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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · OCTOBER 2008

Impact Factor: 12.11 · DOI: 10.1021/ja804453p · Source: PubMed

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J Am Chem Soc. 2008 September 3; 130(35): 11598–11599. doi:10.1021/ja804453p.

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

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Abstract

Tylactone synthase (TYLS) is a modular polyketide synthase that catalyzes the formation of tylactone (**1**), the parent aglycone precursor of the macrolide antibiotic tylosin. TYLS modules 1 and 2 are responsible for the generation of *anti*-diketide and triketide intermediates, respectively, each bound to an acyl carrier protein (ACP) domain. Each module harbors a ketoreductase (KR) domain. The stereospecificity of TYLS KR1 and TYLS KR2 has been determined by incubating each of the recombinant ketoreductase domains with reconstituted ketosynthase—acyltransferase [KS][AT] and ACP domains from the 6-deoxyerythronolide B synthase (DEBS) in the presence of the *N*-acetylcysteamine thioester of *syn*-(2*S*,3*R*)-2-methyl-3-hydroxypentanoate (**6**), methylmalonyl-CoA, and NADPH resulting in the exclusive formation of the ACP-bound (2*R*,3*R*,4*S*,5*R*)-2,4-methyl-3,5-dihydroxyhepanoyl triketide, as established by GC-MS analysis of the TMS ether of the derived triketide lactone **7**. Both TYLS KR1 and KR2 therefore catalyze the stereospecific reduction of the 2-methyl-3-ketoacyl-ACP substrate from the *re*-face, with specificity for the reduction of the (2*R*)-methyl (D) diastereomer. The dehydration that is catalyzed by the dehydratase (DH) domains of TYLS module 2 to give the unsaturated (2*E*,4S,5*R*)-2,4-dimethyl-5-hydroxyhept-2-enoyl-ACP2 is therefore a *syn* elimination of water.

Tylactone synthase (TYLS) from *Streptomyces fradiae* is a modular polyketide synthase (PKS) that catalyzes the formation of tylactone (**1**), the parent 16-membered ring macrolactone aglycone precursor of the important veterinary antibiotic tylosin used in the treatment of pulmonary infections in large animals. TYLS consists of seven homodimeric modules, organized into 5 large proteins of subunit M_D ranging from 170 to 460 kDa, each responsible for a single round of polyketide chain extension and functional group modification (Figure 1).[1]

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Supporting Information Available: Experimental procedures, GC-MS data, and sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

Although the general organization of the tylactone synthase has many features in common with the well-studied modular PKS of erythromycin biosynthesis, 6-deoxyerythronolide B (**2**,6-dEB) synthase (DEBS),[2] there are several interesting differences. 1) Tylactone, in common with all other C-14 substituted 16-membered ring macrolides, is derived from an *anti*-diketide intermediate, in this case (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP generated by TYLS module 1 (Figure 1A). In support of this role is the reported intact incorporation of the corresponding *N*-acetylcysteamine analog **3** into tylactone by cultures of *S. fradiae*[3] as well as the observed diastereospecific reduction of racemic 2-methyl-3-keto diketide-SNAC **4** to the *anti*-(2*R*,3*R*)-diketide **3** catalyzed by recombinant TYLS ketoreductase 1 (KR1) (Scheme 1).[4] By contrast, 12- and 14-membered ring macrolides are derived from an initially-generated *syn*-(2*S*,3*R*)-diketide (Figure 1B).[2,4,5] 2) Tylactone carries a trisubstituted double bond generated by the combined action of the KR2 and DH2 domains of TYLS module 2, unlike 6-dEB which has no double bonds. The predicted mode of action of TYLS module 2 is supported by the intact incorporation of the corresponding unsaturated triketide-SNAC **5** into tylactone.[3] Although the stereochemistry of the corresponding β-hydroxyacyl-ACP triketide intermediate is necessarily cryptic, a (3*R*)-β-hydroxy configuration is predicted based on the presence of a conserved active site LDD sequence in the TYLS reductase KR2.[6,7] We now report the determination of the intrinsic stereospecificity of the KR domains from TYLS modules 1 and 2 and provide the first experimental evidence that the DH domain of a modular PKS catalyzes a *syn*-dehydration.

Incubation of recombinant TYLS KR1, expressed and purified as previously described,[7b] with recombinant DEBS [KS3][AT3] didomain and [ACP3][2,8,9] in the presence of *syn*-diketide **6**, methylmalonyl-CoA, and NADPH, followed by basic hydrolysis and acidification, as previously described,[9,10] gave the *anti*-(2*R*,3*R*)-2-methyl-3-hydroxytriketide lactone **7** as the exclusive product (Scheme 2). The configuration of **7** was unambiguously assigned by capillary GC-MS analysis of the corresponding **7**-TMS derivative and direct comparison with synthetic standards of each of the 4 diastereomeric triketide lactones.[10] The same triketide lactone diastereomer **7** was also obtained by an incubation using DEBS [KS6][AT6] plus [ACP6] in combination with TYLS KR1.

TYLS KR2 differs from other Type I PKS KR domains that have previously been cloned and expressed in active form in that it is bounded immediately upstream by a DH rather than an AT domain. The conserved *N*-terminal YRVEW and *C*-terminal RLAGL boundaries of the KR2 domain[2,4,6a,7b,9] were identified by multiple sequence alignments and the corresponding PCR primers were used to amplify the intervening KR2 domain using DNA originally derived from plasmid pKOS168-190 harboring the entire TYLS gene cluster as template. The resultant amplicon was inserted into pET28a to give pRC18 encoding TYLS KR2 with an appended *N*-terminal His_6-tag. Unexpectedly, recombinant KR2 was found to be catalytically inactive. Multiple sequence comparisons, however, reveal that TYLS KR2 has a Ser-Gly diad in place of the universally conserved Ser-Ser sequence in which the second Ser is an essential part of the active site Ser-Tyr-Lys triad typical of short chain dehydrogenase—reductases.[6a,11,12] We therefore replaced Gly365 with a Ser by site-directed mutagenesis.[13] Incubation of Ni-NTA-purified TYLS KR2(G365S) with either DEBS [KS3][AT3] and [ACP3] or DEBS [KS6][AT6] and [ACP6] in the presence of diketide **6**, [2-^{14}C] methylmalonyl-CoA, and NADPH gave a mixture of the triketide ketolactone **8** and the lactone **7**, as established by TLC-phosphorimaging. GC-MS analysis of the corresponding **7**-TMS derivative (ret. time 7.01 min) established the formation of lactone **7** as the exclusive reduction product (Scheme 2).

We have previously determined that the ACP-bound 2-methyl-3-ketoacyl triketide intermediates generated by both DEBS [KS3][AT3] and [KS6][AT6] have exclusively the D-methyl (2*R*) configuration at C-2, as established by trapping with $NaBH_4$.[10] The finding that

both TYLS KR1 and KR2 reduce this intermediate to give a single *anti*-(**2*R***,**3*R***,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoyl-ACP product corresponding to triketide lactone diastereomer **7** is consistent with the established generation of the *anti*-diketide by the KR1 domain of TLYS module 1. This report is also the first demonstration that the KR1 domain can reduce polyketides longer than the natural diketide 3-ketoacyl thioester substrate. Since recombinant TYLS KR2 is expected to retain the intrinsic diastereoselectivity of the integrated KR2 domain, these results establish for the first time that the *anti*-triketide product is generated by TYLS KR2 as part of the catalytic sequence mediated by TLYS module 2. The subsequent dehydration catalyzed by TYLS DH2 to give (2*E*,4*S*,5*R*)-2,4-dimethyl-5-hydroxyhept-2-enoyl-ACP2 (Figure 1) must therefore involve a *syn* dehydration, consistent with the known *syn* stereospecificity of the yeast fatty acid synthase DH domain.[14] Interestingly, the generation of a D-β-hydroxyl group by TYLS KR2 (*re* specificity) contrasts with that of DEBS KR2 which generates the *syn*-(**2*R***,**3*S***,4*S*,5*R*)-triketide en route to 6-dEB (Figure 1B),[10] but is identical to the D-(3*R*)-β-hydroxyl specificity that we have previously established for the picromycin synthase KR2 and the rapamycin KR4 domains, each of which is paired with a DH domain.[15]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants GM22172 (D.E.C.) and CA66736 (C.K.). R.C. was a recipient of a NSERC postdoctoral fellowship and A.Y.C. was a recipient of a Stanford Graduate Fellowship. pKOS168-190 was a gift from Kosan Biosciences, Inc.

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(13). The resulting TYLS KR2(G365S) reduced the standard model substrate *trans*-1-decalone, with k_{cat} 0.27±0.02 s^{-1} and k_{cat}/K_m 800±270 $M^{-1}s^{-1}$ (cf. k_{cat} 0.12-0.28 s^{-1}, k_{cat}/K_m 10-190 $M^{-1}s^{-1}$ for recombinant DEBS KR domains, ref 4).

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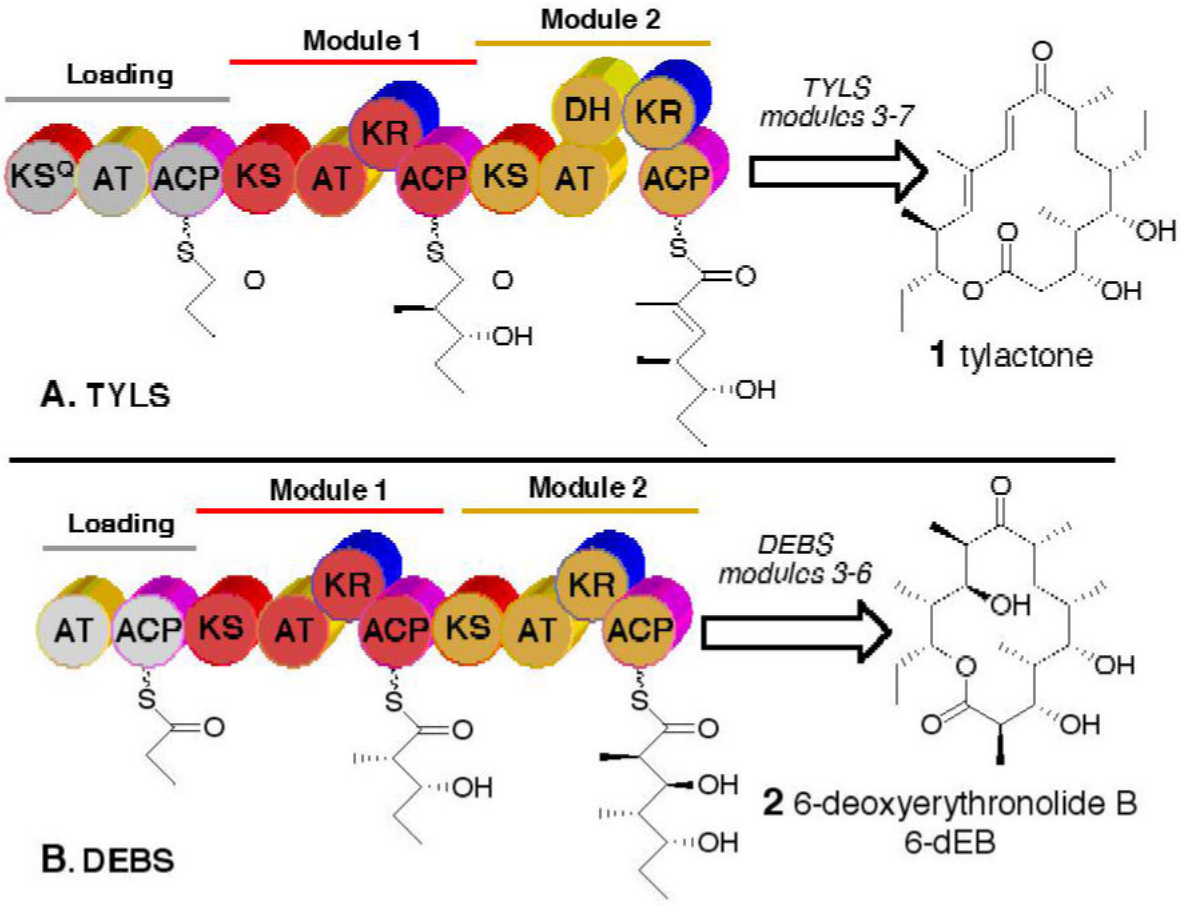
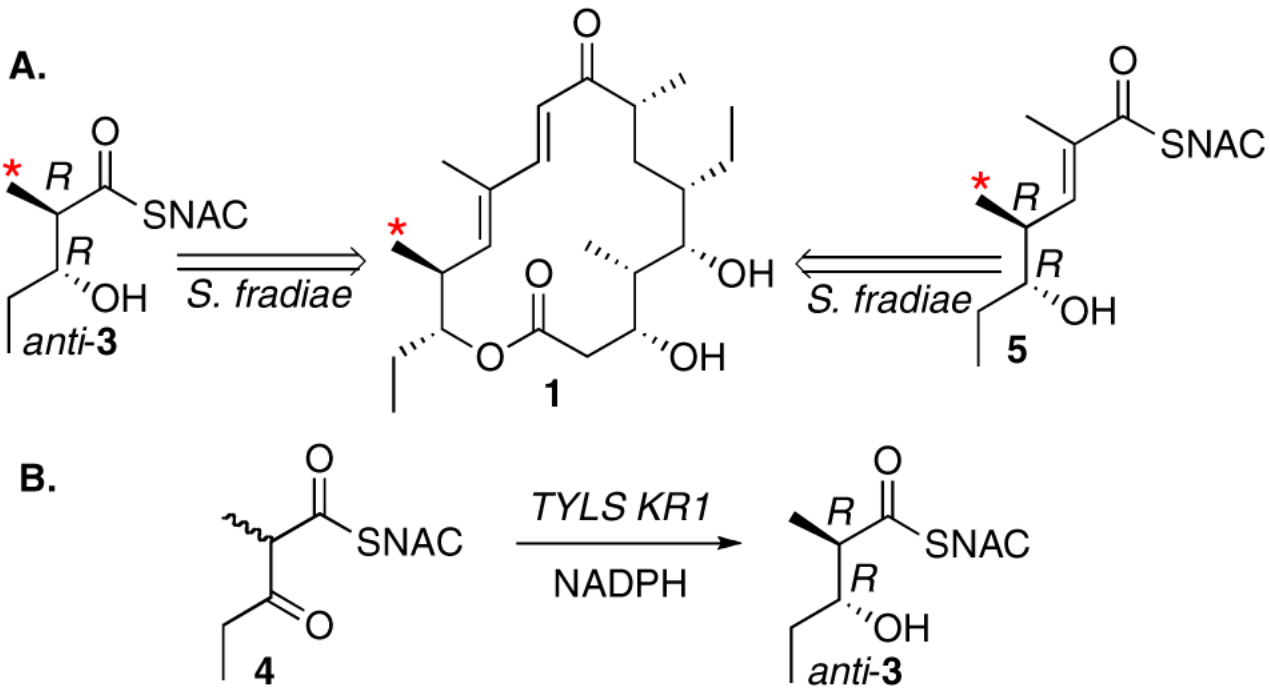


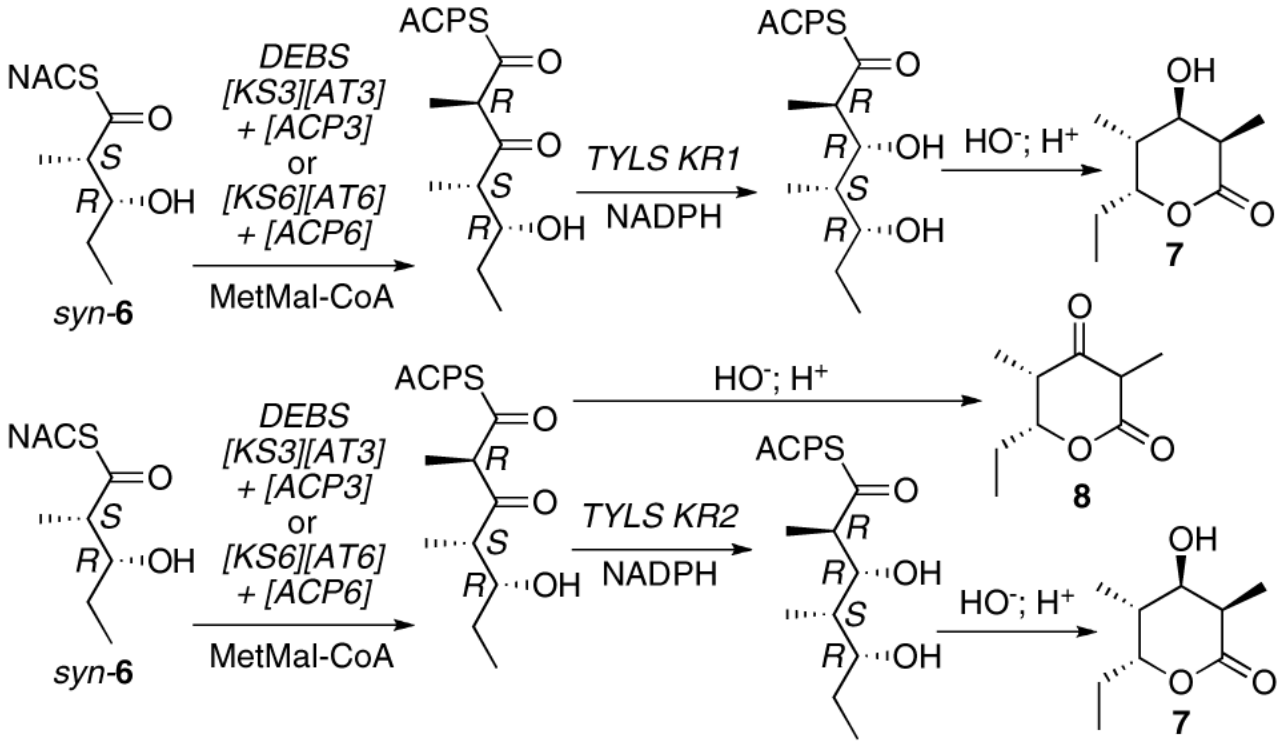
Figure 1.

Modular organization of (A) tylactone synthase (TYLS) and (B) 6-deoxyerythronolide B synthase (DEBS); the loading and first two extension modules of each PKS are shown. In addition to the three core catalytic domains — the β-ketoacyl-ACP synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains — individual extension modules carry specific combinations of tailoring ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER, not shown) domains.



Scheme 1.

(A) Incorporation of [2′-^{13}C]-**3** and [4′-^{13}C]-**5** into tylactone (**1**). (B) Diastereoselective reduction of racemic ketoester **4** to *anti*-(2*R*,3*R*) **3** by recombinant TYLS KR1.



Scheme 2.
Stereochemistry of triketide lactone formation catalyzed by TYLS KR1 and KR2 in combination with dissected DEBS [KS][AT] and [ACP] domains.