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## 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*,4*S*,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).

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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),<sup>2</sup> 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*<sup>3</sup> 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).<sup>4</sup> By contrast, 12- and 14-membered ring macrolides are derived from an initially-generated *syn*-(2*S*,3*R*)-diketide (Figure 1B).<sup>2,4,5</sup> 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.<sup>3</sup> Although the stereochemistry of the corresponding  $\beta$ -hydroxyacyl-ACP triketide intermediate is necessarily cryptic, a (3*R*)- $\beta$ -hydroxy configuration is predicted based on the presence of a conserved active site LDD sequence in the TYLS reductase KR2.<sup>6,7</sup> 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,<sup>7b</sup> with recombinant DEBS [KS3][AT3] didomain and [ACP3]<sup>2,8,9</sup> in the presence of *syn*-diketide **6**, methylmalonyl-CoA, and NADPH, followed by basic hydrolysis and acidification, as previously described,<sup>9,10</sup> 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.<sup>10</sup> 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<sup>2,4,6a,7b,9</sup> 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<sub>6</sub>-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.<sup>6a,11,12</sup> We therefore replaced Gly365 with a Ser by site-directed mutagenesis.<sup>13</sup> 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-<sup>14</sup>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<sub>4</sub>.<sup>10</sup> The finding that

both TYLS KR1 and KR2 reduce this intermediate to give a single *anti*-(**2R,3R,4S,5R**)-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.<sup>14</sup> Interestingly, the generation of a D-β-hydroxyl group by TYLS KR2 (*re* specificity) contrasts with that of DEBS KR2 which generates the *syn*-(**2R,3S,4S,5R**)-triketide en route to 6-dEB (Figure 1B),<sup>10</sup> 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.<sup>15</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

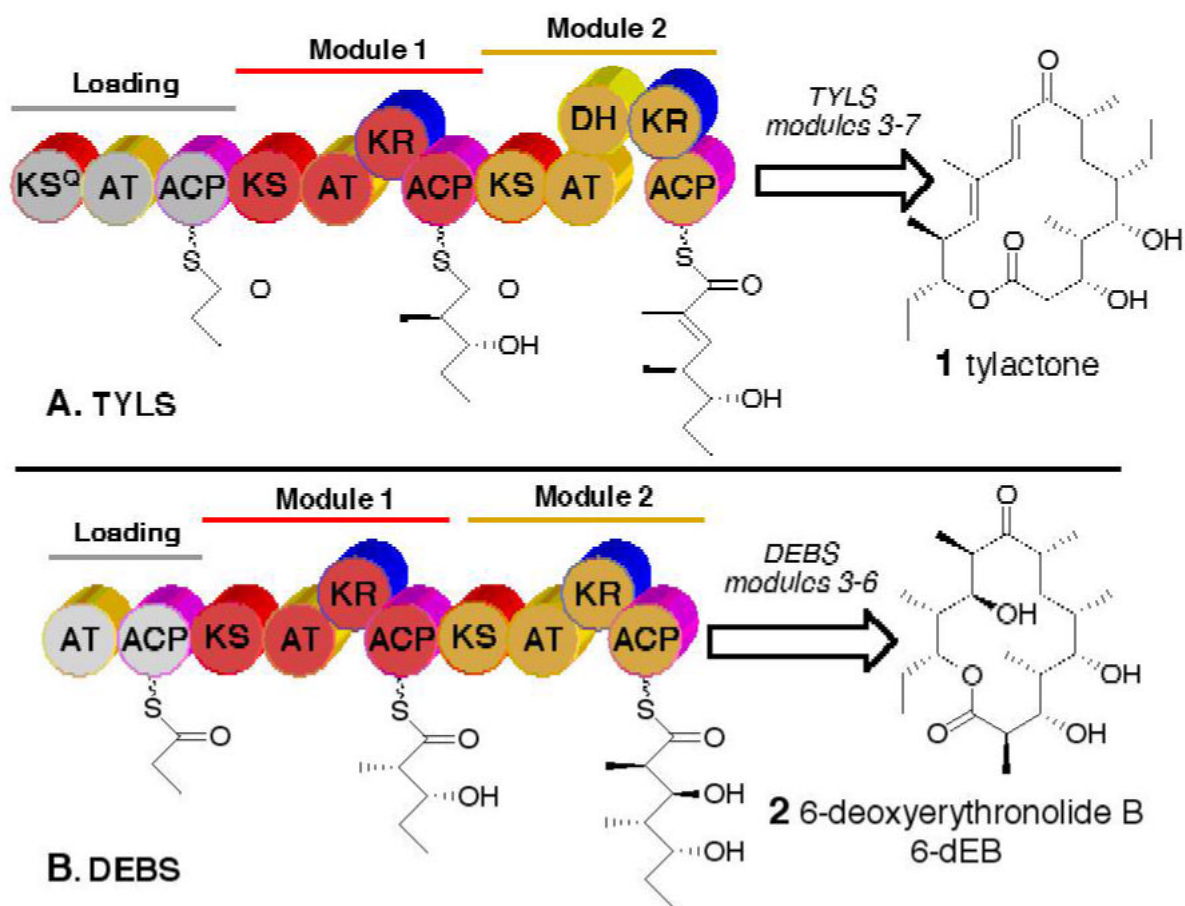
## Acknowledgments

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## References and Notes

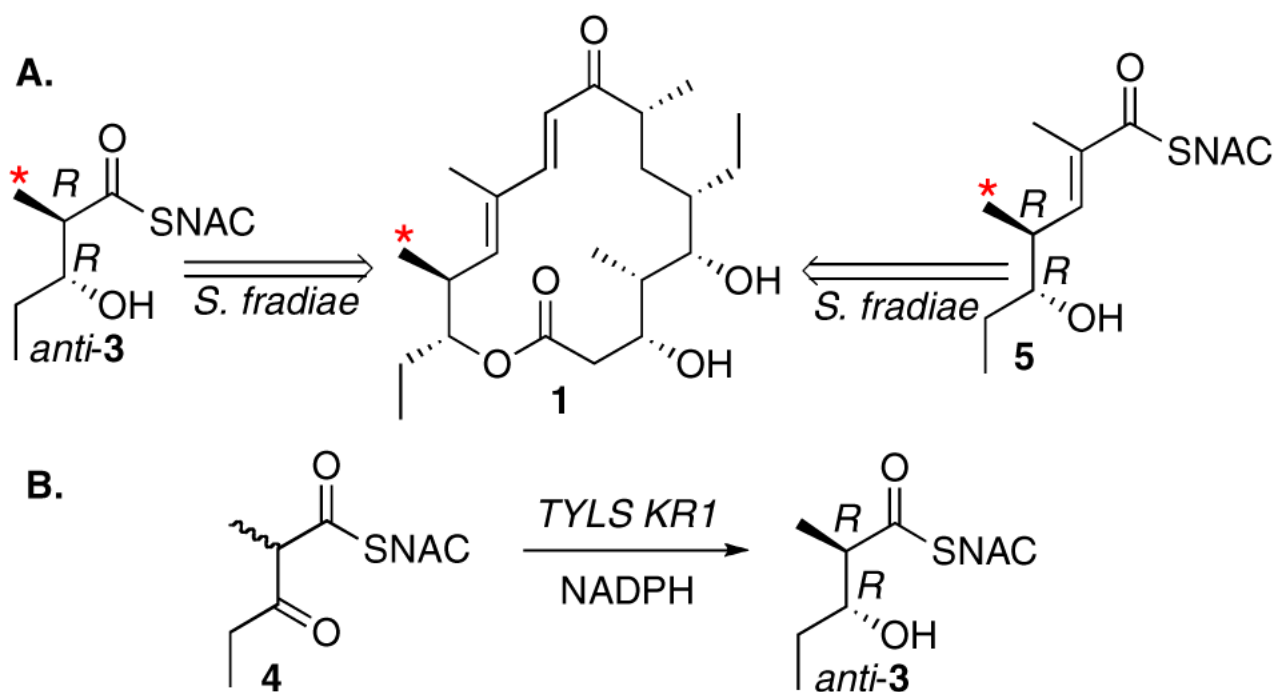
- (1). *tylG* Nucleotide sequence: DeHoffBSSuttonKLRosteckPRJr.Genbank U782891996 *TylG* has been heterologously expressed in *S. venezuelae*: Jung WS, Lee SK, Hong JS, Park SR, Jeong SJ, Han AR, Sohng JK, Kim BG, Choi CY, Sherman DH, Yoon YJ. *Appl. Microbiol. Biotechnol* 2006;72:763–769. [PubMed: 16493552] Cf. the closely related PKS clusters for niddamycin synthase: UniProt ID O30764 and platenolide (spiramycin) synthase: Kuhstoss S, Huber M, Turner JR, Pashal JW, Rao N. *Gene* 1996;183:231–236. [PubMed: 8996112] and BurgettSGKuhstossSARaoRNRRichardsonMARosteckPRJrPlatenolide synthase gene1999 U. S. Patent 5,945,320
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- (12). The variant of recombinant DEBS module 6 with a S2686A mutation in KR6 is reported to show no *in vitro* reductase activity, while the corresponding mutation in the complete DEBS PKS expressed in *S. lividans* retains detectable levels of *in vivo* reductase activity (ref 6a).
- (13). The resulting TYLS KR2(G365S) reduced the standard model substrate *trans*-1-decalone, with  $k_{\text{cat}} 0.27 \pm 0.02 \text{ s}^{-1}$  and  $k_{\text{cat}}/K_{\text{m}} 800 \pm 270 \text{ M}^{-1}\text{s}^{-1}$  (cf.  $k_{\text{cat}} 0.12\text{--}0.28 \text{ s}^{-1}$ ,  $k_{\text{cat}}/K_{\text{m}} 10\text{--}190 \text{ M}^{-1}\text{s}^{-1}$  for recombinant DEBS KR domains, ref 4).
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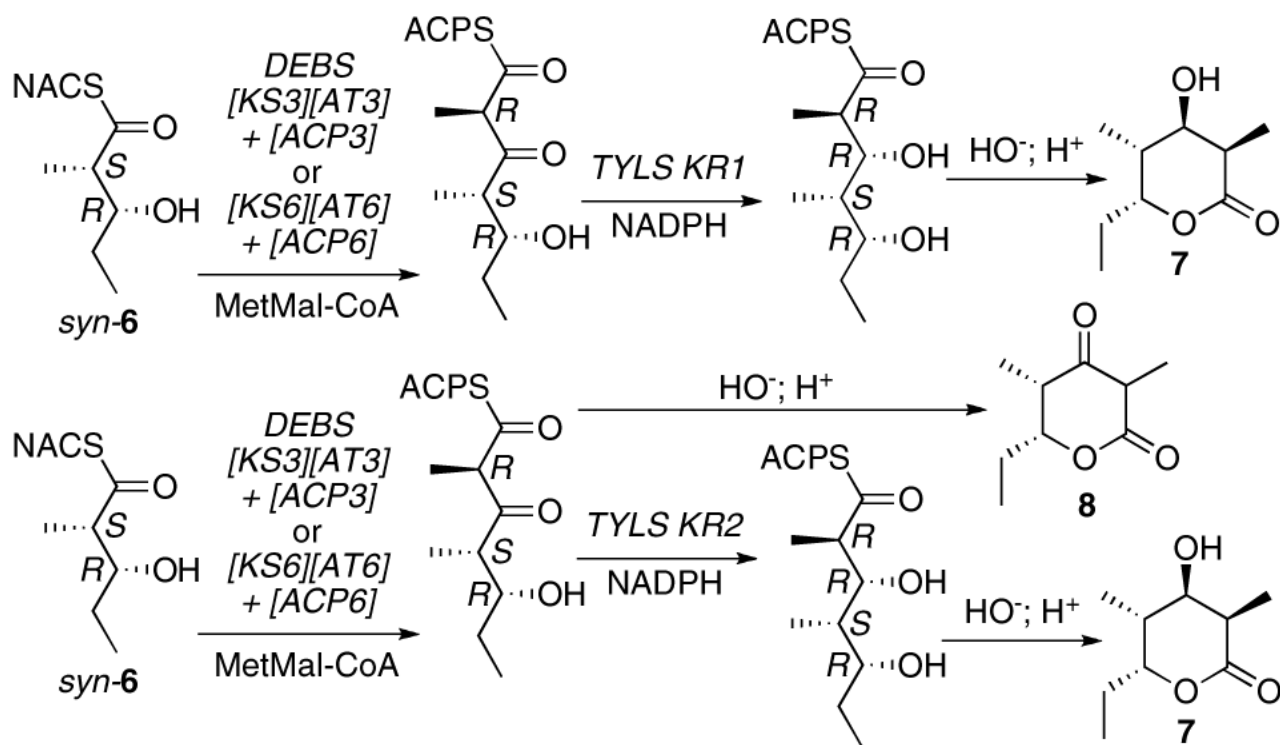


**Figure 1.**

Modular organization of (A) ty lactone 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  $\beta$ -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'-<sup>13</sup>C]-**3** and [4'-<sup>13</sup>C]-**5** into ty lactone (**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.