ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

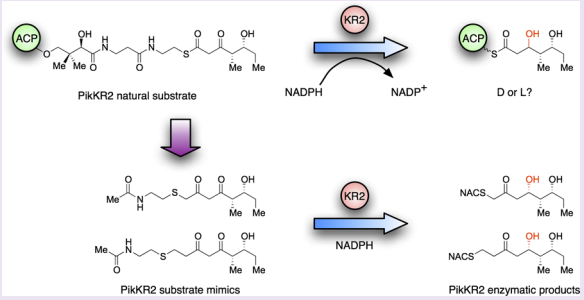
Polyketide Intermediate Mimics as Probes for Revealing Cryptic Stereochemistry of Ketoreductase Domains

Yang Li,[†] William D. Fiers,[†] Steffen M. Bernard,[∥,§] Janet L. Smith,[‡,§] Courtney C. Aldrich,*[,†] and Robert A. Fecik*[,†]

[†]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455, United States
[∥]Chemical Biology Program, [‡]Department of Biological Chemistry, and [§]Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109, United States

Supporting Information

ABSTRACT: Among natural product families, polyketides have shown the most promise for combinatorial biosynthesis of natural product-like libraries. Though recent research in the area has provided many mechanistic revelations, a basic-level understanding of kinetic and substrate tolerability is still needed before the full potential of combinatorial biosynthesis can be realized. We have developed a novel set of chemical probes for the study of ketoreductase domains of polyketide synthases. This chemical tool-based approach was validated using the ketoreductase of pikromycin module 2 (PikKR2) as a model system. Triketide substrate mimics **12** and **13** were designed to increase stability (incorporating a nonhydrolyzable thioether linkage) and minimize nonessential functionality (truncating the phosphopantetheinyl arm). PikKR2 reduction product identities as well as steady-state kinetic parameters were determined by a combination of LC-MS/MS analysis of synthetic standards and a NADPH consumption assay. The D-hydroxyl product is consistent with bioinformatic analysis and results from a complementary biochemical and molecular biological approach. When compared to widely employed substrates in previous studies, diketide **63** and *trans*-decalone **64**, substrates **12** and **13** showed 2−10 fold lower K_M values (2.4 ± 0.8 and 7.8 ± 2.7 mM, respectively), indicating molecular recognition of intermediate-like substrates. Due to an abundance of the nonreducable enol-tautomer, the k_{cat} values were attenuated by as much as 15−336 fold relative to known substrates. This study reveals the high stereoselectivity of PikKR2 in the face of gross substrate permutation, highlighting the utility of a chemical probe-based approach in the study of polyketide ketoreductases.



Bacterial type I polyketide synthases (PKSs) are large, multifunctional, modular proteins that biosynthesize a large class of diverse, polyoxygenated secondary metabolites with important pharmacological and therapeutic activities.[1−3] All modular type I PKSs contain a loading module and several extension modules, each responsible for one round of polyketide chain elongation and β-carbon processing, organized in an assembly line fashion. Each extension module features a minimal set of three domains responsible for a two-carbon extension of the chain elongation intermediate: acyl carrier protein (ACP), acyltransferase (AT), and ketosynthase (KS) domains. All chain elongation intermediates are bound to the noncatalytic ACP domains through thioester bonds with the phosphopantetheinyl arm of the ACP domain. After extension, the newly formed β-keto thioester intermediate is frequently followed by sequential β-carbon processing by ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains. The KR domain is the most abundant β-carbon processing domain throughout all PKSs. It stereoselectively reduces the newly formed β-ketone to an alcohol with NADPH and can also control the stereochemistry of the α-substituent through enzymatic epimerization.[4−8] Following multiple rounds of chain elongation and β-carbon processing, a thioesterase (TE) domain catalyzes intramolecular cyclization or hydrolysis to release a macrolactone or carboxylic acid, respectively. Bacterial type I PKSs such as 6-deoxyerythronolide B synthase (DEBS), the biosynthetic pathway for erythromycins, have been well-studied for their potential in drug discovery by combinatorial biosynthesis and metabolic engineering.[9] We, and others, have studied the pikromycin (Pik) PKS of *Streptomyces venezuelae* (Figure 1) for these purposes based upon its unique ability to produce both 12- and 14-membered macrolactones, including 10-deoxymethynolide (**1**), narbonolide (**2**), methymycin (**3**), and pikromycin (**4**).[10−15]

Each catalytic domain in a PKS conducts its reaction in a highly stereoselective manner. Generally, the stereochemical outcome of the β-carbon processing domains can be readily deduced by examining the absolute stereochemistry of the natural product. For instance, the initial KR domain of the Pik PKS (KR1) reduces the β-ketone to an alcohol, which establishes the configuration at C-11 of methymycin and C-13 of pikromycin

Received: May 16, 2014
Accepted: October 9, 2014
Published: October 9, 2014

   dx.doi.org/10.1021/cb5006883 | ACS Chem. Biol. 2014, 9, 2914−2922

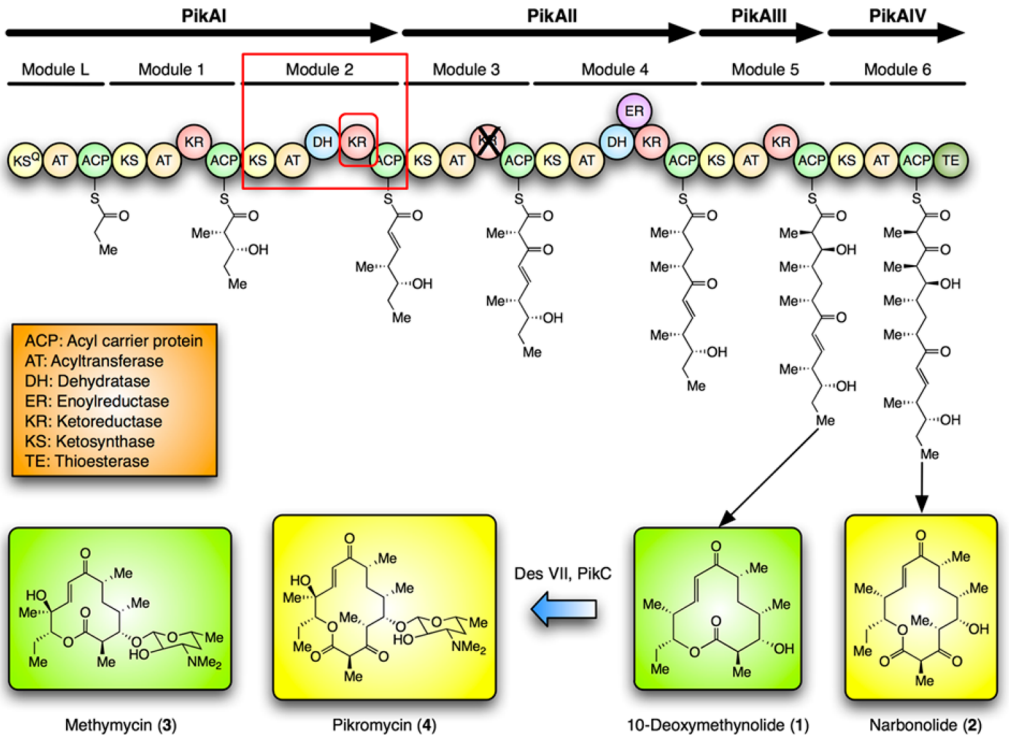


Figure 1. Pikromycin and methymycin biosynthetic pathway in *Streptomyces venezuelae* ATCC 15439.

(Figure 1). However, this observational method cannot be utilized for modules that contain a DH domain since the resulting dehydration eliminates the stereogenic center as seen in modules 2 and 4 of the Pik PKS. This type of "hidden" stereoselectivity, when referral to the mature polyketide gives no implication of the stereochemical outcome of the KR domain of interest, has been termed cryptic stereochemistry.

Biochemical and molecular biological approaches have been widely used to provide valuable insights into the mechanism as well as substrate and stereoselectivity of PKS domains, especially KRs.[16−19] Cane and co-workers established the stereochemical outcome of PikKR2 by engineering Pik module 2 with PikTE and inactivation of PikDH2 by site-directed mutagenesis (Figure 2a).[16] Incubation of diketide thioester **5**, methylmalonyl-CoA, and NADPH with this construct produced triketide lactone **6** exclusively, establishing the D-configuration of the C3-alcohol. As a general approach, this method may require extensive work on protein expression, mutant construction, and product detection. More importantly, most of the substrates used in this and other previous studies are simple diketide analogs, which likely show low affinity and poor stereoselectivity for the study of domains in later, downstream modules due to poor structural similarity to the native substrates.[11] For example, in a recent study with KR domains from the amphotericin, pikromycin, and spinosyn PKSs (AmpKR10, PikKR5, and SpnKR3), multiple diastereomeric products resulted from use of the simple (2*RS*)-methyl-3-oxobutane-SNAC (*N*-acetylcysteamine) thioester as the substrate.[20]

We describe here an alternative, chemical probe-based approach to understand the structural and mechanistic features of KR domains. This method entails incubation of synthetic substrate mimics with excised KR domains and observation of enzymatic products by LC-MS/MS (Figure 2b). Compared to previous approaches, we envision our new method offers unique insights in three aspects. First, this method reduces the

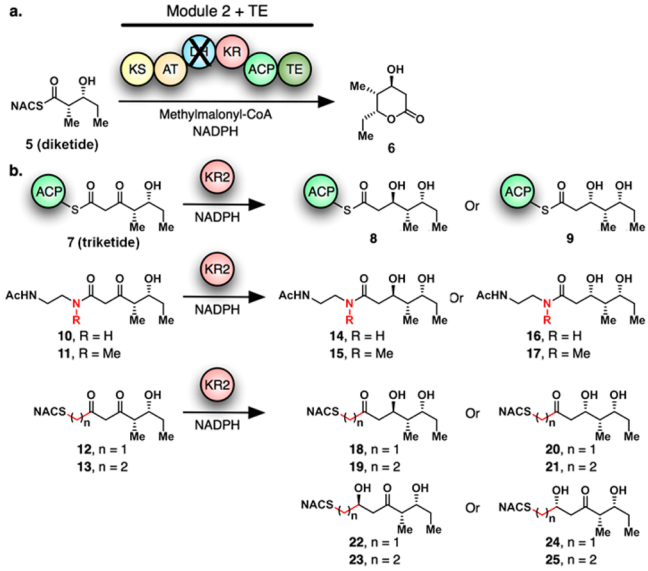


Figure 2. (a) Previous approach to determining the cryptic stereochemistry in PikKR2 using an engineered Pik module 2.[19] (b) Design of PikKR2 substrate mimics **10**−**13** for a KR-based assay to determine cryptic stereochemistry.

biochemical and molecular biological workload in studying cryptic stereochemistry since it requires only a single domain versus an entire multimodular PKS protein. Additionally, the substrate is not tethered to the ACP domain; thus neither a TE domain nor a complicated workup procedure is required to release the enzymatic product. Second, this intermolecular assay design allows for interrogation of the steady-state kinetic parameters of the individual excised domains. Testing of non-natural substrates enables investigation of substrate−protein interactions. It would also be possible to investigate protein−protein interactions if the probes are linked to the ACP domain.

dx.doi.org/10.1021/cb5006883 | *ACS Chem. Biol.* 2014, 9, 2914−2922

Third, compatibility of substrates is guaranteed. Given that the substrate is specifically designed on the basis of the natural substrate, substrate and stereoselectivity is ensured due to nearly identical steric properties.[21,22]

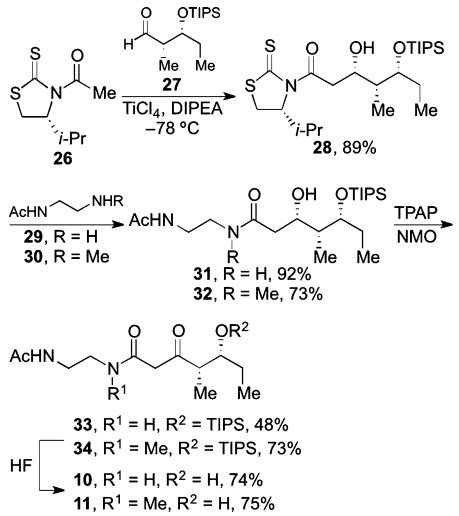
Recent research has shown a strong correlation between the amino acid sequence of KR domains and the stereochemistry of the KR products. In KRs, the fingerprint LDD motif predicts a D-alcohol product, while KRs with a W motif produce an L-alcohol.[23] Moreover, absolute configuration of an α-substituent can be determined through additional motif indicators.[24] Although the amino acid sequence can provide insight into the stereochemistry of uncharacterized KRs, current work has revealed several exceptions. For instance, rifamycin (Rif) KR7 and RifKR10 do not possess the signature W motif, yet they still yield L-alcohol products.[25] Due to the empirical nature of the fingerprint method, the predicted stereochemical outcome of cryptic domains is not absolutely infallible. The geometry of the olefin created by the DH domain may also indicate the stereochemistry of the alcohol precursor, as *trans*-olefins arise from D-alcohols.[22,23] However, the olefin geometry may be reduced by the following ER domain or isomerized later in the biosynthetic pathway. This highlights the need for novel experimental methods to complement signature sequence methods to decipher cryptic stereochemistry.

RESULTS AND DISCUSSION

In light of previous research (vide supra), we chose PikKR2 to validate this chemical probe-based approach. Both PikKR2 substrate and product mimics were designed and synthesized (Figure 2b). The substrate mimic designs were based on the natural substrate of PikKR2 (**7**) with two modifications. First, truncation of the ACP-phosphopantetheinyl arm to a NAC-based substrate was planned to ease synthetic access to the target molecules. Since the ACP-phosphopantetheinyl arm does not affect the stereocontrol of KRs, NAC thioester derivatives have been widely used as surrogates of natural substrates.[20,22,26] Second, since 5-hydroxy triketide thioesters are prone to rapid cyclization to triketide lactones,[21] we sought to replace and mimic the electrophilic thioester of the native PikKR2 intermediate with an amide or thioether bond. Thus, two series of substrate mimics with stabilized structures were designed: (1) incorporation of secondary (**10**) or tertiary (**11**) amides as stable thioester isosteres[27] and (2) thioether analogs **12** and **13**, which are based upon previous work of us and others.[13,28] Although thioethers **12** and **13** have one- and two-carbon spacers between the C1-carbonyl of the triketide and the NAC group, we anticipated that the long phosphopantetheinyl-binding channel of PikKR2 could accommodate this variance. Additional support for this approach was suggested by prior work of others with malonyl CoA analogs where the thioester was replaced with a ketone. These malonyl CoA analogs, when added to fermentation broths, underwent chain elongation, β-processing, and chain termination to release off-loaded intermediates.[29−33] For analysis of the enzymatic reaction of substrate mimics **10**−**13**, synthesis of both potential diastereomeric products **14**−**21** of each substrate mimic was also required. Since thioether substrate mimics **12** and **13** have two ketones that could potentially be reduced by PikKR2, we also synthesized diols **22**−**25** (as diastereomeric mixtures). Diols **22**−**25** would result from reduction of the C1-ketone (polyketide numbering) instead of expected reduction of the C3-ketone. As C1 reduction is an unnatural event, and does not occur in the biosynthetic pathway, the C1-epimeric product mixture was deemed sufficient for these studies.

Synthesis of Substrate Mimics. Synthesis of secondary and tertiary amide substrate analogs **10** and **11** commenced with the acetate aldol reaction of thiazolidinethione **26**[34,35] with aldehyde **27**[36,37] to give alcohol **28** as the major diastereomer (Scheme 1).

Scheme 1. Synthesis of Amide Substrate Analogs 10 and 11



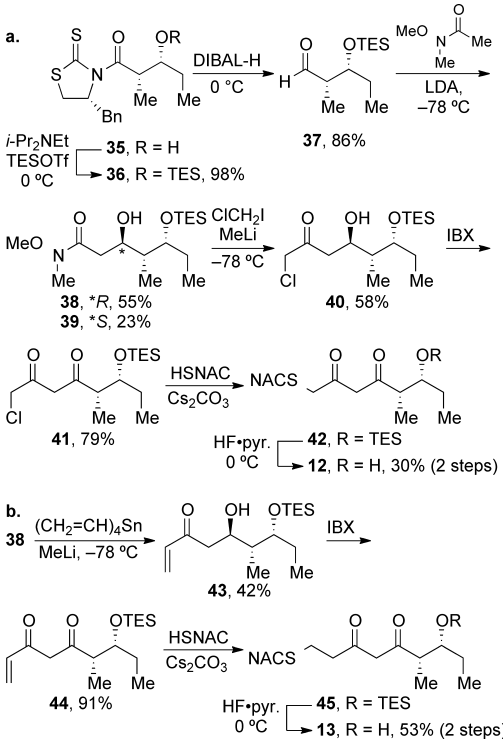
Direct aminolysis of the chiral auxiliary with amine **29** or *N*-methylamine **30** (synthesized in three steps from *N*-(2-aminoethyl)acetamide, Supporting Information Scheme 1) yielded amide **31** and *N*-methylamide **32**, respectively. TPAP/NMO oxidation and deprotection afforded amide substrate mimics **10** and **11**. The stereochemistry of aldol product **28** was established by deprotection of amide **31** and conversion to its acetonide, which was subject to Rychnovsky's acetonide analysis (Supporting Information Scheme 2).[38,39]

For the synthesis of thioether substrate mimics **12** and **13**, *N*-acyl thiazolidinethione **35**[37] was protected and reduced to aldehyde **37** (Scheme 2a), followed by acetate aldol reaction with *N*-methoxy-*N*-methylacetamide to give Weinreb amides **38** and **39** (dr = 7:3, separated by chromatography). Weinreb amide **38** was directly converted to chloromethyl ketone **40**, followed by IBX oxidation of the secondary alcohol, S_N2 reaction of the chloromethyl ketone with *N*-acetylcysteamine, and deprotection to afford thioether substrate mimic **12**.[40,41] The synthesis of thioether substrate mimic **13** was accomplished in an analogous fashion from Weinreb amide **38** by nucleophilic addition of vinyl lithium to produce vinyl ketone **43** (Scheme 2b). IBX oxidation, Michael addition of *N*-acetylcysteamine, and deprotection furnished thioether substrate analog **13**. The stereochemistry at C-3 of aldol products **38** and **39** was determined by conversion to the products' acetonides followed by Rychnovsky's acetonide analysis (Supporting Information Scheme 3).[38,39]

Synthesis of Product Mimics. All potential products from PikKR2 reduction of thioether substrate mimics **12** and **13** were synthesized as authentic standards for analytical comparison to the enzymatic products (Scheme 3). C3-Hydroxy products of substrate mimic **12**, diols **18** and **20**, were synthesized from Weinreb amides **38** and **39** by protection, conversion of the Weinreb amides to the chloromethyl ketones, S_N2 displacement with *N*-acetylcysteamine, and deprotection (Scheme 3a). Synthesis of the potential products resulting from C1-reduction of substrate mimic **12**, C1-alcohols **22** and **24**, began with

dx.doi.org/10.1021/cb5006883 | ACS Chem. Biol. 2014, 9, 2914−2922

Scheme 2. Synthesis of Thioether Substrate Analogs (a) 12 and (b) 13



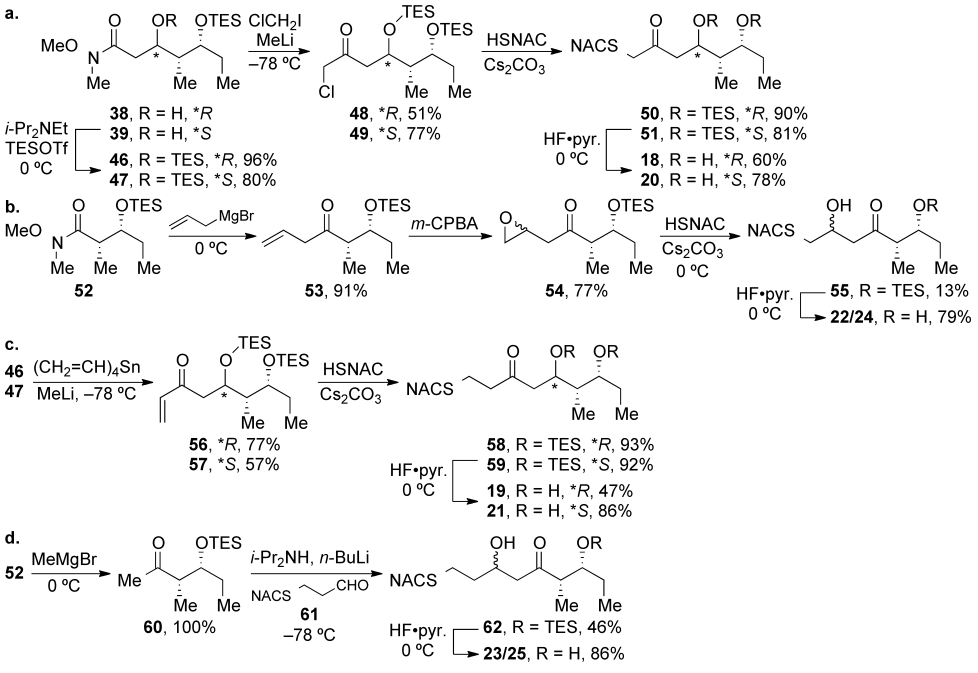
Grignard addition of allyl magnesium bromide to Weinreb amide **52** (synthesized in two steps from thiazolidinethione **35**, Supporting Information Scheme 4) to afford allyl ketone **53** (Scheme 3b).[42] Nonselective epoxidation of **53** with *m*-CPBA, epoxide opening with *N*-acetylcysteamine, and deprotection gave C1 products **22**/**24** as a distereomeric mixture. The reaction of epoxide **54** with *N*-acetylcysteamine was low-yielding due to competitive formation of the C-2 addition product.

The potential C3-reduction products of thioether substrate mimic **13**, diols **19** and **21**, were synthesized from protected Weinreb amides **46** and **47** in an analogous fashion to **18** and **20** (Scheme 3c). Synthesis of the potential C1-reduction products of substrate mimic **13**, diols **23** and **25**, began with addition of methylmagesium bromide to Weinreb amide **52**, followed by aldol reaction of the resulting methyl ketone **60** with aldehyde **61** (synthesized in one step from *N*-acetylcysteamine and acrolein, Supporting Information Scheme 5) to give alcohol **62** (dr = 2:1, inseparable by chromatography), and deprotection to provide diols **23**/**25** as a diastereomeric mixture.

Cloning, Expression, and Purification of PikKR2. The KR2 domain of PikAI was identified by sequence comparison with other type I PKS KR domains. The PikAI sequence encoding KR2 (residues 3880−4355) was cloned from the cosmid pLZ51 into expression vector pMCSG7 and verified by sequencing.[43,44] The N-terminal hexa-His tagged protein was overexpressed in *E. coli* BL21(DE3) cells. The protein was purified by sequential nickel affinity and gel filtration chromatography to afford approximately 45 mg of purified enzyme per liter of culture that was >95% pure as judged by SDS-PAGE (Supporting Information Figure 1). The molecular weight determined by SDS-PAGE is approximately 55 kDa, while the monoisotopic mass of PikKR2 determined by electrospray ionization (ESI) mass spectrometry is 51 430 Da, also consistent with the calculated value from the amino acid sequence of the PikKR2 domain and the histidine tag (51 431 Da). A Superdex 200 gel filtration, calibrated using a Gel Filtration Calibration Kit LMW (GE Healthcare), was used to estimate the molecular weight of the native enzyme as 95 kDa, suggesting the enzyme exists as a homodimer. The cloning, expression, and purification of PikKR2-DH2 didomain was conducted in an analogous fashion.

Substrate Specificity of PikKR2. To initially test whether the synthesized substrate mimics **10**−**13** were competent substrates, PikKR2 (5 μM) was incubated overnight (12−16 h) with **10**, **11**, **12**, or **13** (1 mM) and NADPH (2 mM) in

Scheme 3. Synthesis of All Possible Thioether Products (a) 18 and 20, (b) 22/24, (c) 19 and 21, and (d) 23/25



dx.doi.org/10.1021/cb5006883 | *ACS Chem. Biol.* 2014, 9, 2914−2922

sodium phosphate buffer (100 mM, pH 7.1). After incubation, each enzymatic reaction was quenched via the addition of MeCN, and precipitated protein was removed by centrifugation. The supernatant was analyzed by ESI mass spectrometry to detect enzymatic reduction products (as seen by the increase of [M+2] peak), compared to control reaction (either without enzyme or without NADPH). The reactions of amides **10** and **11** did not show any appreciable increase of the [M+2] peaks, nor consumption of the parent ion peaks (data not shown). In contrast, thioethers **12** and **13** demonstrated substantial turnover with large increases of the [M+2] peaks (Supporting Information Figure 2). Exclusion of either NADPH or PikKR2 abolished product formation with **12** and **13**. This assay indicated that thioethers **12** and **13** are substrates for PikKR2 while amides **10** and **11** are not turned over by the enzyme.

Several factors may contribute to the lack of reactivity of amides **10** and **11**. Unlike the thioester, amides may form either an unfavorable N−H hydrogen bond (secondary amide **10**) or harbor excess steric bulk (tertiary amide **11**), impeding access into the narrow active site channel. The amide linkage's restricted conformations (i.e., *s-trans* for **10** and *s-cis* for **11**) may also not enable the substrate mimics to adopt the required catalytically active conformation. Computational docking by others of a ketide-bound ACP substrate to a KR domain indicated that the thioester linkage may not be planar, lending support to the latter hypothesis.[24]

Confirmation of Reaction Products by LC-MS/MS. Due to its intrinsic selectivity and sensitivity, LC-MS/MS was chosen for assignment of the enzymatic products of PikKR2. We synthesized all plausible constitutional isomer reduction products of thioethers **12** and **13** as authentic standards. These synthetic standards are readily resolved by LC-MS/MS by their unique HPLC retention times and/or by their parent and daughter ions in MS/MS (Supporting Information Figure 3 and Supporting Information Table 1). Standards **19** and **21** are observed with two retention times by HPLC (Supporting Information Figure 3), which we hypothesize is caused by the ring−chain equilibration between the open chain keto tautomer and the closed ring lactol tautomer. Standards **22**/**24** and **23**/**25** are mixtures of two diastereomers, also resulting in two HPLC retention times.

Incubation of thioether **12** with PikKR2 using conditions described above resulted in two reaction products with retention times of 4.00 min (red trace, transition m/z 292 → 216) and 4.70 min (blue trace, transition m/z 314 → 198) corresponding to the C1-reduction product (**22** or **24**) and C3-reduction product **20**, respectively (Figure 3a). The normalized product ratio of **22**/**20** or **24**/**20** is 1:4 based on a standard curve generated from the authentic standards. The larger peak for **22** or **24** at 4.00 min actually represents the minor product, a consequence of its high ionization efficiency. Similarly, incubation of thioether **13** with PikKR2 resulted in two reaction products. The peaks at 5.88 and 6.93 min (blue trace, transition m/z 328 → 212) correspond to standard **21**, while the peak at 5.68 min (red trace, transition m/z 306 → 230) corresponds to one of the diastereomers in standard **23**/**25** (Figure 3b). The normalized product ratio of (**23** or **25**):**21** is 1:6. These results indicate that PikKR2 preferentially reduces the C3-ketone in substrates **12** and **13** with exquisite stereoselectivity to afford the D-configured alcohol products **20** and **21**, respectively. Intriguingly, only a single peak was observed by LC-MS/MS for C1-reduction products, which corresponds to **22** or **24** (Figure 3a) and **23** or **25** (Figure 3b), indicating even

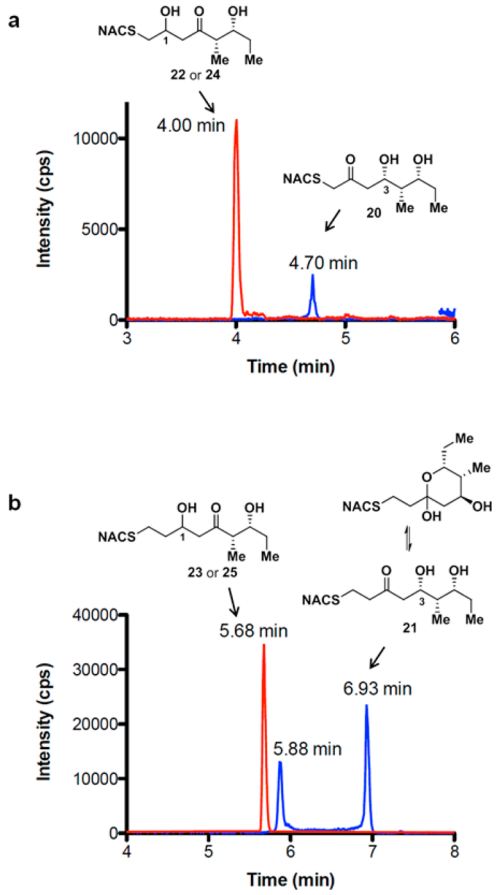


Figure 3. LC-MS/MS analysis of PiKKR2 activity. (a) LC-MS/MS enzymatic product analysis of substrate **12**; red trace represents MRM (m/z 292 → 216); blue trace represents MRM (m/z 314 → 198). (b) LC-MS/MS analysis for enzymatic product of substrate **13**; red trace represents MRM (m/z 306 → 230); blue trace represents MRM (m/z 328 → 212).

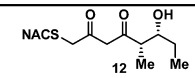
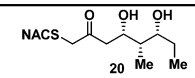
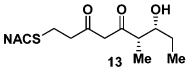
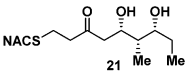
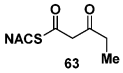
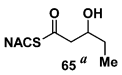
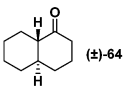
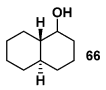
the minor reduction (C1 reduction) is conducted in a complete diastereoselective fashion.

To investigate the stereoselectivity of KR in a more native context, substrate **13** was incubated with the PikKR2-DH2 didomain and analyzed in an analogous way. The LC-MS/MS trace of didomain products showed exactly the same retention times as observed by the single domain, demonstrating the same enzymatic products, D-alcohol at C3-reduction and one single diastereomer at C1-reduction (Supporting Information Figure 4). The ratio of C3 reduction (natural event) to C1 reduction (unnatural event) by PikKR2-DH2 didomain was increased to 18:1, suggesting that larger portions of the PKS module preferably conduct the natural reaction (C3 reduction) over the competitive side reactions (C1 reduction).

Steady-State Kinetic Analysis of PikKR2. To study the substrate specificity of PikKR2, the steady-state kinetic parameters of the triketide substrate mimics **12** and **13**, as well as widely used substrates diketide **63** and *trans*-decalone **64**, were determined (Table 1 and Supporting Information Figure 5).[6,45] Incubations were carried out with PikKR2 (5 μM), saturating NADPH (0.5 mM), and varying substrate concentrations under initial velocity conditions. Apparent kinetic parameters for NADPH (Table 1) were determined with 40 mM **63** (this is nonsaturating as a result of the limited solubility of this substrate). The initial rates, v_0, at a given [S] were determined

dx.doi.org/10.1021/cb5006883 | *ACS Chem. Biol.* 2014, 9, 2914−2922

Table 1. Steady-State Kinetic Parameters for PikKR2 Substrates

Substrate	Product	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}M^{-1}$)
 **12**	 **20**	2.4 ± 0.8	0.029 ± 0.004	12.1 ± 5.5
 **13**	 **21**	7.8 ± 2.7	0.113 ± 0.023	14.5 ± 7.9
 **63**	 **65**[a]	26.1 ± 4.8	1.71 ± 0.16	66 ± 18
 (±)-**64**	 **66**	15.2 ± 6.0	10.92 ± 2.62	718 ± 456
NADPH	$NADP^+$	$(65.4 \pm 12.4) \times 10^{-3}$	1.54 ± 0.10	$(23.5 \pm 5.9) \times 10^3$

[a] **65** was isolated as a 4:1 mixture of D- and L-alcohols.

by single-time point stopped-time incubations at 12 min for **12** and **13** (the reaction velocity remained linear up to 30 min). The reaction products **20** and **21** were quantified by LC-MS/MS employing a standard curve generated from synthetic standards. For substrates **63** and **64**, whose turnover was substantially faster than **12** and **13**, we were able to employ a continuous assay that monitors consumption of NADPH by a decrease in absorbance at 340 nm. The enzymatic products of diketide **63** were identified as a mixture of D- and L-alcohols, in a ratio of 4 to 1, by chiral HPLC (Supporting Information Figure 6).

The specificity constants (k_{cat}/K_M) of the triketide substrate mimics **12** and **13** are 12.1 and 14.5 min^{-1} M^{-1}, respectively while the k_{cat}/K_M values of diketide **63** is 66 min^{-1} M^{-1} and *trans*-decalone **64** is 718 min^{-1} M^{-1}. Thus, the native substrate mimics **12** and **13** are processed between 4−60 fold less efficiently than the unnatural substrates **63** and **64**. Examination of the kinetic parameters reveals that **12** and **13** possess improved K_M values that are 2−10 fold lower than **63** and **64**, likely due to their similarity to the natural acyl chain. Consequently, the lower specificity constants for **12** and **13** are exclusively caused by the greatly reduced turnover of these substrates. Indeed the k_{cat} values for **12** and **13** are 15−336 fold lower than **63** and **64**. To explain the slow turnover of substrates **12** and **13**, we initially tested for product inhibition (see Supporting Information). However, product inhibition was not observed, thus we considered an alternative rationale for attenuation of k_{cat} values. We believe the low activity of the thioether substrates **12** and **13** may be caused by their β-diketone moieties, which exist to a large extent in the nonreducable enol forms (35−85% observed in ^1H NMR ($CDCl_3$), see Supporting Information) as a result of the greater acidity of this functional group relative to a β-ketothioester.

Structure and Mechanism of A- and B-type KRs. KRs can be classified into two types based on the stereochemistry of the reduction product. A-type KRs generate L-hydroxyacyl products and contain a highly conserved tryptophan (W motif) while B-type KRs yield D-hydroxyacyl products and harbor a LDD motif (or often LXD motif). After aligning the protein sequence of PikKR2 with other characterized KRs, an LSD motif (a variation of the LDD motif) is found (Figure 4a), indicative of a B-type KR with a D-hydroxyl product. The results here substantiate the prediction for PikKR2, which also agrees with the experimental approach using an engineered Pik module 2.[16]

To rationalize the relationship between observed stereochemical outcome and the empirically deduced amino acid

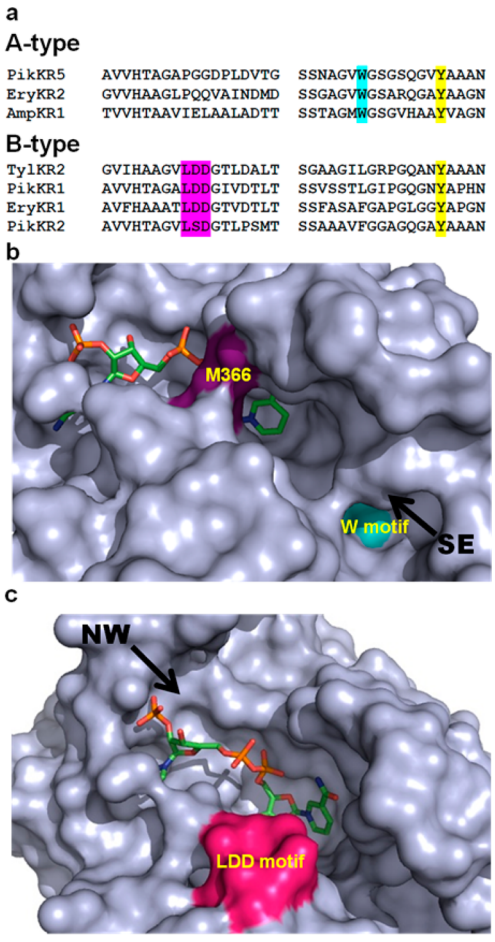


Figure 4. Signature motif comparison of A- and B-type KRs. (a) Sequence alignment of PikKR5, EryKR2, AmpKR1, TylKR2, PikKR1, EryKR1, and PikKR2; conserved catalytic tyrosine shown in yellow; W motif in cyan; LDD motif in pink. (b) Active site of A-type PlmKR1 with $NADP^+$ bound; W motif shown in cyan; M366 shown in purple. (c) Active site of B-type EryKR1 with $NADP^+$ bound; LDD motif shown in pink. Parts b and c were created in Pymol using PDB codes 4HXY[25] and 2FR0.[41]

motifs, there have been many efforts to obtain KR domain structural information. Recent crystallographic studies have shown that all KRs bind the cofactor NADPH in the same orientation, which transfers the 4-*pro*-*S*-hydride to the β-carbonyl

dx.doi.org/10.1021/cb5006883 | ACS Chem. Biol. 2014, 9, 2914−2922

carbon in the polyketide intermediate.[21,22,24,46,47] As a result, the difference in stereochemical outcome must arise from a reversed presentation of the β-ketoacyl substrate in the active site, exposing *re* or *si* faces of the β-keto group to NADPH.[24] One hypothesis is that the W motif promotes entry of the polyketide intermediate through the southeast opening of the active site groove in A-type KR (Figure 4b), yielding a product with an L orientation. In an analogous way, in B-type KR, the LDD motif directs the substrate to access the active site groove from the northwest side to generate D-hydroxyl product (Figure 4c). However, the W and LDD motifs are not the only factors contributing to stereocontrol based on the distance from the active site (W) and inability to occlude active site openings (LDD).[21,22,48] Analysis of crystal structures of both A- and B-type KRs led to a corollary to the direction-of-entry hypothesis by noting that cofactor binding (NADPH or $NADP^+$) in A-type KRs may generate a tight and well-ordered conformation at the active site through a hydrogen bonding network.[22] In this catalysis-ready conformation, Met366 is pushed into the active site to block the entry from the northwest. Thus, the substrate could penetrate the active site groove only from the southeast (Figure 4b). In B-type KRs, cofactor binding is loose, allowing for polyketide entry through both channel openings. It is proposed that only binding of substrate from the northwest side could set a tight, catalysis-ready conformation at the active site (Figure 4c).[22] Further biochemical and structural experiments may be needed to better understand the mechanism of the stereoselectivity of KRs.

Application in Chemoenzymatic Synthesis. Excised enzymes from natural product biosynthetic pathways have shown great promise in chemoenzymatic synthesis providing diverse natural product analogs.[20,49−51] Due to their promiscuity for a variety of substrates, these enzymes are able to accept unnatural substrates and conduct highly stereoselective reactions, which are usually difficult to accomplish by purely synthetic methods. Enzymes provide an economically efficient and environmentally friendly strategy in organic synthesis by eliminating the need for organic solvents, chiral auxiliaries, or expensive chiral ligands. More importantly, enzymes can catalyze asymmetric chemical reactions in excellent yield and enantiomeric (or diastereomeric) purity under mild conditions and can reduce the number of reactions in a synthetic sequence.[52] Asymmetric ketoreduction is a promising area for biocatalyst development as many pharmaceuticals contain stereogenic hydroxyl groups. PikKR2, in this study, has shown relaxed substrate flexibility toward unnatural substrates by reducing diketones to alcohols while affording an extremely high level of ketoreduction stereoselectivity at both C1 and C3 keto groups.

Combinatorial Biosynthesis. A deeper mechanistic understanding of polyketide biosynthesis can aid the efficient production of "unnatural" natural product libraries for drug discovery by combinatorial biosynthesis.[53,54] This topic has drawn much attention and continuous efforts over the past three decades. Although tremendous breakthroughs have been made to create libraries of natural product analogs, full exploitation of combinatorial biosynthesis remains a largely unattained goal.[55] One challenging aspect is the loss of specificity and activity of unnatural substrates or altered domains. For instance, deletion of KR2 in DEBS PKS led to the shutdown of the biosynthesis as module 3 could not accept the non-native, unreduced β-keto intermediate.[55] In another example, when the stereochemistry of the C2-methyl group established by AmpKR2 was altered by mutagenesis, the polyketide intermediate skipped the ketoreduction step and resulted in an unexpected product due to the competition between the slower, unnatural KR domain and more rapid downstream enzymes.[56] Clearly, prerequisite knowledge of the mechanism, kinetic parameters, stereoselectivity, and substrate specificity of each domain is crucial for applications in combinatorial biosynthesis and metabolic engineering.

Conclusion. This study demonstrates that chemical-based approaches to the study of PKS KR domains provide access to valuable information crucial for understanding the limitations of current approaches in combinatorial biosynthesis. Utilizing a small number of probes designed as substrate analogs, we were able to expose a limitation in accepted substrate linkages (secondary and tertiary amides) and a flexible catalytic reduction site (C1 vs C3 reduction in diketone thioether substrates), indicating a stringent preference in linkage region with a flexible reduction locale in the active site, respectively. The extremely high stereoselectivity of PikKR2 when presented with a variety of substrates exhibits its potential use in combinatorial biosynthesis and chemoenzymatic synthesis. This technique is expected to be broadly applicable to other PKS KR domains, hopefully unveiling fundamental knowledge required for the rational engineering of PKS systems for drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

Methods are reported in the Supporting Information, which contains Supporting Information Schemes 1−5, Supporting Information Figures 1−6, Supporting Information Table 1, general biology procedures, cloning, protein expression and purification, NADPH consumption kinetic assay, kinetic assay and analysis of PikKR2 reaction products by LC-MS/MS, general chemistry procedures and experimental procedures, spectral data, and copies of ^1H and ^{13}C NMR spectra for all new compounds. This material is available is available free of charge via the Internet at http:/pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: aldri0015@umn.edu.
*E-mail: fecik001@umn.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank B. Witthuhn (Center for Mass Spectrometry and Proteomics, University of Minnesota) for assistance with LC-MS/MS and N. Bleeker (Institute for Therapeutics Discovery and Development, University of Minnesota) for the help with HPLC. Financial support from NIH GM081544-01 (to J.L.S. and R.A.F.), the Department of Medicinal Chemistry (University of Minnesota), and support from a Chemistry Biology Interface training grant (to S.M.B.) is gratefully acknowledged.

■ REFERENCES

(1) Staunton, J., and Weissman, K. J. (2001) Polyketide biosynthesis: a millennium review. *Nat. Prod. Rep. 18*, 380−416.
(2) Kwan, D. H., and Schulz, F. (2011) The stereochemistry of complex polyketide biosynthesis by modular polyketide synthesase. *Molecules 16*, 6092−6115.
(3) Keatinge-Clay, A. T. (2012) The structures of type I polyketide synthases. *Nat. Prod. Rep. 29*, 1050−1073.

(4) Kao, C. M., McPherson, M., McDaniel, R. N., Fu, H., Cane, D. E., and Khosla, C. (1998) Alcohol stereochemistry in polyketide backbones is controlled by β-ketoreductase domains of modular polyketide synthase. *J. Am. Chem. Soc. 120*, 2478−2479.
(5) Zheng, J., and Keatinge-Clay, A. T. (2013) The status of type I polyketide synthase ketoreductases. *Med. Chem. Commun. 4*, 34−40.
(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., and Leadlay, P. F. (2005) Molecular basis of Celmer's rules: stereochemistry of catalysis by isolated ketoreductase domains for modular polyketide synthases. *Chem. Biol. 12*, 1145−1153.
(7) Valenzano, C. R., Lawson, R. J., Chen, A. Y., Khosla, C., and Cane, D. E. (2009) The biochemical basis for stereochemical control in polyketide biosynthesis. *J. Am. Chem. Soc. 131*, 18501−18511.
(8) Garg, A., Khosla, C., and Cane, D. E. (2013) Coupled methyl group epimerization and reduction by polyketide synthase ketoreductase domains. Ketoreductase-catalyzed equilibrium isotope exchange. *J. Am. Chem. Soc. 135*, 16324−16327.
(9) Cane, D. E. (2010) Programming of erythromycin biosynthesis by a modular polyketide synthase. *J. Biol. Chem. 285*, 27517−27523.
(10) Beck, B. J., Aldrich, C. C., Fecik, R. A., Reynolds, K. A., and Sherman, D. H. (2003) Structure recognition and channeling of monomodules from the pikromycin polyketide synthase. *J. Am. Chem. Soc. 125*, 12551−12557.
(11) Aldrich, C. C., Beck, B. J., Fecik, R. A., and Sherman, D. H. (2005) Biochemical investigation of pikromycin biosynthesis employing native penta- and hexaketide chain elongation intermediates. *J. Am. Chem. Soc. 127*, 8441−8452.
(12) Akey, D. L., Kittendorf, J. D., Giraldes, J. W., Fecik, R. A., Sherman, D. H., and Smith, J. L. (2006) Structural basis for macrolactonization by the pikromycin thioesterase. *Nat. Chem. Biol. 2*, 537−542.
(13) Giraldes, J. W., Akey, D. L., Kittendorf, J. D., Sherman, D. H., Smith, J. L., and Fecik, R. A. (2006) Structural and mechanisitic insights into polyketide macrolactonization from polyketide-based affinity labels. *Nat. Chem. Biol. 2*, 531−536.
(14) Sherman, D. H., Li, S., Yermalitskaya, L. V., Kim, Y., Smith, J. A., Waterman, M. R., and Podust, L. M. (2006) The structural basis for substrate anchoring, active site selectivity, and product formation by P450 PikC from *Streptomyces venezuelae*. *J. Biol. Chem. 281*, 26289−26297.
(15) Hansen, D. A., Rath, C. M., Eisman, E. B., Narayan, A. R., Kittendorf, J. D., Mortison, J. D., Yoon, Y. J., and Sherman, D. H. (2013) Biocatalytic synthesis of pikromycin, methymycin, neomethymycin, novamethymycin and ketomethymycin. *J. Am. Chem. Soc. 135*, 11232−11238.
(16) Wu, J., Zaleski, T. J., Valenzano, C., Khosla, C., and Cane, D. E. (2005) Polyketide double bond biosynthesis. Mechanistic analysis of the dehydratase-containing module 2 of the picromycin/methymycin polyketide synthase. *J. Am. Chem. Soc. 127*, 17393−17404.
(17) Castonguay, R., He, W., Chen, A. Y., Khosla, C., and Cane, D. E. (2007) Stereospecificity of ketoreductase domains of the 6-deoxyerthronolide B synthase. *J. Am. Chem. Soc. 129*, 13758−13769.
(18) Castonguay, R., Valenzano, C. R., Chen, A. Y., Keatinge-Clay, A., Khosla, C., and Cane, D. E. (2008) Stereospecificity of ketoreductase domains 1 and 2 of the tylactone modular polyketide synthase. *J. Am. Chem. Soc. 130*, 11598−11599.
(19) Guo, X., Liu, T., Valenzano, C. R., Deng, Z., and Cane, D. E. (2010) Mechanism and stereospecificity of a fully saturating polyketide synthase module: Nanchangmycin synthase module 2 and its dehydratase domain. *J. Am. Chem. Soc. 1132*, 14694−14696.
(20) Piasecki, S. K., Taylor, C. A., Detelich, J. F., Liu, J., Zheng, J., Komosoukaniants, A., Siegel, D. R., and Keatinge-Clay, A. T. (2011) Employing modular polyketide synthase ketoreductases as biocatalysts in the preparative chemoenzymatic syntheses of diketide chiral building blocks. *Chem. Biol. 18*, 1331−1340.
(21) Zheng, J., Taylor, C. A., Piasecki, S. K., and Keatinge-Clay, A. T. (2010) Structural and functional analysis of A-type ketoreductases from the amphotericin modular polyketide synthase. *Structure 18*, 913−922.
(22) Bonnett, S. A., Whicher, J. R., Papireddy, K., Florova, G., and Smith, J. L. (2013) Structural and stereochemical analysis of a modular polyketide synthase ketoreductase domain required for the generation of a *cis*-alkene. *Chem. Biol. 20*, 772−783.
(23) Caffrey, P. (2003) Conserved amino acid residues correlation with ketoreductase stereospecificity in modular polyketide synthases. *ChemBioChem 4*, 654−657.
(24) Keatinge-Clay, A. T. (2007) A tylosin ketoreductase reveals how chirality is determined in polyketides. *Chem. Biol. 14*, 898−908.
(25) You, Y., Khosla, C., and Cane, D. E. (2013) Stereochemistry of reductions catalyzed by methyl-epimerizing ketoreductase domains of polyketide synthases. *J. Am. Chem. Soc. 135*, 16324−16327.
(26) Holzbaur, I., Harris, R., Bycroft, M., Cortes, J., Bisang, C., Staunton, J., Rudd, B. A., and Leadlay, P. F. (1999) Molecular basis of Celmer's rules: the role of two ketoreductase domains in the control of chirality by the erythromycin modular polyketide synthase. *Chem. Biol. 6*, 189−195.
(27) Zamudio-Vázquez, R., Albericio, F., Tulla-Puche, J., and Fox, K. R. (2014) Thioester bonds of thiocoraline can be replaced with *N*Me-amide bridges without affecting its DNA-binding properties. *ACS Med. Chem. Lett. 5*, 45−50.
(28) Leggans, E. K., Akey, D. L., Smith, J. L., and Fecik, R. A. (2010) A general scheme for synthesis of substrate-based polyketide labels for acyl carrier proteins. *Bioorg. Med. Chem. Lett. 20*, 5939−5942.
(29) Spiteller, D., Waterman, C. L., and Spencer, J. B. (2005) A method for trapping intermediates of polyketide biosynthesis with a non-hydrolyzable malonyl-coenzyme A analogue. *Angew. Chem., Int. Ed. 44*, 7079−7082.
(30) Tosin, M., Spiteller, D., and Spencer, J. B. (2009) Malonyl carba(dethia)- and malonyl oxa(dethia)-coenzyme A as tools for trapping polyketide intermediates. *ChemBioChem 10*, 1714−1723.
(31) Tosin, M., Betancor, L., Stephens, E., Li, W. M. A., Spencer, J. B., and Leadlay, P. F. (2010) Synthetic chain terminators off-load intermediates from a type I polyketide synthase. *ChemBioChem 11*, 539−546.
(32) Tosin, M., Smith, L., and Leadlay, P. F. (2011) Insights into lasalocid A ring formation by chemical chain termination *in vivo*. *Angew. Chem., Int. Ed. 50*, 11930−11933.
(33) Tosin, M., Demydchuk, Y., Parascandolo, J. S., Blasco Per, C., Leeper, F. J., and Leadlay, P. F. (2011) *In vivo* trapping of polyketide intermediates from an assembly line synthase using malonyl carba-(dethia)-*N*-acetyl cysteamine. *Chem. Commun. 47*, 3460−3462.
(34) Nagao, Y., Hagiwara, Y., Kumagai, T., and Ochiai, M. (1986) New C4-chiral 1,3-thiazolidine-2-thiones: Excellent chiral auxiliaries for highly diastereocontrolled Aldol-type reactions of acetic acid and α,β−unsaturated aldehydes. *J. Org. Chem. 51*, 2391−2393.
(35) Nagao, Y., Dai, W., Ochiai, M., Tsukagoshi, S., and Fujita, E. (1990) Highly diastereoselective alkylation of chiral Tin (II) enolates onto cyclic acyl imines. An efficient asymmetric synthesis of bicyclic alkaloids bearing a nitrogen atom ring juncture. *J. Org. Chem. 55*, 1148−1156.
(36) Crimmins, M. T., King, B. W., Tabet, E. A., and Chaudhary, K. (2001) Asymmetric aldol additions: use of titanium tetrachloride and (−)-sparteine for the soft enolization of *N*-acyl oxazolidinones, oxazolidinethiones, and thiazolidinethiones. *J. Org. Chem. 66*, 894−902.
(37) Crimmins, M. T., and Slade, D. J. (2006) Formal synthesis of 6-deoxyerythronolide B. *Org. Lett. 8*, 2191−2194.
(38) Rychnovsky, S. D., and Skalitzky, D. J. (1990) Stereochemistry of alternating polyol chains: ^{13}C NMR analysis of 1,3-diol acetonides. *Tetrahedron Lett. 31*, 945−948.
(39) Evans, D. A., Rieger, D. L., and Gage, J. R. (1990) ^{13}C NMR chemical shift correlations in 1,3-diol acetonides. Implications for the stereochemical assignment of propionate-derived polyols. *Tetrahedron Lett. 31*, 7099−7100.
(40) Groesbeek, M., and Smith, S. O. (1997) Synthesis of 19-fluororetinal and 20-fluororetinal. *J. Org. Chem. 62*, 3638−3641.
(41) Bartlett, S. L., and Beaudry, C. M. (2011) High-yielding oxidation of β-hydroxyketones to β-diketones using *o*-iodoxybenzoic acid. *J. Org. Chem. 76*, 9852−9855.

(42) Makino, K., Kimura, K., Nakajima, N., Hashimoto, S., and Yonemitsu, O. (1996) Toward the total synthesis of hygrolidin: Stereocontrolled construction of the C1-C17 seco-acid fragment and the C18-C25 masked hemiacetal subunit. *Tetrahedron Lett. 37*, 9073−9076.
(43) Xue, Y., Zhao, L., Liu, H. W., and Sherman, D. H. (1998) A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity. *Proc. Natl. Acad. Sci. U.S.A. 95*, 12111−12116.
(44) Donnelly, M. I., Zhou, M., Millard, C. S., Clancy, S., Stols, L., Eschenfeldt, W. H., Collart, F. R., and Joachimiak, A. (2006) An expression vector tailored for large-scale, high-throughput purification of recombinant proteins. *Protein Expr. Purif. 47*, 446−454.
(45) Bali, S., and Weissman, K. J. (2006) Ketoreduction in mycolactone biosynthesis: insight into substrate specificity and stereocontrol from studies of discrete ketoreductase domains *in vitro*. *ChemBioChem 7*, 1935−1942.
(46) Keatinge-Clay, A. T., and Stroud, R. M. (2006) The structure of a ketoreductase determines the organization of the β-carbon processing enzymes of modular polyketide synthases. *Structure 14*, 737−748.
(47) Zheng, J., Gay, D. C., Demeler, D., White, M. A., and Keatinge-Clay, A. T. (2012) Divergence of multimodular polyketide synthases revealed by a didomain structure. *Nat. Chem. Biol. 8*, 615−621.
(48) Kwan, D. H., Tosin, M., Schläger, N., Shulz, F., and Leadlay, P. F. (2011) Insights into the stereospecificity of ketoreduction in a modular polyketide synthase. *Org. Biomol. Chem. 9*, 2053−2056.
(49) Kohli, R. M., Walsh, C. T., and Burkart, M. D. (2002) Biomimetic synthesis and optimization of cyclic peptide antibiotics. *Nature 418*, 658−661.
(50) Ding, Y., Seufert, W. H., Beck, Z. Q., and Sherman, D. H. (2008) Analysis of the cryptophycin P450 epoxidase reveals substrate tolerance and cooperativity. *J. Am. Chem. Soc. 130*, 5492−5498.
(51) Bali, S., O'Hare, H. M., and Weissman, K. J. (2006) Broad substrate specificity of ketoreductases derived from modular polyketide synthases. *ChemBioChem 7*, 478−484.
(52) Desai, A. A. (2011) Sitagliptin manufacture: A compelling tale of green chemistry, process intensification, and industrial asymmetric catalysis. *Angew. Chem., Int. Ed. 50*, 1974−1976.
(53) Weissman, K. J., and Leadlay, P. F. (2011) Combinatorial biosynthesis of reduced polyketides. *Nat. Rev. Microbiol. 3*, 925−936.
(54) Wong, F. T., and Khosla, C. (2012) Combinatorial biosynthesis of polyketides − a perspective. *Curr. Opin. Chem. Biol. 16*, 117−123.
(55) McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M., and Ashley, G. (1999) Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc. Natl. Acad. Sci. U.S.A. 96*, 1846−1851.
(56) Zheng, J., Piasecki, S. K., and Keatinge-Clay, A. T. (2013) Structural studies of an A2-type modular polyketide synthase ketoreductase reveal features controlling α-substituent stereochemistry. *ACS Chem. Biol. 8*, 1964−1971.