Organic LETTERS

Letter

pubs.acs.org/OrgLett

Open Access on 07/14/2015

Enediyne Polyketide Synthases Stereoselectively Reduce the β-Ketoacyl Intermediates to β-D-Hydroxyacyl Intermediates in Enediyne Core Biosynthesis

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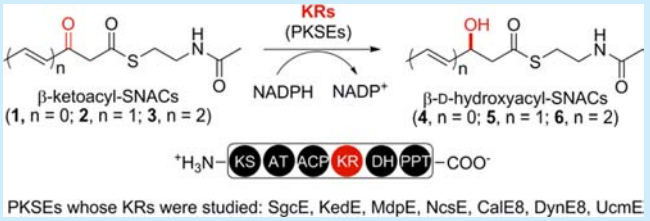
Supporting Information

ABSTRACT: PKSE biosynthesizes an enediyne core precursor from decarboxylative condensation of eight malonyl-CoAs. The KR domain of PKSE is responsible for iterative β-ketoreduction in each round of polyketide chain elongation. KRs from selected PKSEs were investigated in vitro with β-ketoacyl-SNACs as substrate mimics. Each of the KRs reduced the β-ketoacyl-SNACs stereoselectively, all affording the corresponding β-D-hydroxyacyl-SNACs, and the catalytic efficiencies (k_{cat}/K_M) of the KRs increased significantly as the chain length of the β-ketoacyl-SNAC substrate increases.



Polyketides are a structurally diverse family of natural products with a broad range of biological activities. While their biosynthesis is mechanistically similar to that of fatty acids, reductive modifications of the β-keto groups to different extents at each cycle of polyketide chain elongation differ from that for fatty acid biosynthesis and result in polyketides bearing a variety of functionalities at the β-positions. Among polyketides, the enediyne natural products are some of the most potent antitumor antibiotics known to date.[1] Common to the enediyne natural products is a 9- or 10-membered carbocyclic enediyne core. Biosynthesis of the carbon skeleton of enediyne cores from eight molecules of malonyl-CoA is catalyzed by the enediyne polyketide synthase (PKSE).[2,3] PKSEs are iterative type I PKSs that are characterized with six domains, ketoacyl synthase (KS), acetyltransferase (AT), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), and phosphopantetheinyl transferase (PPT) (Figure 1A).[2,3] While this domain organization is quite distinct from other type I PKSs, it is absolutely conserved among all PKSEs known to date (Figure S1, Supporting Information (SI)).

While the indispensable role PKSE plays in enediyne natural product biosynthesis has been unambiguously established,[2] it remains elusive how the enediyne cores are constructed and what determines the biosynthetic divergence between 9- versus 10-membered enediynes.[3] Based on phylogenetic analysis of the known PKSEs, we had previously proposed that the PKSEs for 9- and 10-membered enediyne cores were distinct, and this distinction might be critical to the alternate folding and early modification steps that lead to the divergence of the 9- and 10-membered enediynes.[3a,b] This proposal was supported by in vivo studies, in which *Streptomyces globisporus* SB1005, a Δ*sgcE* mutant strain of the C-1027 producer *S. globisporus*, and *Streptomyces carzinostaticus* SB5002, a Δ*ncsE* mutant strain of the neocarzinostatin (NCS) producer *S. carzinostaticus*, can be functionally complemented to restore C-1027 or NCS production, respectively, by PKSE genes from selected 9-

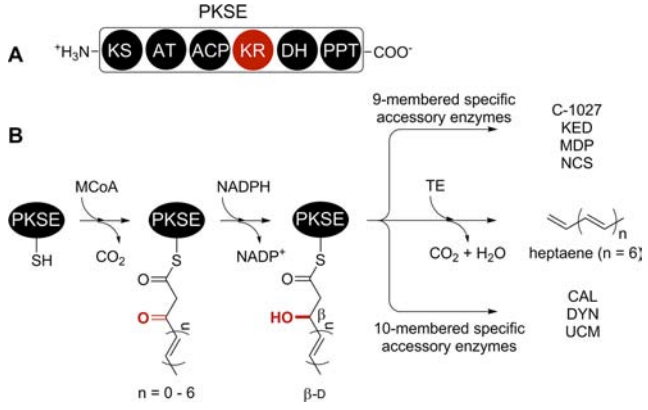


Figure 1. Enediyne polyketide synthase (PKSE) and its proposed roles in 9- and 10-membered enediyne biosynthesis. (A) Domain organization of PKSEs: KS, ketoacyl synthase; AT, acetyltransferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; and PPT, phosphopantetheinyl transferase. (B) A unified model for enediyne biosynthesis featuring common PKSE chemistry with accessory enzyme-directed pathway divergence to 9- and 10-membered enediyne cores. The KR domain and its catalyzed chemistry are highlighted in red. MCoA, malonyl-CoA; TE, thioesterase. See Figure S1 (SI) for structures of C-1027, calicheamicin (CAL), dynemicin (DYN), kedarcidin (KED), maduropeptin (MDP), neocarzinostatin (NCS), and uncialamycin (UCM).

Received: June 18, 2014
Published: July 14, 2014

   dx.doi.org/10.1021/ol501767v | *Org. Lett.* 2014, 16, 3958–3961

membered, but not 10-membered, enediyne pathways.[3c] Subsequent in vitro studies of selected PKSEs, alone or in combination with the associated thioesterases (TEs), revealed that PKSEs most likely biosynthesized a common intermediate that was then morphed into the 9- and 10-membered scaffolds by pathway-specific accessory enzymes, and the reconstituted PKSE-TE of both 9- and 10-membered enediyne core origin afforded enzymatic synthesis of the same set of products, most notably a heptaene (Figure 1).[4] These in vitro findings were further supported by (i) heptaene production upon expressing selected *pksE* genes of both 9- and 10-membered enediyne core origin with their cognate and noncognate TE genes in both *E. coli* and *Streptomyces* and (ii) detection of heptaene from selected native producers of both nine- and ten-membered enediyne natural products (Figure 1).[3c,d]

Despite these advances, it is far from certain that the stereochemistry of the double bonds deduced from the isolated heptaene could be directly correlated to the PKSE-tethered precursor that is ultimately morphed into the 9- or 10-membered enediyne cores. The KR domain catalyzes the iterative β-ketoreduction in each round of polyketide chain elongation, affording the PKSE-tethered β-hydroxyacyl intermediates that are subsequently dehydrated by the DH domain to the double bonds (Figure 1B). For bacterial noniterative type I PKSs, the stereochemistry of β-hydroxyacyl intermediates destines the DH domain in the ensuing dehydration step to afford a cis- or trans-double bond in the growing polyketide products.[5,6] Thus, it is tempting to speculate that the stereochemistry of PKSE-tethered β-hydroxyacyl intermediates could dedicate the geometry of the resultant double bonds, the difference of which in the PKSE-tethered full length polyene products could impact on eventual formation of the 9- or 10-membered enediyne core by the pathway-specific accessory enzymes.

Herein, we report in vitro characterization of KRs from seven selected PKSEs using β-ketoacyl-*N*-acetylcysteamines (SNACs) with varying acyl chain lengths as substrate mimics. Remarkably, each of the KRs reduced the β-ketoacyl-SNACs stereoselectively, all affording the corresponding β-D-hydroxyacyl-SNACs, and the catalytic efficiencies (k_{cat}/K_M) of the KRs increased significantly as the chain length of the β-ketoacyl-SNAC substrate increases.

The KR domains from four nine-membered [C-1027, kedarcidin (KED), maduropeptin (MDP), and neocarzinostatin (NCS)] and three 10-membered [calicheamicin (CAL), dynemicin (DYN), and uncialamycin (UCM)] PKSEs were selected in this study (Figure S1, SI).[2] While little is known about the KR domain of PKSE, KRs of type I PKSs have been extensively characterized mechanistically and structurally.[5−7] These KR domains, averaging ∼466 amino acids, catalyze stereoselective β-keto reduction of the PKS-tethered β-ketoacyl intermediates (via the ACP domain), utilizing NADPH. The KR domains have been further classified into A- or B-type. A-type KRs, featuring a conserved Trp, generate a β-L-hydroxyacyl intermediate, and B-type KRs, featuring an LDD motif, afford a β-D-hydroxyacyl intermediate (Figure 2A); the presence of the LDD motif in the B-type KRs and the absence of the LDD motif, accompanied by the presence of the conserved Trp, in the A-type KRs have often been used to discriminate the two types of KRs.[6,7] The KR domains from PKSEs show high sequence homology among each other, as well as to known KRs of type I PKSs, possessing the critical catalytic residues (Lys, Ser, and Tyr) and the conserved Gly-rich motif for

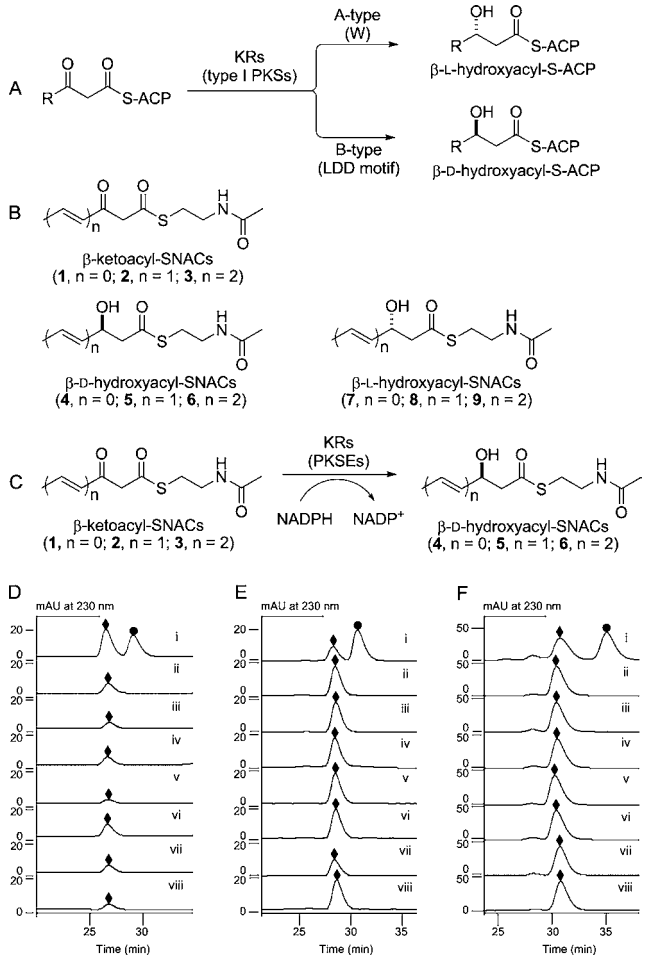


Figure 2. In vitro assay of KRs of PKSEs for both 9- and 10-membered enediyne core biosynthesis using β-ketoacyl-SNACs as substrate mimics revealing the same intrinsic stereoselectivity to afford the β-D-hydroxyacyl-SNAC products. (A) A- and B-type KRs of type I PKS affording β-L- or β-D-hydroxyacyl intermediates in polyketide biosynthesis featuring the conserved residue and diagnostic motif (i.e., W for A-type and LDD for B-type).[6,7] ACP, acyl carrier protein. (B) The synthesized β-ketoacyl-SNACs as substrate mimics (**1**−**3**) and β-D-hydroxyacyl-SNACs (**4**−**6**) and β-L-hydroxyacyl-SNACs (**7**−**9**) as authentic standards of the reduced products. (C) In vitro assay of KRs of PKSEs using β-ketoacyl-SNACs as substrate mimics in the presence of NADPH. Chiral HPLC analysis of KR-catalyzed stereoselective reduction of the β-ketoacyl-SNAC substrates **1** (D), **2** (E), and **3** (F) to the corresponding β-D-hydroxyacyl-SNAC products **4**, **5**, and **6**. (i) Authentic standards of the β-D-hydroxyacyl-SNAC (♦) and β-L-hydroxyacyl-SNAC (●) products; (ii) KR of SgcE; (iii) KR of KedE; (iv) KR of MdpE; (v) KR of NcsE; (vi) KR of CalE8; (vii) KR of DynE8; (viii) KR of UcmE.

NADPH binding. However, they lack both the LDD motif and the conserved Trp residue, the absence of both of which makes it difficult to classify the KRs of PKSEs into A- or B-type KRs, thereby predicting the stereochemistry of the resultant β-hydroxyacyl products (Figure S2, SI).[6,7]

The boundaries of the KRs of PKSEs were defined by following those established for the KRs of type I PKSs.[5−7] The seven KRs, i.e., KRs from SgcE (residues 1018−1452), KedE (residues 1008−1448), MdpE (residues 1033−1466), NcsE (residues 1055−1488), CalE8 (residues 1025−1448), DynE8 (residues 1011−1437), and UcmE (residues 1003−1444), were overproduced in *E. coli* BL21(DE3) (Figures S1, S2, and Table

dx.doi.org/10.1021/ol501767v | *Org. Lett.* 2014, 16, 3958−3961

S1, SI) as N-terminal His_6-tagged fusion proteins. Each of the KRs was purified to homogeneity and showed as a single band upon SDS-PAGE analysis consistent with the predicted molecular weight of each protein (Figure S3, SI).

Three β-ketoacyl-SNACs with varying chain lengths (i.e., **1**, **2**, and **3**) were next synthesized as substrates to directly test the activity of KRs in vitro (Figure 2B and Figures S4−S9, SI). β-Ketoacyl-SNACs are known mimics of the ACP-tethered growing polyketide intermediates.[5−7] The three substrates were designed to mimic the nascent polyketide intermediates in the early rounds of chain elongation in enediyne core biosynthesis, and **1**, **2**, and **3** mimic the diketide, triketide, and tetraketide intermediate, respectively (Figure 1B). Since the stereoselectivity of the selected KRs of PKSEs cannot be predicted a priori, we also prepared both enantiomers of the corresponding reduced products, i.e., β-L-hydroxyacyl-SNACs (**4**−**6**) and β-D-hydroxyacyl-SNACs (**7**−**9**) (Figure 2B and Figures S10−S15, SI), as authentic standards for chiral HPLC analysis, and the absolute stereochemistry of β-hydroxyacyl-SNACs was independently established by Mosher's method (Figures S16−S21, SI).

The enzymatic activity for each of the seven KRs was investigated by in vitro assay of the three substrate mimics in the presence of NADPH (Figure 2C). The enzymatic reaction courses were followed by HPLC analysis for the disappearance of the substrate (**1**, **2**, or **3**) and appearance of the products (**4**, **5**, or **6**) and by continuous photometric measurement at 340 nm for the consumption of NADPH (SI). Thus, upon addition of the KR proteins to reaction mixtures containing the substrate mimics and NADPH, the absorbance at 340 nm began to decline immediately, indicative of NADPH consumption (Figure S22, SI), and HPLC analysis of the assay mixtures confirmed time-dependent reduction of the substrates to specific products. Close examination of the reduction products by chiral HPLC revealed that the seven KRs all reduced the β-keto group in **1**, **2**, or **3** efficiently to the corresponding β-D-hydroxyacyl-SNACs **4**, **5**, or **6**, respectively, the identities of which were unambiguously established by LCMS and chiral HPLC analysis in comparison with synthetic standards (Figure 2D−F). These findings provide direct experimental evidence, supporting that the KRs in PKSEs can iteratively reduce the β-ketoacyl intermediates in each round of chain elongation, and revealing for the first time that, regardless of the origin of the PKSEs, the KRs exhibit the same intrinsic stereoselectivity toward the growing β-ketoacyl intermediates, affording the β-D-hydroxyacyl intermediates in the biosynthesis of both 9- and 10-membered enediyne cores.

The three β-ketoacyl-SNACs with varying chain lengths also provided an excellent opportunity to probe the relative rates for the reductive modification steps at each round of chain elongation, and such rates could have fundamental impact on the net metabolic pathway flux according to the processive mechanism for polyketide biosynthesis. Although poor substrate solubility prevented us from using substrate concentrations well above the expected K_M values to determine the single-substrate kinetic constants, the apparent k_{cat}/K_M values for each of the KRs were obtained from the linear fit of the limiting case of the Michaelis−Menten model ($v = (k_{cat}/K_M)[S][E]_0$) for low substrate concentration (where $[S] \ll K_M$).[5d] Thus, each of the KRs was assayed with the three substrate mimics, and the enzymatic reactions were followed spectrophotometrically by continuously following the absorbance at 340 nm to measure NADPH consumption (Figure S23, SI). The calculated k_{cat}/K_M for each of the KRs was summarized in Table 1. While each of the KRs showed similar catalytic efficiencies (k_{cat}/K_M) toward the same substrate, the catalytic efficiencies of the KRs all increased significantly as the chain length of the β-ketoacyl-SNAC substrates increases. Such properties in fact would be consistent with the processive mechanism of polyketide biosynthesis so that all elongating intermediates could rapidly proceed to completion without stalling the biosynthetic machinery. It would be very interesting to investigate if the other domains (KS and DH) of PKSE have similar intrinsic catalytic properties.

Table 1. Pseudo-First-Order Kinetic Parameters of KRs of the Selected PKSEs toward the Three β-Ketoacyl-SNACs

	k_{cat}/K_M (s^{-1} M^{-1})[a] (β-ketoacyl-SNACs)		
KR (PKSE)	**1**	**2**	**3**
SgcE	12 ± 2	17 ± 5	240 ± 34
KedE	6.1 ± 2.0	27 ± 3	110 ± 12
MdpE	5.7 ± 0.8	15 ± 2	140 ± 8
NcsE	8.4 ± 1.5	22 ± 2	170 ± 20
CalE8	15 ± 2	28 ± 2	210 ± 24
DynE8	4.7 ± 0.6	13 ± 1	160 ± 34
UcmE	3.1 ± 0.7	18 ± 1	133 ± 31

[a]Based on the linear fit of the limited case of the Michaelis−Menten model ($v = (k_{cat}/K_M)[S][E]_0$) for low substrate concentration (where $[S] \ll K_M$).[5d] Data are reported as means of triplicate experiments (Figure S23, SI).

Classification of KRs into A- or B-type according to the conserved residues and motifs (Figure 2A) was based mainly on bioinformatics analysis of noniterative type I PKSs of bacterial origin.[6,7] Each KR acts once, during each cycle of chain elongation, to furnish a β-D- or β-L-hydroxy group in the growing polyketide intermediate, and this model has been supported by site-directed mutagenesis studies and X-ray structural analysis of several KRs. The conserved Trp in the A-type KRs and the LDD motif in the B-type KRs reside on opposite sides of the active site groove and control the entrance of substrates into the active site from different sides. The guided entrance of substrates positions the β-keto group in opposite orientations relative to the pro-4S hydride of NADPH and, as such, stereoselective attack of the β-keto group by the hydride results in a β-D- or β-L-hydroxyacyl product.[6,7c,d] PKSEs are iterative type I PKSs that are of bacterial origin,[3] and iterative type I PKSs for aromatic polyketide biosynthesis in bacteria are also known.[8] Although the KRs of iterative PKSs show significant sequence homology to KRs of their noniterative counterparts, the diagnostic Trp residue and LDD motif for A- or B-type classification are not conserved (Figure S2, SI). The stereochemistry of KRs of iterative type I PKSs therefore cannot be similarly predicted according to the model of KRs of noniterative type I PKSs, as exemplified by the current study of the KRs of PKSEs. Similar findings have also been observed for fungal iterative type I PKSs.[9] It would therefore surely be rewarding to compare and contrast the KR structures of iterative and noniterative type I PKSs, thereby deciphering the molecular control of the β-hydroxy stereochemistry in polyketide biosynthesis.

KR of iterative type I PKS must act iteratively to catalyze multiple rounds of β-ketoreduction in polyketide biosynthesis.[3,8,9] Although one would expect each ketoreduction to occur with the same stereochemistry, as supported by the

dx.doi.org/10.1021/ol501767v | Org. Lett. 2014, 16, 3958−3961

current study of the KRs of PKSEs for the first three rounds of chain elongation, caution has to be taken not to overinterpret this finding, and substrate-dependent stereoselectivity has indeed been reported for a KR of fungal iterative PKS.[9] For type I PKSs, it is the stereochemistry of β-hydroxyacyl intermediates, rather than the cognate DH domain, that has been correlated to the double bond geometry of the resultant elongating polyketide products[5,6,7c,d] If this would be the same for PKSEs, the β-D-hydroxyacyl products from all the KRs examined in the current study would suggest polyene intermediates with all double bonds in trans configuration in the biosynthesis of both nine- or ten-membered enediyne core, a provocative prediction that begs for experimental verification.

Finally, the current study unambiguously established that the KRs of PKSEs of both 9- and 10-membered enediynes generate identical β-D-hydroxyacyl intermediates at least in the first three rounds of chain elongation (Figures 1B and 2C–F). These findings further support the previous conclusion that PKSE chemistry does not direct biosynthetic divergence between 9- and 10-membered enediynes.[3,4]

■ ASSOCIATED CONTENT

Supporting Information

Complete description of materials and methods, Table S1, Figures S1–S23. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Y. Li, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing, China, for the wild-type *Streptomyces globisporus* strain that produces C-1027 and Dr. Julian Davies, University of British Columbia, Vancouver, Canada, for the wild-type *Streptomyces uncialis* DCA2648 strain that produces uncialamycin. This work was supported in part by NIH Grant No. CA78747.

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