Supporting Information

Elucidation of the Cryptic Epimerase Activity of Redox-Inactive Ketoreductase Domains from Modular Polyketide Synthases by Tandem Equilibrium Isotope Exchange

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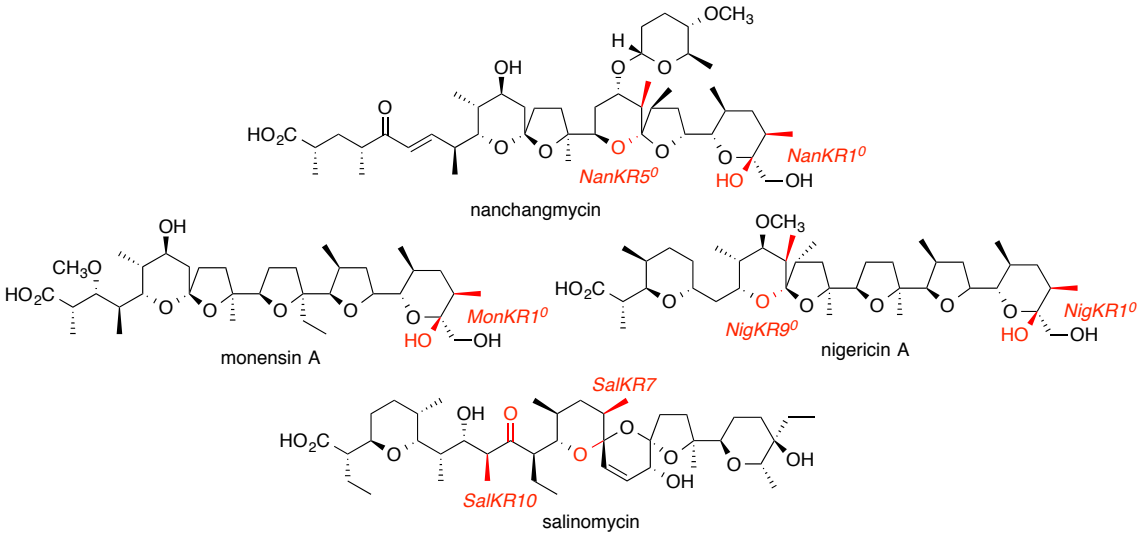

Figure S1. Representative polylketides produced by synthases harboring redox-inactive KR^0 domains. nanchangmycin synthase (Sun et al)[1]; monensin synthase (Oliynyk et al)[3]; nigericin synthase (Harvey et al)[3]; salinomycin synthase (Jiang et al, Yurkovich et al)[4,5].

Materials

Isopropylthio-β-D-galactopyranoside (IPTG), ampicillin, kanamycin, and Phusion Flash High-Fidelity PCR Master Mix were purchased from Thermo Scientific. All other chemical reagents were purchased from Sigma-Aldrich and utilized without further purification. DNA primers were synthesized by Integrated DNA Technologies. Competent *E. coli* 5-alpha and BL21(DE3) cloning and expression strains were purchased from New England Biolabs Inc (NEB). Restriction enzymes and T4 DNA ligase were purchased from NEB and used according to the manufacturer's specifications. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15 and Amicon Ultra-4, 30,000 MWCO) were purchased from Millipore. Pre-charged 5 mL HisTrap™ FF columns were purchased from GE Healthcare Life Sciences. The construction and sequence information of plasmid containing DNA encoding EryKR1 protein was previously described. Recombinant EryACP6, EryKR1, EryKR6, TylKR1, and Sfp were each expressed and purified as previously described.[6-9] The expression vector for $PicKR3^0$, which has been previously described, was expressed in *E. coli* BL21(DE3) and purified using the same protocols used for EryKR1.[10] The ACP-bound substrate (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-EryACP6 was prepared as previously described.[11] Reference standards of methyl (2*S*,3*R*)-2-methyl-3-hydroxypentanoate,

methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate, methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate, and methyl (2*R*,3*R*)-2-methyl-3-hydroxypentanoate, prepared as previously described,[9] were used as standards for chiral GC-MS analysis and for comparison with the corresponding [2-^{2}H]-2-methyl-3-hydroxypentanoates.[9,11]

Methods. General methods were as previously described.[9,12] Growth media and conditions used for *E. coli* strains and standard methods for handling *E. coli* in *vivo* and in *vitro* were those described previously,[12] unless otherwise noted. All DNA manipulations performed following standard procedures.[12] DNA sequencing was carried out at the U. C. Davis Sequencing Facility, Davis, CA. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford,[13] using a Tecan infinite M200 Microplate Reader with bovine serum albumin as the standard. Protein purity and size was estimated using SDS-PAGE and visualized using Coomassie Blue stain and analyzed with a Bio-Rad ChemiDoc MP System. Protein accurate molecular weight was identified by Agilent 6530 Accurate-Mass Q-TOF LC/MS. NADPH binding assay and reductase activity assay were carried out on the Tecan Microplate Reader. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III HD Ascend 600 MHz spectrometer. Chiral GC-MS analysis was performed on a GC–MS Hewlett-Packard Series 2 GC-MSD, 70 eV EI in positive ion mode with a Varian CP-Chirasil-DEX CB capillary column, 25 m × 0.32 mm. A Thermo LXQ equipped with Surveyor HPLC system and a Phenomenex Jupiter C4 column (150 mm × 2 mm, 5.0 μm) was utilized for analysis of diketide-ACP compounds. HPLC-ESI-MS-MS analysis was carried out in positive ion mode for analysis of pantetheinate ejection fragments.

EryKR3^0. Using pKOS422-51-1, harboring a *E.* coli codon-optimized, synthetic DEBSII, as a template,[14] the DNA encoding EryKR3 was PCR-amplified with primers 5′-GCAGATATA*CATATG*GCTGCCGCGAGCGATGAACTG-3′ and 5′-GTGGTG*CTCGAG*TCATCAGGCTTCTGCCTGACCCCCGCG-3′ (NdeI and XhoI restriction sites in italics, stop codon underlined). The insert was digested and ligated into pET28b (Novagen). The expression plasmid was transformed into *E. coli* BL21(DE3) cells. Expression and purification of N-terminal His_6-tag-EryKR3 utilized the same protocols previously described for EryKR1 and EryKR6.[6,8,9]

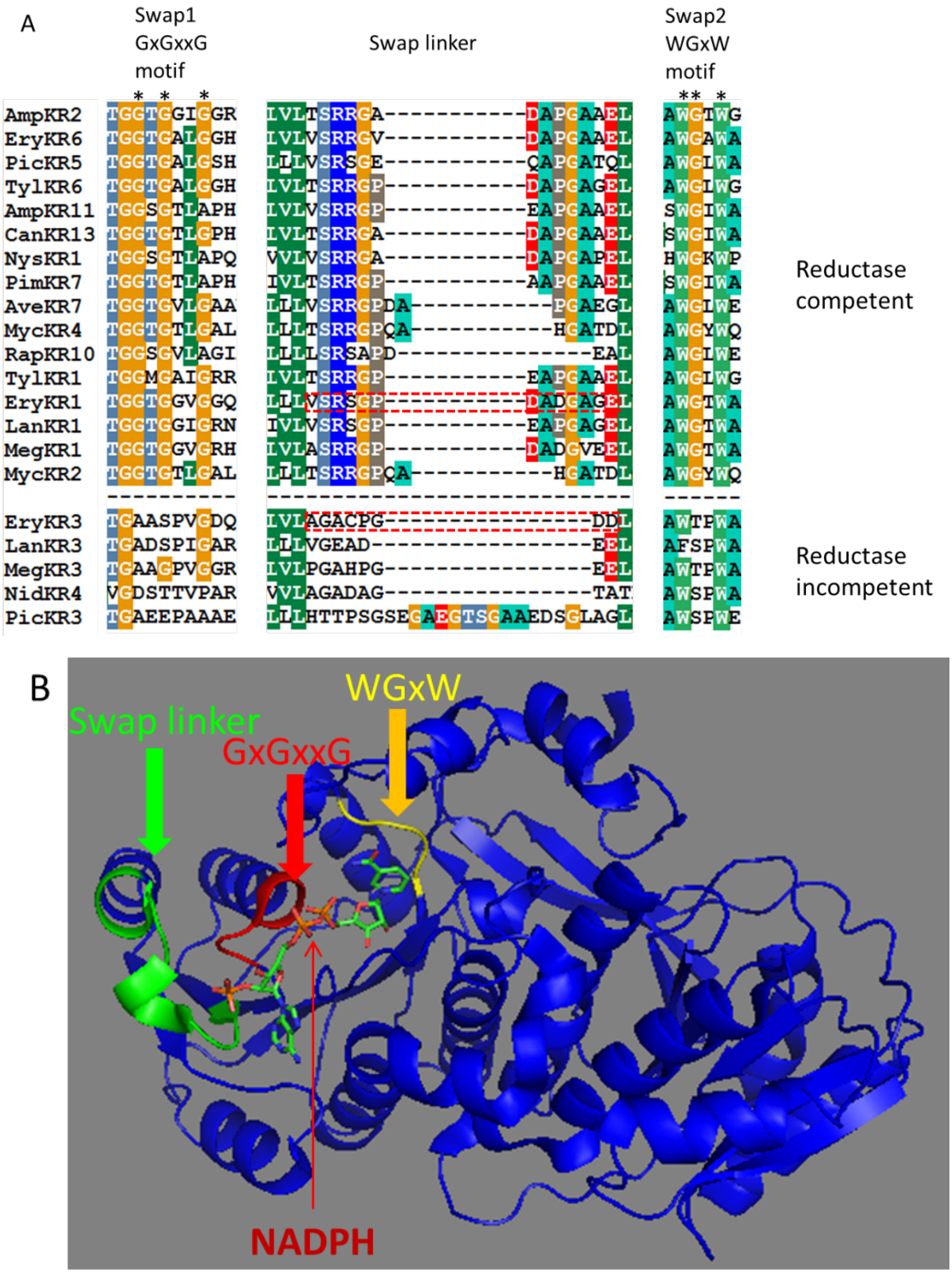

Figure S2. A. Mega3.0 (http://www.megasoftware.net) sequence alignment of PKS KR domains including reductase-active and reductase-inactive domains. The NADPH binding site in reductase-competent KR domains includes three conserved regions: a GxGxxG motif, a Swap linker and a WGxW motif. PKS source: Amp, amphotericin; Ave,

avermectin; Can, candicidin; Ery, erythromycin; Lan, lankamycin; Meg, megalomicin; Myc, mycolactone; Nid, niddamycin; Nys, nystatin; Pic, picromycin; Pim, pimaricin; Rap, rapamycin; Tyl, tylosin. B. Structure of EryKR1 with bound NADPH (PDB ID 2FR0). The conserved NADPH-binding motifs are highlighted as GxGxxG (red), WGxW (yellow), and Swap linker (green).

Engineering of redox-inactive EryKR1 mutants by disruption of the NADPH binding site. Plasmid pAYC59 harboring the DNA sequence encoding the EryKR1 domain[9] served as the template for mutation of EryKR1 by Overlap Extension PCR.[15] To generate the plasmid encoding mutant EryKR1^0-eG in which the **G**T**G**GV**G** motif of wild-type EryKR1 had been replaced by the corresponding AASPVG motif from EryKR3, the mutagenic DNA target was amplified by two cycles of PCR with Phusion Flash High-Fidelity PCR Master Mix. A pair of primers, EryKR1-NdeI/Swap1R (EryKR1-NdeI: 5′-AAAAAACATATGGACGAGGTTTCCGCGCTGCGC-3′, NdeI restriction site underlined; and Swap1/EryKR3R: 5′-CGCGCGATCTGCCCGCCGAC**GGGGCTCGCCGC**ACCGGTGACCAGC-3′, mutated sites in bold) was designed to amplify a 710-bp DNA fragment to generate one of the templates for the second cycle of PCR. A complementary pair of primers, EryKR1-EcoRI/Swap1F (EryKR1-EcoRI: 5′-TTTTGAATTCACCGCGCCCACCCGCGGTTCGGC-3′, EcoRI restriction site underlined; and Swap1/EryKR3F: 5′-GCTGGTCACCGGT**GCGGCGAGCCCC**GTCGGCGGGCAGATCGCGCG-3′, mutated sites in bold) was used to amplify an overlapping 793-bp DNA fragment for the second cycle of PCR. The two first-cycle DNA segments were purified from agarose gel, then mixed in a 1:1 molar ration and used as template for the second round of PCR amplification by the two outside primers (EryKR1-NdeI and EryKR1-EcoRI) to generate the 1458-bp mutagenized DNA. The purified 1458-bp fragment was digested with NdeI and EcoRI, inserted into the corresponding NdeI and EcoRI sites of cloning vector pUC18, and transformed into *E. coli* DH5 (NEB). A single colony of the resultant *E. coli* DH5 transformants harboring the plasmid with the inserted 1442-bp fragment was inoculated into Luria-Bertani (LB) media containing 100 mg/ml ampicillin and incubated at 37 °C overnight. The plasmid isolated from this overnight cell culture was digested by NdeI and EcoRI and the purified 1458-bp fragment was ligated into the corresponding

NdeI and EcoRI sites of expression vector pET28a to give. the plasmid encoding mutant EryKR1^0-eG. The ligated plasmid was transformed into *E. coli* DH5 cells for DNA isolation and sequencing. Additional sets of primers were designed to construct the corresponding swap mutations using the same protocol (Table S1). The EryKR1^0-eG mutant plasmid DNA was also used as the template for two further rounds of overlap extension PCR amplification by two pairs of primers (EryKR1-NdeI/EcoRI and Swap linkerF/R) to construct the double mutant EryKR1^0-eGeL, in which the GTGGVG motif of EryKR1 was replaced by the AASPVG motif from EryKR3 and the linker sequence VSRSGPDADGAGE from EryKR1 was replaced by the sequence AGACPGDD from EryKR3. Plasmid DNA was purified (Qiagen mini-prep kit or Thermo Scientific GeneJET Plasmid mini-prep kit) in each case from cultures derived from a single colony, and DNA sequencing confirmed the sequences of all mutant plasmid inserts.

Table S1. Mutagenic Primers Utilized to Generate EryKR1^0 Mutants

Mutation	F or R	Primer sequence 5′-3′
Swap1/PicKR3 (EryKR1^0-pG)	F	GCTGGTCACCGGT**GCCGAGGAGCCTGCGGCC**GGGCAGATCGCGCG
	R	CGCGCGATCTGCCC**GGCCGCAGGCTCCTCGGC**ACCGGTGACCAGC
Swap linker (EryKR1^0-eL)	F	CCCCACCTGCTGCTG**GCCGGAGCCTGTCCGGGCGACGAC**CTGGTCGCCGAGCTC
	R	GAGCTCGGCGACCAG**GTCGTCGCCCGGACAGGCTCCGGC**CAGCAGCAGGTGGGG

F, forward primer; R, reverse primer. Bold letters represent the introduced mutant codons.

Expression and purification of mutant EryKR1^0 proteins. The confirmed mutant plasmids were each transformed into competent cells of *E. coli* BL21(DE3) and the single colonies were incubated in LB media containing 50 mg/L Kanamycin at 37 °C overnight. The 10-mL seed cultures were each inoculated into 500 mL LB, Super Broth (SB) or Terrific Broth (TB) media containing 50 mg/L Kanamycin at 37 °C, which was grown to an OD_{600} of 0.5-0.7. The broth was cooled to 16 °C for 30 min and induced with 0.2 mM ITPG. After 40 h at 16 °C, cells were harvested by centrifugation and washed and resuspended in lysis buffer (50 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, 10% glycerol, pH 8.0). The suspended cells were stored at -80 °C until

use. The stored cells in lysis buffer were thawed and lysed by sonication (40 x 5 s cycles) or French Press. Cell supernatant and cell debris were separated by centrifugation (23,000*g* for 50 min). The supernatant was poured over a previously Lysis-buffer-equilibrated, pre-charged 5 mL HisTrap^{TM} FF column (GE Healthcare). The column was washed with 25 mL lysis buffer and then 25 mL washing buffer (50 mM sodium phosphate, 500 mM NaCl, 60 mM imidazole, 10% glycerol, pH 7.6). The protein was eluted by elution buffer (50 mM sodium phosphate, 50 mM NaCl, 150 mM imidazole, 10% glycerol, pH 7.4). The elution fractions were concentrated by ultrafiltration (Amicon, 30,000 MWCO), the buffer was exchanged with exchange buffer (50 mM sodium phosphate, 10% glycerol, pH 7.2), and the protein was concentrated by ultrafiltration and stored at -80 °C. Analysis by SDS-PAGE analysis by Bio-Rad Image Lab Software indicated that the purity of all mutant proteins was >90% and that the relative M_r values were all similar (Figure S3). The molecular mass M_D of each mutant protein was verified by Agilent Technologies Q-TOF LC-MS and matched the predicted values (Table S2).

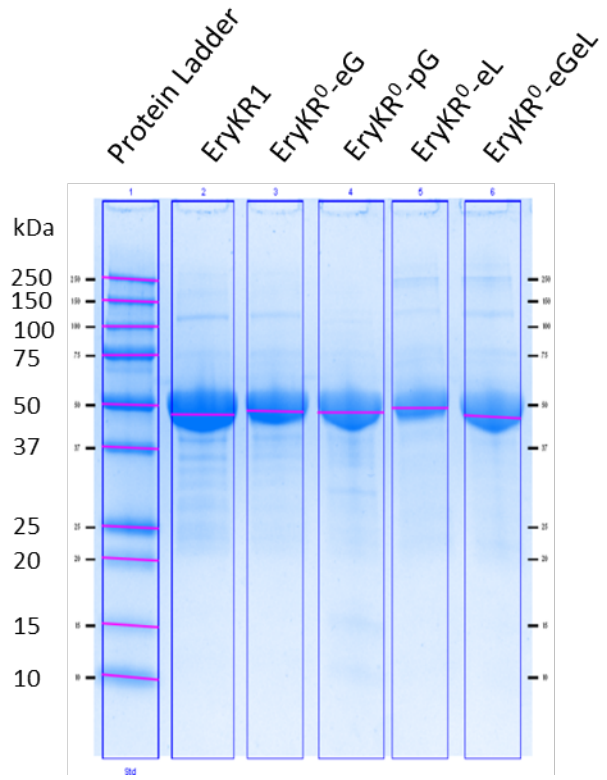

Figure S3. SDS PAGE of wild-type and mutant EryKR1.

Table S2. Predicted MW and observed M_r and M_D of wild-type and mutant EryKR1.

Protein	EryKR1-wt	EryKR1^0-eG	EryKR1^0-pG	EryKR1^0-eL	EryKR1^0-eGeL
MW (cal, Da)	54794	54848	54934	54281	54335
SDS PAGE (M_r)	63358	58769	60493	57758	64607
Q-TOF (M_D)	54794	54848	54935	54280	54336

Fluorescence enhancement assay of NADPH binding by EryKR1 mutants. The loss of NADPH binding activity in mutant EryKR1 proteins was evaluated by fluorescence enhancement assay[16] in 96-well plates (Thermo Scientific Optical Bottom Black Polystyrene Plate) with a Tecan microplate reader. Each mutant EryKR1 protein, as well as wild-type EryKR1 as positive control, and PicKR3^0 and EryKR3^0 as negative controls, was prepared at 1 mg/mL in 50 mM sodium phosphate, 10 % glycerol, pH7.2. Successive 1-μL portions of NADPH solution were added into 96 wells containing 100

μL of 1 mg/ml protein solution or buffer alone. The final NADPH concentration in the assay ranged from 0 to 20 μM (0, 2, 4, 8, 12, 16, 20 μM). The plate was incubated for 20 min at room temperature. NADPH fluorescence was excited at 340 nm (5 nm slit width) and monitored between 440 nm and 470 nm (6 nm slit width). Each assay was conducted in duplicate. NADPH fluorescence enhancement was calculated from the difference of NADPH fluorescence intensities in the presence and absence of proteins ($F_{en}=F_{pre}-F_{ab}$; where F_{en} is fluorescence enhancement, F_{pre} is fluorescence intensity of NADPH in the presence of protein, F_{ab} is fluorescence intensity of NADPH in the absence of protein; F_{pre} and F_{ab} were measured at the same concentration of NADPH). Protein binding of cofactor NADPH results in an increased fluorescence enhancement with increasing concentration of NADPH over the range of the assay. NADPH fluorescence was enhanced in the presence of the positive control EryKR1 protein but showed no increase in the presence of negative control EryKR3^0 and PicKR3^0 protein (Figure S4). There was no increase in NADPH fluorescence intensity in presence of all mutant proteins.

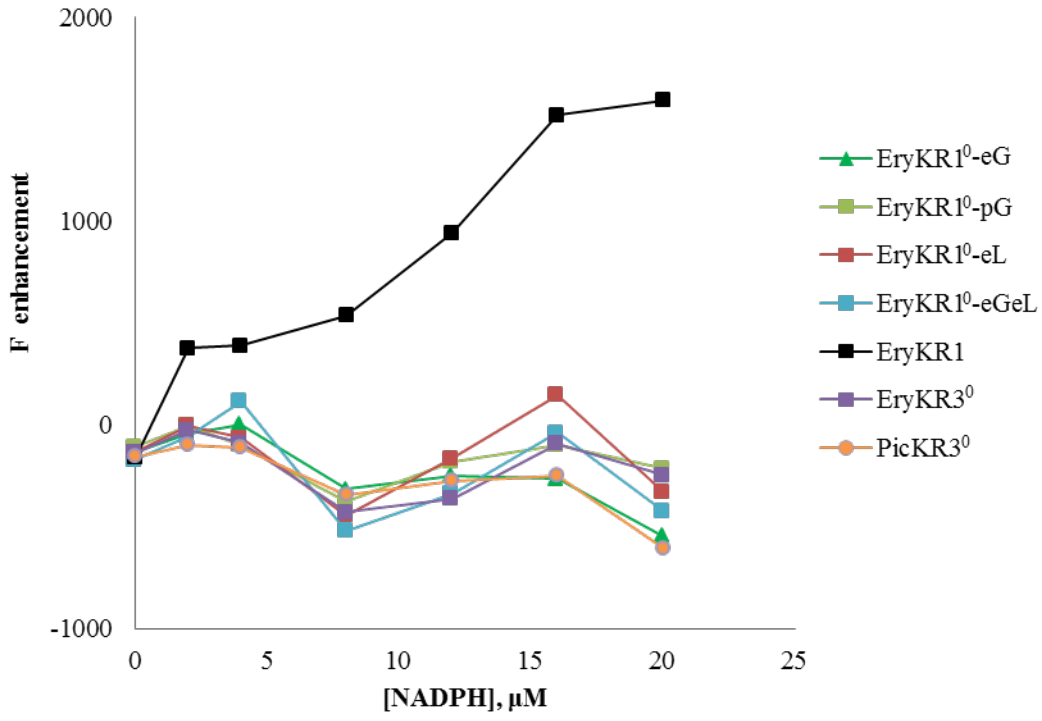

Figure S4. Characterization of the NADPH binding by EryKR1 mutants by fluorescence enhancement: EryKR1^0-eG, GTGGVG motif of EryKR1 replaced by AASPVG from

EryKR3^0; EryKR1^0-pG, GTGGVG motif of EryKR1 replaced by AEEPAA from PicKR3^0; EryKR1^0-eL, swaplinker motif VSRSGPDADGAGE of EryKR1 replaced by AGACPGDD from EryKR3^0; EryKR1^0-eGeL, double mutant, GTGGVG and swaplinker replaced by corresponding sequences from EryKR3^0, plus wild-type EryKR1, EryKR3^0, and PicKR3^0 proteins. Proteins at 1 mg/mL were titrated with NADPH. Fluorescence enhancement was determined as described in the assay procedure.

Assay of ketoreductase activity of EryKR1 mutants. The reductase activity of mutant and wild-type KR proteins was tested in 96-well plates using the standard assay substrate *trans*-1-decalone.[17] Each KR protein (5 μM) was incubated with 1 mM NADPH in 50 mM sodium phosphate, pH 7.2, at 30 °C for 20 min. The *trans*-1-decalone (final concentration 0-8.0 mM) was added in a total volume of 200 μL and the consumption of NADPH was measured with the Tecan plate reader by monitoring the decrease in the absorbance at 340 nm for 30 min at 30 °C (Figure S5). None of the four mutant EryKR1 proteins exhibited detectable reductase activity

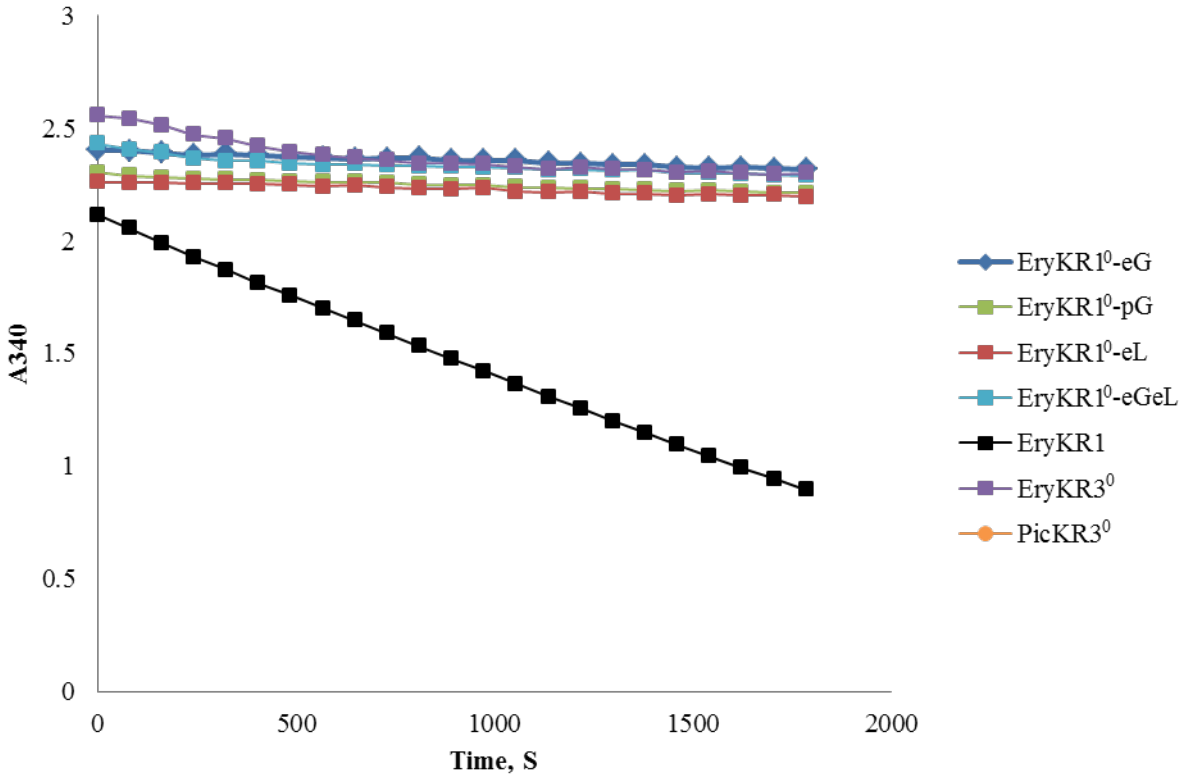

Figure S5. Assay of KR-catalyzed reduction of 8 mM *trans*-1-decalone by EryKR1^0 mutants and wild type EryKR1, EryKR3, PicKR3, with monitoring of the consumption of NADPH by the absorbance at 340 nm.

Tandem equilibrium isotope exchange assay of redox-inactive KR proteins. For each incubation, [2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**3b**, 1 equiv.) was incubated with a catalytic amount of $NADP^+$ (0.05 or 0.10 equiv) and a mixture of 0.25 equiv. of redox-active EryKR6 and 0.25 equiv. of redox-inactive KR^0 domain. Thus in a typical incubation, [2-^{2}H]-**3b** (90 μL of 500 μM solution, 45 nmol, final conc 300 μM), EryKR6 (11.25 nmol, 75 μM), $EryKR3^0$ (11.25 nmol, 75 μM), and $NADP^+$ (1.5 μL of 1.5 mM soln, 2.25 nmol, final conc 15 μM) were incubated in 50 mM phosphate buffer (pH 7.2) (tot vol 150 μL) at room temp. Samples were withdrawn at periodic intervals up to 60 min and frozen in liq N_2, before direct analysis of the pantetheinate ejection fragments by LC-ESI(+)-MS-MS, as previously described.[11,18,19]

Chiral GC-MS analysis of recovered (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (3b). A tandem incubation of [2-^{2}H]-**3b** with EryKR6, $EryKR3^0$, and cat. $NADP^+$, carried out with the same concentrations of enzymes and cofactor as above in a total volume 500 μL, was quenched after 20 min by addition of 200 μL of 0.5 M NaOH, followed by incubation at 65 °C for 20 min., followed by acidification with dil HCl. and extraction with ethyl acetate. After evaporation of the solvent, the residue was dissolved in 100 μL of methanol, followed by addition of TMS-CHN_2. The derived methyl ester was analyzed by chiral GC-MS as noted above using the previously described column and temperature program (Figure S6).[9,11] Direct comparison with authentic standards confirmed that the recovered methyl ester was exclusively methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate.

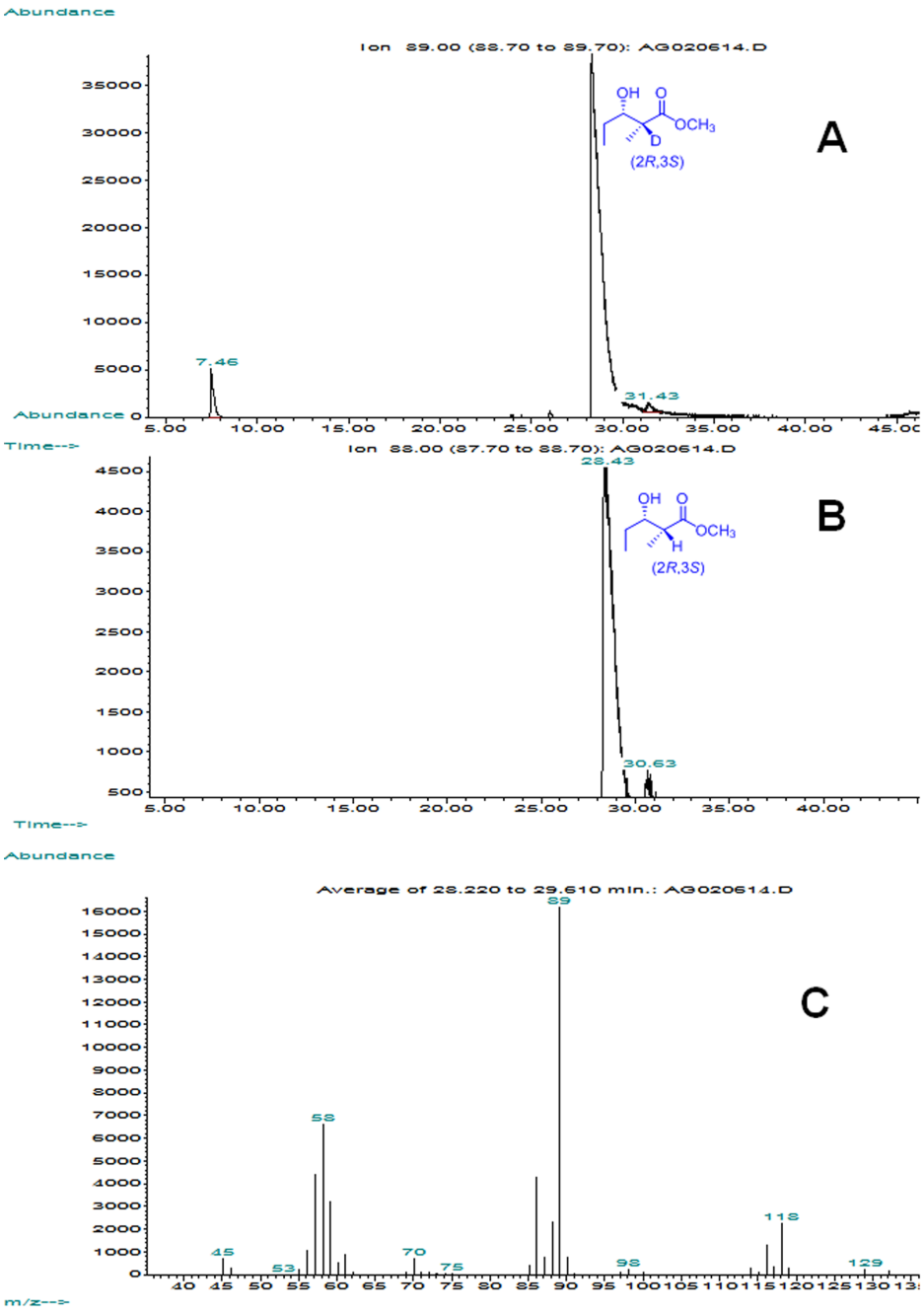

Figure S6. Chiral GC-MS analysis of methyl (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate from 20 min tandem equilibrium isotope exchange of [2-^{2}H]-**3b** with EryKR6 and EryKR3^{0}, hydrolysis, and methylation. A. Methyl (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate, XIC, *m/z* 89. B. XIC, *m/z* 88. C. MS of methyl (2*R*,3*S*)-[2-^{2}H/^{1}H]-2-methyl-3-hydroxypentanoate.

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