of the handles of 2-methyl-3-oxopentanoyl substrates and evaluated the effects in structure-activity relationship (SAR) fashion.

Four enzymes - EryKR1, TylKR1, the 2nd KR from the amphotericin PKS (AmpKR2), and the 7th KR from the rifamycin PKS (RifKR7) - were selected as representative B2-, B1-, A1-, and A2-type KRs, respectively, since they retain their stereochemical fidelity with **1** and are active in the presence of DMSO[5,12] (20% v/v DMSO is necessary to observe activity with the hydrophobic substrate **3**). Reactions combining a KR, **1-3**, and an NADPH regeneration system[5] (Scheme 1) were run overnight and analyzed by chiral chromatography. To establish the elution order of the four stereoisomeric products that resulted from both the reduction of **2** and **3**, synthetic standards were generated using stereospecific aldol chemistry[5,12] (Scheme S1). The standards confirmed that each

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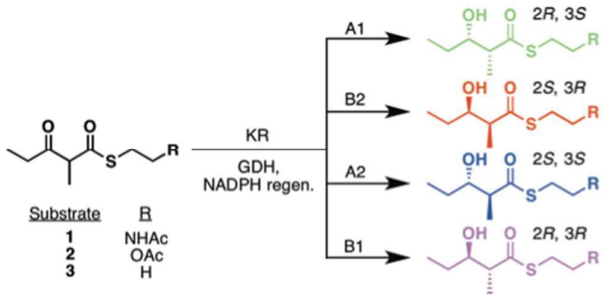
KR produced the predicted stereoisomer as the major product (Figure S1).



Scheme 1. KR-mediated reduction of 2-methyl-3-oxopentanoyl substrates with linkages to NAC (**1**), thioethylacetate (**2**), and ethanethiol (**3**) handles. GDH, glucose dehydrogenase.

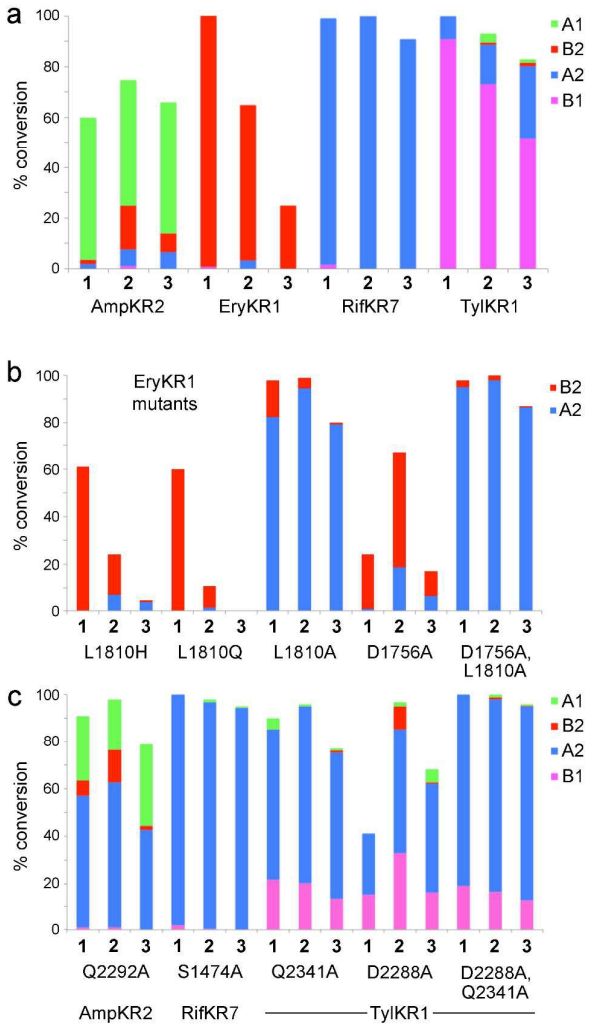


Figure 1. Percent conversion and stereoisomeric product ratios of (a) unmutated KRs, (b) EryKR1 point mutants and (c) AmpKR2, RifKR7, and TylKR1 point mutants. See Figures S1 and S2 for chromatograms. Colors from Scheme 1.

The amide NH in the NAC handle was probed first. Hypothesizing that it forms hydrogen bonds with KRs to help orient the 2-methyl-3-oxopentanoyl moiety within KR active sites, it was substituted with an oxygen to yield **2**. Although this replacement is conservative, the ester functionality is distinct from the amide in that it lacks a hydrogen bond donor, is less polar, and is more flexible. Surprisingly, the effects of this substitution on stereocontrol were relatively subtle. AmpKR2 was most affected, with 31% of its products being other than the anticipated A1 product, compared to 6% from the reduction of **1** (Figure 1a). TylKR1 and EryKR1 largely retained their stereocontrol, but generated more A2 product than when reducing **1**. RifKR7 only generated its expected A2 product (Figure 1a). Hypothesizing that the amide carbonyl forms more significant interactions than the amide NH, we generated and assayed substrate **3**, which only possesses an ethyl group as a handle. Incredibly, the results were similar to those with **2** (Figure 1a).

The retention of stereocontrol when KRs reduce **3** indicates that stereoselectivity is primarily mediated by interactions between KRs and the diketide moiety. Although this result was unexpected, it is in agreement with a hypothesis presented by Leadlay and coworkers that proposes KR stereocontrol is predominantly enforced by subtle stereoelectronic effects mediated by active site residues.[8,9] To test this, the well-studied EryKR1 was selected as a model KR, and the leucine (L1810) three residues before its catalytic tyrosine, was chosen for mutation[4,13] (Figure 2). The identity of the residue in this position often aids in predicting product stereochemistries: in B2-type KRs it is a usually leucine, in A2-type KRs it is usually a histidine, and in B1- and A1-type KRs it is a glutamine[4] (Figure S5). This residue has also been experimentally implicated in mediating stereocontrol - the analogous residue in AmpKR2, Q364, was one of two that, when mutated, completely reversed KR stereospecificity.[14] Accordingly, the L1810H, L1810Q, and L1810A point mutants of EryKR1 were generated and assayed.

The L1810H and L1810Q mutants showed both decreased activity and stereocontrol compared to unmutated EryKR1 when incubated with each of **1**-**3** (Figure 1b). With **1**, both mutants generated the natural B2 product, whereas with **2**, stereocontrol was significantly eroded (28% and 12% A2 product was generated by L1810H and L1810Q, respectively). With **3,** little to no activity was observed. In contrast, the L1810A mutant was both highly active and stereoselective towards each of **1**-**3**. By percent conversion, the L1810A mutant was more active than unmutated EryKR1. Remarkably, its stereoselectivity is opposite that of unmutated EryKR1. Also, diastereomeric excess [*de* = (A2-B2)/(A2+B2); only trace quantities of the A1 and B1 products were observed] was found to be greater for substrates containing worse mimics of the phosphopantetheinyl arm (for **1**, **2**, and **3**, the *de* values were 69%, 91%, and 98%, respectively) (Figure 1b, Table S4).

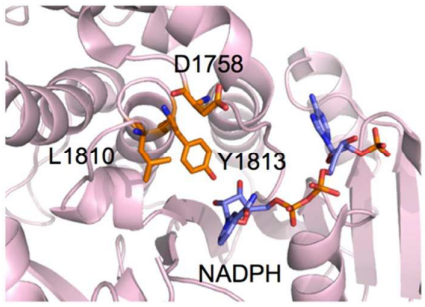


Figure 2. The EryKR1 active site reveals the proximity of D1758 and L1810 to the catalytic tyrosine, Y1813, and NADPH (PDB code: 2FR1).

KR stereoselectivity arises from differences in the orientation of the polyketide substrate (all KR-types bind NADPH in the same orientation).[3,4,13-19] The leucine-aspartate-aspartate (LDD) motif of B-type KRs has been hypothesized to interact with the phosphopantetheinyl arm. This motif is near the active site and could form hydrogen bonds with the aforementioned amide to appropriately position the β-keto group for reduction. In the crystal structure of the related oxidoreductase PhaB bound to acetoacetyl-CoA, a hydrogen bond is formed between an aspartate equivalent to the second D of the LDD motif and the amide NH (PDB code: 4N5M; Figure S4).[20] This interaction could explain the differences in stereocontrol observed for the L1810A mutant towards **1**-**3**: Hydrogen bonding between the amide NH and the aspartate would account for the minor production of the B2 product from **1**; that an ester cannot form a hydrogen bond with the aspartate would explain why less B2 product is generated from **2**; the lack of a functional group on the handle to interact with the aspartate also would explain why virtually no B2 product was generated from **3**.

To test this hypothesis, we mutated the second D of the LDD motif (D1758), generating both a D1758A point mutant and a D1758A/L1810A double mutant. The B2 stereoisomer remained the major product in assays of the D1758A mutant with each of **1**-**3** (Figure 1b), indicating that L1810 has a greater role enforcing stereocontrol than D1758. This is consistent with the retention of stereocontrol demonstrated by unmutated EryKR1 towards **2** and **3,** which are unable to form a hydrogen bond with D1758 through their handles (Figure 1b). Gratifyingly, the D1758A and L1810A mutations were synergistic, with the double mutant generating a larger *de* than the L1810A mutant when incubated with **1** (95% vs. 69%; Figure 1b and Table S4). Both the L1810A mutant and the D1758A/L1810A double mutant are more active than unmutated EryKR1 (Figure 1b).

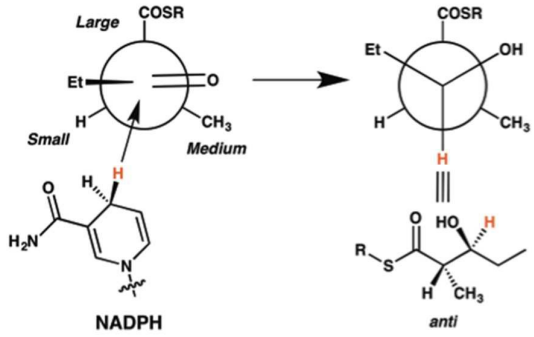
Prior mutational studies performed with AmpKR2, EryKR1, and EryKR2 yielded mutants that primarily generate A2 products.[8,9,14,15] The EryKR1 mutants presented here with enlarged active sites (previously reported EryKR1 mutants contain the natural L1810[8,9]) also generate a greater proportion of A2 product. These findings led us to speculate that A2 products result from the most intrinsically energetically-favored pathway available for the reduction reaction. This is reaffirmed by the observation that the A2-type RifKR7, in contrast to the other KRs examined here, does not lose stereocontrol when incubated with the less natural substrates **2** and **3**. To further probe this hypothesis, we sought to determine how general this phenomenon was by generating and assaying analogous alanine point mutations in AmpKR2, RifKR7, and TylKR1.

Consistent with our hypothesis, the Q2292A mutant of A1-type AmpKR2 primarily produced the A2 stereoisomer when reducing **1**-**3** (the A1 stereoisomer is second-most abundant product at 30%, 22%, and 40% from **1**-**3**) (Figure 1c). As expected, the stereocontrol demonstrated by the S1474A mutant of the A2-type RifKR7 (A2-type KRs from modules also harboring a dehydratase often possess a residue other than histidine at this position[4]) is essentially unchanged from that of the unmutated enzyme (Figure 1c). Q2341A, D2288A, and D2288A/Q2341A mutants were generated for the B1-type TylKR1, analogous to the three mutants generated for the B2-type EryKR1 (Figure 1c). As with the L1810A mutant of EryKR1, the Q2341A mutant of TylKR1 produces more A2 product with substrates that are worse mimics of the phosphopantetheinyl arm (42%, 56%, and 63% *ee* for **1**-**3**) (Figure 1c). The D2288A mutant also generates more A2 product, but to a lesser degree than the Q2341A mutant (Figure 1c). As with the D1758A/L1810A double mutant of EryKR1 that shows synergism between the individual mutations, the D2288A/Q2341A double mutant of TylKR1 produces more A2 product with **1**-**3** than either of the TylKR1 point mutants. However, this reversal of stereoselectivity in the TylKR1 mutants is not as complete (66, 63, and 71% *ee* for **1**-**3**) as the analogous reversal demonstrated by the EryKR1 double mutant (Figure 1c). As with the EryKR1 mutants, each of the TylKR1 mutants is more active than the unmutated enzyme. Thus the stereocontrol and activity demonstrated by each of the TylKR1 mutants are also consistent with the hypothesis that the A2 stereoisomer is produced through a default, low-energy pathway.

The observed, intrinsic *anti* diasteroselectivity can be rationalized through the Felkin-Ahn model.[21] Under Felkin-Ahn selectivity, the nucleophile (the NADPH hydride) will approach the electrophile (the β-keto carbon) in the least sterically-hindered fashion when it is in the least torsionally-strained conformation (Scheme 2). In the absence of favorable interactions between the substrate and KR residues that naturally guide substrates for B-type reduction (such as the residue three before the catalytic tyrosine and the conserved aspartate), the A2 *anti* product is favoured over the B1 *anti* product. The importance of the conserved aspartate for B-type reduction is apparent from the KRs of *trans*-acyltransferase PKSs: all B-type KRs possess this aspartate, whereas no conserved residue exists for A-type KRs.[19] Notably, *anti* selective KRs (usually B1-type, but sometimes A2-type) are present in PKS modules harboring an active dehydratase.[4,16-19,22] It may make evolutionary sense in modules wherein stereochemical information is lost (dehydration of the hydroxyl group yields an olefin) for KR-

mediated reductions to proceed through intrinsically energetically-favorable pathways. The TylKR1 mutants support this hypothesis: that the reversal of stereocontrol they displayed was not as large as for the EryKR1 mutants may be due to the formation of the natural *anti* product of the B1-type TylKR1 being more intrinsically favorable than the natural *syn* product of the B2-type EryKR1.

As such, the next challenge will be to convert a KR that generates *anti* products into one that generates *syn* products. This should be achievable, as kinetic data with **1** indicate that the energetic differences that lead to different stereochemical outcomes in KRs are small.[9] Indeed, with unnatural substrates such as **1**-**3**, KRs are not acting as evolutionarily optimized catalysts but as loosely bound chiral catalysts aiding asymmetric induction, where 95% *ee* can result from an energetic difference of ~2 kcal/mol.[23]



Scheme 2. Felkin-Ahn analysis the reduction of a diketide by NADPH, resulting in *anti* selectivity.

In summary, we employed a substrate SAR approach to elucidate interactions utilized by PKS KRs to set chiral centers. The realization that contacts between KRs and the β-ketoacyl moiety are the most critical in mediating stereocontrol enabled us to completely reverse the stereoselectivity of EryKR1 through two judicious point mutations. This result demonstrates the utility of applying simple physical organic models to both rationalize and engineer enzymatic stereocontrol.

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