Supporting Information

Stereospecificity of Ketoreductase Domains 1 and 2 of the Tylactone Modular Polyketide Synthase

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Materials and Methods

General methods for protein expression, purification and DNA manipulation were as described.[1] DEBS [KS3][AT3], [ACP3], [KS6][AT6] and [ACP6] were expressed and purified as previously described.[2] TYLS KR1 was expressed and purified as previously described.[3] Reference standards of each of the 4 diastereomers of triketide lactone **7** and the corresponding TMS-derivative were prepared and analyzed as described.[1] DNA sequences were determined by the U. C. Davis Sequencing Facility, Davis, CA.

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NIDS_KR2      LHEATLGHP--LRAFVLFSAAAGLLGRPGQASYAAANAVLDAFARLRRAEGLPAVSLAWG 58
SRMG_KR2      -HEATLDRP--LRAFVLFSAAAGLLGRPGQASYAAANAVLDALAGARRAAGLPAVSLAWG 57
TYLS_KR2      -HELTRTSP--LSVFAVFSGAAGILGRPGQANYAAANTFLDALAQHRRAHGLPAVSLAWG 57
TYLS_KR3      LHELTQGHA--LSAFILYSSAAGVLGSAGQSGYAAANAYLDSFAVWRRSRGLPAVSLGWG 58
TYLS_KR1      -HQLTADIK-GLDAFVLFSSVTGTWGNAGQGAYAAANAALDALAERRRAAGLPATSVAWG 58
DEBS_KR2      LDELCSDA----ELFLLFSSGAGVWGSARQGAYAAGNAFLDAFARHRRGRGLPATSVAWG 56
PICS_KR2      LDELTSTPAYDLAAFVMFSSAAAVFGGAGQGAYAAANATLDALAWRRRAAGLPALSLGWG 60
PICS_KR1      LDELTRDLD--LDAFVLFSSVSSTLGIPGQGNYAPHNAYLDALAARRRATGRSAVSVAWG 58
DEBS_KR1      LHELTRELD--LTAFVLFSSFASAFGAPGLGGYAPGNAYLDGLAQQRRSDGLPATAVAWG 58
Consensus       .:            * ::*. :.  * .  . **. *: **.:*  **. * .* ::.**
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Figure S1. CLUSTAL W2 sequence alignment of representative PKS KR domains. TYLS KR2 has a Gly in place of the consensus active site Ser at position 17 (corresponding to aa 365 in recombinant TYLS KR2). The closely related niddamycin synthase (NIDS) and platenolide synthase (spiramycin synthase SRMG) KR2 domains carry an Ala at this position, while all other PKS KR domains, whether associated with DH domain (PICS KR2, TYLS KR3) or not, have the conserved Ser. (CLUSTAL W2: European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/es/cgi-bin/clustalw2).

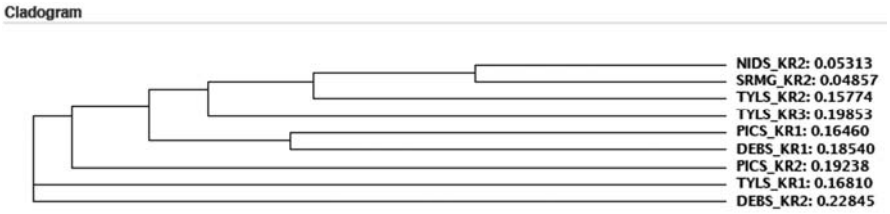


Figure S2. Cladogram from CLUSTALW2 alignment of PKS KR2 domains.

Comment on the TYLS G365S mutation. The active TylKR2(G365S) mutant reduces the ACP-bound 2-methyl-3-ketotriketide to generate a single diastereomer of the product. While it is possible that the TylKR2(G365S) mutation slightly alters substrate positioning during catalysis, it is extremely unlikely that this point mutation completely alters how the stereochemistry of the reduction, either with regard to the α-methyl or β-hydroxy stereochemistry. Both Reid and Caffrey have reported extensive sequence alignments of more than 70 PKS KR domains that show a strong correlation between reduction stereochemistry and specific sequence motifs, all of which are independent of the conserved active site Ser residue, which forms part of a catalytic triad with conserved Lys and Tyr residues typical of the short chain dehydrogenase-reductase superfamily.[4] Keatinge-Clay has also analyzed the structures of both DEBS KR1 and TYLS KR1 and discussed the relationship between between conserved residues and the diastereoselectivity of reduction.[3] Attempts to reverse the observed stereospecificity of DEBS KR1 and KR2 domains by directed mutagenesis through directed mutagenesis involving these conserved amino acid motifs had no affect on the observed stereochemistry

of the methyl group in the resulting reduction products.[5] There is thus no evidence that the G365S mutation in TYLS KR2 will affect the stereospecificity of the reduction in any way.

Cloning of TYLS KR2. Plasmid pRC42, which harbors the coding sequence of TLYS module 2, and which was prepared by subcloning from pKOS168-190 containing the entire tylactone PKS gene cluster (data not shown), was used as the template for amplification and cloning of the TYLS KR2 domain. The primers KR-FWD3: 5′-CACACACATATGGGCGAACGGTCGCTGTACCGCGTC-3′ (NdeI restriction site underlined) and KR-REV3: 5′-CACACAGAATTC*TTA*GAGCCCGGCCAGCCGCTCG-3′ (EcoRI, stop codon in italics) were used with *Pfu* Turbo DNA polymerase. The resulting PCR amplicon was digested with NdeI and EcoRI and inserted into the corresponding restriction sites of pET28a to give pRC18, encoding the N-terminal His_6-tagged TYLS KR2. Plasmid pRC18 was the template for the G365S mutation using primers SDM-KR1: 5′-CGGTCTTCTCG**A**GCGCTGCCGGCATC-3′ and SDM-KR2: 5′-GATGCCGGCAGCGC**T**CGAGAAGACCG-3′ (mutated sites in bold) using the QuikChange Site-Directed Mutagenesis kit to generate plasmid pRC18M. The sequences of all plasmid inserts, including the wild-type and mutant TYLS KR2, were verified by resequencing.

Expression and purification of TYLS KR2 and TYLS KR2(G365S). Plasmids pRC18 and pRC18M were each transformed into *E. coli* BL21(DE3) and the transformants were incubated in LB medium overnight. The seed cultures were then used for the inoculation of 500 mL Super Broth (SB) medium at 37 °C which was grown to an OD (600 nm) of 0.6. The cells were cooled to 18 °C and induced with 0.2 mM IPTG for 16 h, then harvested, washed and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 10 % glycerol, pH 8.0) supplemented with 2 μg/mL pepstatin and leupeptin. Lysozyme (1 mg/mL) was added and the cells were incubated at 4 °C for 30 min. The cells were lysed by sonication (3 × 30 s cycles) and centrifuged. The supernatant was mixed with previously equilibrated Ni-NTA resin (Qiagen, 2 mL/L culture) and slowly agitated at 4 °C for 1.5 h. The resin was then poured into a fritted column and washed with lysis

buffer (10 col. vol.), lysis buffer with 70 mM imidazole (5 col. vol.) and the protein was eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) (10 col. vol.). The protein was then concentrated by ultrafiltration (Amicon, 50,000 MWCO), the buffer was exchanged on a PD-10 column with storage buffer (100 mM sodium phosphate, 10 % glycerol, pH 7.2) and the protein was concentrated by ultrafiltration and frozen at -78 °C. SDS-PAGE analysis indicated that both KR2 and KR2(G365S) were free of contaminating proteins except for 30-40% of *E. coli* GroEL (UniProt ID P0A6F5) that copurified with each preparation. MALDI-TOF analysis – KR2: 53531±50 (obsvd), 53509 (calcd); KR2-Met: 53381±50 (obsvd), 53377 (calcd); KR2(G365S) 53570±50 (obsvd), 53539 (calcd); KR2(G365S)-Met: 53412±50 (obsvd), 53408 (calcd).

UV kinetic assay of TYLS KR2 and TYLS KR2(G365S) with *trans*-1-decalone.[6] TYLS KR2 (75 μM) or TYLS KR2(G365S) (5 μM) was incubated with 2.0 mM NADPH in 100 mM sodium phosphate, pH 7.2, for 15 min at 30 °C with a final DMSO content of 5.3 %. *trans*-1-decalone (0.2-8.0 mM from a stock solution of 150 mM in DMSO) was added and the reaction was monitored on plate reader for 30 min at the same temperature at 340 nm in a total volume of 110 μL with an initial orbital shaking of 8 s, followed by orbital shaking of 3 s before every reading. The linear slope in the first 4 min was used to calculate the rate of consumption of NADPH. A standard curve for NADPH was measured under the same conditions and gave a linear extinction coefficient of 1700 $M^{-1}cm^{-1}$. The results acquired on a Tecan plate reader (96-well plates controlled by XFluor software) were measured in duplicate. Steady-state kinetic parameters were determined by using Kaleidagraph software and fitting the data to the Michaelis-Menten equation by non-linear least-squares regression (Table S1). Reported standard deviations in the steady-state kinetic parameters represent the calculated statistical errors in the non-linear, least squares regression analysis.

GC-MS kinetic assay of TYLS KR2 and TYLS KR2(G365S) with *trans*-1-decalone. To verify that KR-dependent consumption of NADPH was due to reduction of *trans*-1-decalone, the reaction was also monitored by GC-MS analysis of the derived *trans*-1-decalol. TYLS KR2 (50 μM) or TYLS

KR2(G365S) (5 μM) with 2.5 mM NADPH in 100 mM sodium phosphate, pH 7.2 was pre-incubated for 15 min at 30 °C with a final DMSO content of 5.3 % in a typical volume of 200 μL. A series of *trans*-1-decalone concentrations similar to those described above were then added and the reaction was quenched by adding 500 μL ethyl acetate after 5 min for TYLS KR2(G365S) (up to 6 h or overnight for TYLS KR2). The reaction mixture was extracted with ethyl acetate (2 × 500 μL) and the organic phase was concentrated *in vacuo*. The residue was then derivatized by treatment with 100 μL BSTFA for 10 min under nitrogen at room temp. The derivatized product was then analyzed by GC-MS [Hewlett Packard HP G1800 GCD System Series II using a HP-5MS capillary column (30 m × 0.25 mm) with the injector port at 250 °C and a temperature program of 50 °C for 2 min, then 20 °C/min up to 280 °C, and 280 °C]. A standard calibration curve was generated using *cis*-1-decalol. Steady-state kinetic parameters (Table S1) were calculated as above.

Table S1. Assay of TYLS KR2(G365S) with trans-1-decalone. Steady state kinetic parameters obtained by UV and GC-MS analysis for reduction of *trans*-1-decalone by TYLS KR2(G365S) and NADPH.

Kinetic constants	UV assay	GC-MS assay
K_m	0.34 ± 0.11 mM	1.27 ± 0.26 mM
k_{cat}	0.274 ± 0.02 s^{-1}	0.44 ± 0.04 s^{-1}
k_{cat}/K_m	800 ± 268 $M^{-1}s^{-1}$	347 ± 78 $M^{-1}s^{-1}$

Incubation of recombinant KR domains and TLC-phosphorimaging of enzymatic reaction products. [KS3][AT3] or [KS6][AT6] (40 μM, in 100 mM phosphate [pH 7.2]) was incubated with 5 mM (2S,3R)-2-methyl-3-hydroxypentanoyl-N-acetylcysteamine thioester (**6**) and 5 mM of TCEP for 1 h at room temp. TYLS KR2 or TYLS KR2(G365S) (300 μM), [ACP3] or [ACP6] (200 μM), NADPH (2 mM) and DL-[2-methyl-^{14}C]-methylmalonyl-CoA (300 μM) were added and allowed to react for an additional hour at room temp in a final volume of 25 μL. The reaction was quenched by addition of 10 μL 0.5 M NaOH and heating the mixture at 65 °C for 20 min. Hydrochloric acid (5 μL of 1.5 M) was then added. Ethyl acetate extraction (2 × 100 μL) and solvent removal by Speed-Vac allowed isolation

of the organic product. The residue was redissolved in 20 μL ethyl acetate and spotted onto a silica gel TLC plate. A 60:40 ethyl acetate:hexane mixture was used for TLC. Radiolabeled products were visualized using a BioRad FX-Pro Plus Molecular Imager (Figure S3).

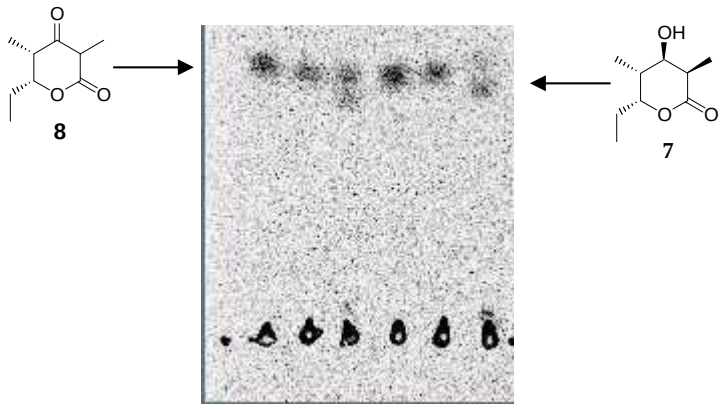


Figure S3. Radio-TLC of the incubation of [KS][AT], [ACP] and TYLS KR2 or TYLS KR2(G365S) with NDK-SNAC, [^{14}C]-methylmalonyl-CoA and NADPH. Lanes 1-3: [KS3][AT3] and [ACP3] fragments. Lanes 4-6: [KS6][AT6] and [ACP6] fragments. Lanes 2 and 5 used TYLS KR2 and lanes 3 and 6, TYLS KR2(G365S).

Incubation of recombinant KR domains with DEBS domains and GC-MS analysis of enzymatic products.[1] In a typical incubation [KS][AT] didomain (40 μM) was incubated with *syn*-diketide **6** (5 mM) and TCEP (5 mM) in 100 mM sodium phosphate, pH 7.2, at room temp for 1 h in a volume of 500 μL. [ACP] (200 μM), TYLS KR1 (300 μM) or TYLS KR2(G365S) (300 μM), methylmalonyl-CoA (300 μM) and NADPH (2 mM) were added and the mixture incubated at room temp for 1 h. The triketide was hydrolytically released from the ACP by addition of 200 μL of aq. 0.5 M NaOH and incubation at 65 °C for 20 min, followed by addition of 100 μL of 1.5 M HCl. After extraction with ethyl acetate (4 × 700 μL), the organic phase was concentrated in vacuo and the residue was derivatized with 100 μL BSTFA and 1 μL TMS-imidazole for 10 min at room temp under nitrogen. Derivatized product was analyzed by GC-MS [JEOL JMS-600H mass spectrometer using an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) with the injector port at 250 °C and a temperature program of 60 °C for 1 min, then 25 °C/min up to 280 °C, and 280 °C for 5 min] (Figures S4-S6) and the retention time was compared with that of triketide lactone reference standards. (Table S2).

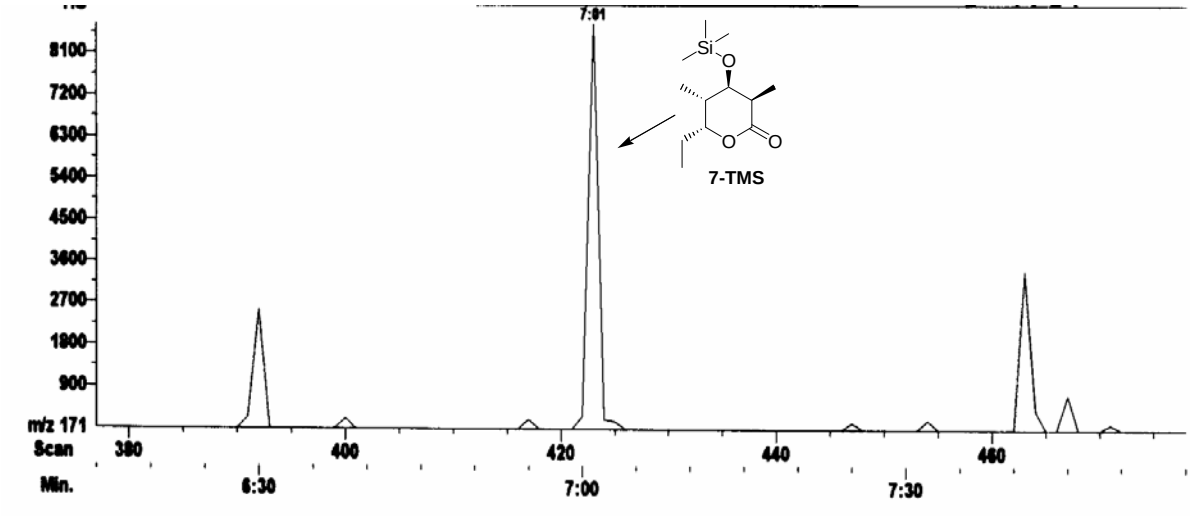


Figure S4. GC-CI-MS (XIC at *m/z* 171) of TMS-derivatized triketide lactone **7–TMS** product of incubation of [KS3][AT3], [ACP3] and TYLS KR1.

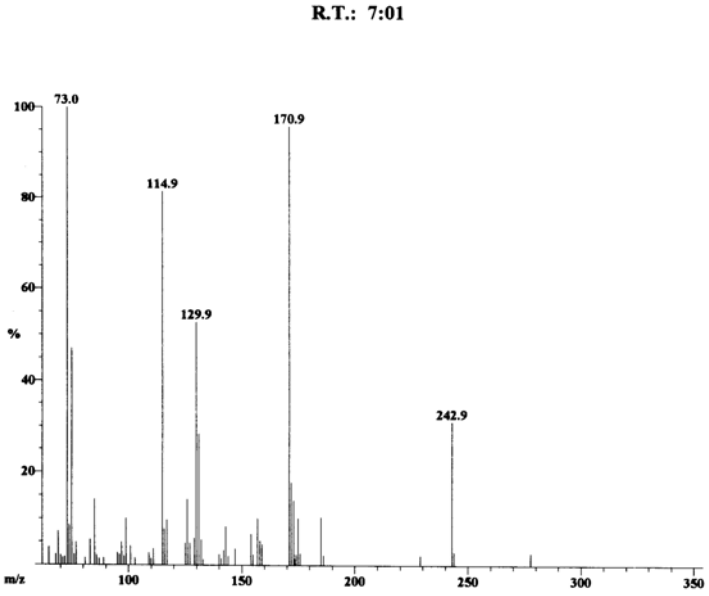


Figure S5. Mass fragmentation pattern of the peak at a retention time of 7.01, indicative of the triketide lactone diasteromer **7-TMS**.

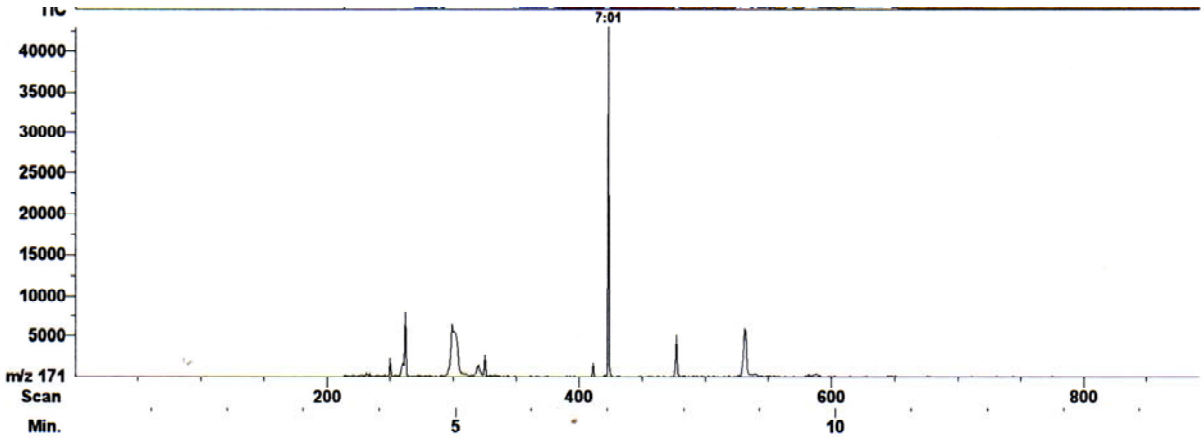
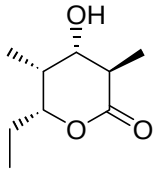
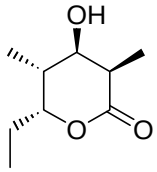
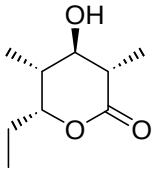
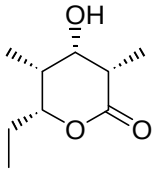


Figure S6. GC-CI-MS (XIC at *m/z* 171) of TMS-derivatized triketide lactone **7–TMS** product of [KS3][AT3], [ACP3] incubation with TYLS KR2(G365S).

Table S2. Structure of the 4 diastereomers of triketide lactone **7** and previously reported retention times of the corresponding TMS derivatives.[1]

(2*R*,3*S*)-triketide lactone	(2*R*,3*R*)-triketide lactone **7**	(2*S*,3*R*)-triketide lactone	(2*S*,3*S*)-triketide lactone
			
Retention time (min)	Retention time (min)	Retention time (min)	Retention time (min)
6:55	7:01	6:49	7:17

References

(1) Castonguay, R.; He, W.; Chen, A. Y.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2007**, *129*, 13758-13769.

(2) Chen, A. Y.; Schnarr, N. A.; Kim, C. Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2006**, *128*, 3067-3074. Chen, A. Y.; Cane, D. E.; Khosla, C. *Chem. Biol.* **2007**, *14*, 784-792. Gokhale, R. S.; Hunziker, D.; Cane, D. E.; Khosla, C. *Chem. Biol.* **1999**, *6*, 117-125.

(3) Keatinge-Clay, A. T. *Chem. Biol.* **2007**, *14*, 898-908.

(4)) a) Reid, R.; Piagentini, M.; Rodriguez, E.; Ashley, G.; Viswanathan, N.; Carney, J.; Santi, D. V.; Hutchinson, C. R.; McDaniel, R. *Biochemistry* **2003**, *42*, 72-79. b) Caffrey, P. *ChemBioChem* **2003**, *4*, 654-657.

(5) Baerga-Ortiz, A.; Popovic, B.; Siskos, A. P.; O'Hare, H. M.; Spiteller, D.; Williams, M. G.; Campillo, N.; Spencer, J. B.; Leadlay, P. F. *Chem. Biol.* **2006**, *13*, 277-285.

(6) Siskos, A. P.; Baerga-Ortiz, A.; Bali, S.; Stein, V.; Mamdani, H.; Spiteller, D.; Popovic, B.; Spencer, J. B.; Staunton, J.; Weissman, K. J.; Leadlay, P. F. *Chem. Biol.* **2005**, *12*, 1145-1153.