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Stereospecific Formation of *Z*-Trisubstituted Double Bonds by the Successive Action of Ketoreductase and Dehydratase Domains from *trans*-AT Polyketide Synthases

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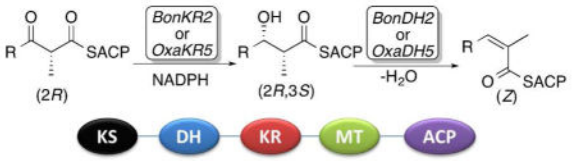
Abstract

Incubation of (±)-2-methyl-3-ketobutyryl-SNAC (**3**) and (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) with BonKR2 or OxaKR5, ketoreductase domains from the bongkrekic acid (**1**) and oxazolomycin (**2**) polyketide synthases, in the presence of NADPH gave in each case the corresponding (2*R*, 3*S*)-2-methyl-3-hydroxybutyryl-SNAC (**5**) or (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC (**6**) products, as established by chiral GC-MS analysis of the derived methyl esters. Identical results were obtained by BonKR2- and OxaKR5-catalyzed reduction of chemoenzymatically prepared (2*R*)-2-methyl-3-ketopentanoyl-EryACP6, (2*R*)-2-methyl-3-ketobutyryl-BonACP2 (**12**), and (2*R*)-2-methyl-3-ketopentanoyl-BonACP2 (**13**). The paired dehydratase domains, BonDH2 and OxaDH5, were then shown to catalyze the reversible *syn* dehydration of (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**) to give the corresponding trisubstituted (*Z*)-2-methylbutenoyl-BonACP2 (**16**).

Graphical abstract



Double bonds are ubiquitous structural features found in thousands of known bacterial polyketide natural products. While the vast majority are disubstituted or trisubstituted *E* double bonds, numerous polyketides harbor isomeric *Z* double bonds, such as those found in

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Notes

The authors declare no competing financial interest.

Supporting Information

Experimental methods, including sequence alignments, KR and DH design and expression, kinetic assays, and GC-MS and ESI-MS analysis of KR and DH incubations. This material is available free of charge on the ACS Publications website at DOI:10.1021/acs.biochem********

bongkrekic acid (**1**)[1] and oxazolomycin A (**2**)[2] (Figure 1), as well as in fostriecin,[3–5] phoslactomycin,[6] borrellidin, [7, 8] bacillaene,[9, 10] curacin,[11,12] difficidin,[13] chivosazol A,[14] disorazol A,[14] lactimidomycin,[14] and macrolactin, [14] among many others. Modular polyketide synthases (PKSs) typically generate each double bond by the coupled action of a ketoreductase (KR) domain, which carries out the stereospecific, NADPH-dependent reduction of a 3-ketoacyl-acyl carrier protein (ACP) or a 2-methyl-3-ketoacyl-ACP chain elongation intermediate, and a paired dehydratase (DH) domain, which catalyzes the *syn* dehydration of the reduced 3-hydroxyacyl-ACP or 2-methyl-3-hydroxyacyl-ACP intermediate.

A variety of DH domains from modular PKSs have been expressed as discrete proteins and their reactions characterized biochemically. Thus EryDH4, from module 4 of the erythromycin PKS,[15] and NanDH2, from module 2 of the nanchangmycin PKS,[16] each catalyze the *syn* dehydration of a (2*R*,3*R*)-2-methyl-3-hydroxyacyl-ACP substrate to the corresponding trisubstituted (*E*)-2-methylenoyl-ACP. By contrast, RifDH10, from module 10 of the rifamycin PKS, catalyzes the analogous *syn* dehydration of the diastereomeric (2*S*, 3*S*)-2-methyl-3-hydroxyacyl-ACP to an (*E*)-2-methylenoyl-ACP product, in spite of the fact that the derived trisubstituted double bond in the ultimately formed rifamycin has the *Z* configuration, suggesting that the geometry must be altered subsequent to double bond formation.[17] PicDH2, from module 2 of the picromycin PKS,[18, 19] and FosKR1, from module 1 of the fostriecin PKS,[5] each catalyze the dehydration of a (3*R*)-3-hydroxyacyl-ACP substrate to the corresponding disubstituted (*E*)-2-enoyl-ACP. The predicted *syn* stereochemistry of the latter two dehydration reactions has yet to be confirmed experimentally. We recently reported that FosDH2, from module 2 of the fostriecin PKS, catalyzes the reversible dehydration of a (3*S*)-3-hydroxyacyl-ACP to the corresponding disubstituted (*Z*)-2-enoyl-ACP product.[5] This report was the first to document the in vitro DH-catalyzed formation of any (*Z*)-enoyl-thioester product. Indeed, for a variety of reasons, all prior attempts to demonstrate the predicted formation of disubstituted or trisubstituted (Z)-double bonds by recombinant PKS DH domains had been unsuccessful, resulting instead in the exclusive generation of the isomeric (*E*)-double bonds.[7, 11, 12, 17, 20]

We now report that BonDH2, from module 2 of the bongkrekic acid *trans*-AT PKS, and OxaDH5, from module 5 of the oxazolomycin *trans*-AT PKS (Figure 1), each catalyze the stereospecific *syn* dehydration of (2*R*,3*S*)-2-methyl-3-hydroxyacyl-ACP substrates, generated by the paired ketoreductases, BonKR2 and OxaKR5, respectively, to give the corresponding trisubstituted (*Z*)-2-methylenoyl-ACP products.

BonKR2 and BonDH2, from *Burkholderia gladioli* pathovar *cocovenenans*, and OxaKR5 and OxaDH5, from *Streptomyces albus*, were each expressed in *Escherichia coli* as *N*-terminal His_6-tagged proteins using codon-optimized synthetic genes, based on consensus PKS domain boundaries (Figures S1–S4). Each of these four recombinant proteins was purified to homogeneity by immobilized Ni^{2+} affinity chromatography. The purity and M_r of each recombinant protein were assessed by SDS-PAGE and confirmed by LC–ESI(+)–MS analysis (Figure S5 and Table S1).

In common with the vast majority of polyketide synthase KR domains, all of which belong to the superfamily of short chain dehydrogenase/reductase (SDR) proteins, [21, 22] BonKR2 and OxaKR5 each harbor the highly conserved active site triad of Ser, Tyr, and Lys residues (Figure 2).[23, 24] On the other hand, both KR domains, along with other KR domains from *trans*-AT PKSs such as BaeKR9, from module 9 of the bacilllaene PKS, and DifKR6, from module 6 of the difficidin PKS, lack the diagnostic sequence markers, such as the conserved Trp residue (A-Type KR domain) or Leu-Asp-Asp motif (B-Type KR domain), that are normally correlated with the respective (3*S*)- or (3*R*) configuration of the resultant 3-hdyroxy acyl thioester reduction product (Figure 2).[23–26] Moreover, none of the additional conserved KR sequence features that are normally diagnostic of the (2*R*)- or (2*S*)-methyl configuration of the reduced 2-methyl-3-hydroxyacyl-thioester (KR subtypes 1 or 2) are evident in the amino acid sequences of these *trans*-AT KR domains. As a consequence, the intrinsic stereospecifity of BonKR2 or OxaKR5 cannot be deduced from simple consensus sequence alignments. (Although the Leu-Val-Asp triad of OxaKR5 might have suggested classification of OxaKR5 as a B-Type KR domain which would be predicted to generate a (3*R*)-3-hydroxyacyl group, the experiments described below establish firmly that OxaKR5 is an A1-Type KR.)

The reductase activities of BonKR2 and OxaKR5 were each confirmed and the steady-state kinetic parameters were determined using the model *N*-acetylcysteamine thioester substrates (±)-2-methyl-3-ketobutyryl-SNAC (**3**) and (±)-2-methyl-3-ketopentanoyl-SNAC (**4**)[27] and NADPH (Scheme 1a, Figures S6 and S7, Table S2). The stereochemistry of the resulting (2*R*,3*S*)-2-methyl-3-hydroxyacyl-SNAC products **5** and **6** was then established by chiral GC-MS analysis of the derived methyl esters **7-Me** and **8-Me**, including direct comparison with authentic synthetic standards of all four diastereomers of each product (Figures S8–S11, Tables S3 and S4).[28] We also used ACP-bound substrates to confirm the stereospecificity of the BonKR2- and OxaKR5-catalyzed reductions. Thus, in one set of experiments, (2*R*)-2-methyl-3-ketopentanoyl-EryACP6, generated by incubation of propionyl-SNAC with Ery[KS6][AT6], the ketosynthase-acyltranferase didomain from module 6 of the erythromycin PKS, and EryACP6 plus methylmalonyl-CoA,[28] was reduced in separate experiments with BonKR2 or OxaKR5 in the presence of NADPH to generate (2*R*,3*S*)-**9** (Scheme 1b). After basic hydrolysis and methylation with $TMSCHN_2$, chiral GC-MS analysis confirmed the exclusive formation of methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate (**8-Me**) (Figures S12 and S13, Table S3).

Finally, in a complementary series of incubations, a mixture of BonKS2, the ketosynthase from module 2 of the bongkrekic acid PKS, and either acetyl-SNAC or propionyl-SNAC, was combined with malonyl-BonACP2, generated in situ as previously described by treatment of *apo*-BonACP2 with malonyl-CoA and the surfactin pantetheinyl transferase Sfp, so as to yield 3-ketobutyryl-BonACP2 (**10**) or 3-ketopentanoyl-BonACP2 (**11**) (Scheme 1c).[29] Stereospecific methylation of **10** or **11** was achieved as previously described[29] by treatment with a mixture of BonMT2, the *C*-methyl transferase from module 2 of the bongkrekic acid PKS, and *S*-adenosyl methionine (SAM), in the presence of *S*-adenosylhomocysteine (SAH) nucleosidase to prevent potent product inhibition by the co-product SAH, yielding (2*R*)-2-methyl-3-ketobutyryl-BonACP2 (**12**) or (2*R*)-2-methyl-3-

ketopentanoyl-BonACP2 (**13**). In the presence of NADPH, incubation of **12** with either BonKR2 or OxaKR5 gave **14**, while reduction of **13** with BonKR2 gave **15**. Chiral GC-MS analysis of the derived methyl esters **7-Me** and **8-Me**, confirmed the exclusive formation of the corresponding (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**) or (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-BonACP2 (**15**) (Figures S14–S16, Tables S3 and S4).

Having firmly established the stereochemistry of the BonKR2- and OxaKR5-catalyzed reductions, we next determined the stereospecificity of the paired dehydratase reactions. Chemoenzymatically prepared (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**) was incubated in separate experiments with BonDH2 or OxaDH5 (Scheme 2a). Hydrolysis of the resultant acyl-ACP thioester (*Z*)-**16**, the product of *syn* dehydration, by treatment with PICS TE, the thioesterase from the picromycin PKS,[30] gave exclusively (*Z*)-2-methylbutenoic acid (**17**), as established by GC-MS analysis and direct comparison with authentic standards of both (Z)- and (*E*)-2-methylbutenoic acid (Figures S17 and S18). In a negative control, NigDH1, from module 1 of the nigericin PKS, did not dehydrate **14**.[31] (Although NigDH1 naturally acts only as a 2-methyl-3-ketobutyryl-ACP epimerase, we have shown that it also harbors a cryptic dehydratase activity capable of converting (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP to (*E*)-2-methyl-2-pentenoyl-ACP.)[31] BonDH2 and OxaDH5 also catalyzed the reverse reaction, resulting in the stereospecific hydration of chemoenzymatically prepared (*Z*)-2-methylbutenoyl-BonACP2 (**16**) to yield exclusively the *syn* hydration product (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**) (Scheme 2b). LC-ESI(+)-MS analysis after removal of the DH proteins confirmed the addition of water to (*Z*)-**16**, as established by the increase in mass of M+18 of each of the acyl-ACP products (Figure S19, Table S5). Finally, chiral GC-MS analysis established the exclusive formation of the derived methyl (2*R*,3*S*)-2-methyl-3-hydroxybutyrate (**7-Me**) (Figure S20). In the negative control, treatment of (*Z*)-**16** with NigDH1 did not result in the formation of detectable hydration product.

The above-described experiments establish conclusively that the dehydratase domains from the bongkrekic acid and oxazolomycin polyketide synthases, BonDH2 and OxaDH5, each catalyze the *syn* dehydration of (2*R*,3*S*)-2-methyl-3-hydroxyacyl-ACP substrates, which are exclusively generated by their respective paired BonKR2 and OxaKR5 domains, to give the corresponding (*Z*)-2-methylenoyl-ACP products. These findings constitute the first experimental demonstrations of the DH-catalyzed formation of a *Z*-trisubstituted double bond, in spite of a number of unsuccessful prior efforts, cited above, that have been directed toward this surprisingly elusive goal. Protein sequence alignments indicate that all of these dehydratases retain a conserved set of active site His and Asp residues but harbor no obvious DH sequence motifs that can be correlated with the *E*- or *Z*-configuration of their characteristic disubstituted or trisubstituted enoyl-ACP products (Figure S21).[32] All PKS DH-catalyzed dehydration and/or hydration reactions for which the stereochemistry has been determined involve a reversible *syn* elimination/addition of water,[15–17] in common with the established stereospecificity of the closely related FabA and FabZ dehydratase domains of *E. coli* fatty acid biosynthesis[33, 34] as well as the analogous action of the yeast fatty acid synthase.[35] The ultimate *E* or *Z* enoyl-ACP product geometry must reflect

important differences in the active site conformation of the bound 3-hydroxyacyl-ACP substrates that are not obviously correlated with any conserved amino acid sequence motifs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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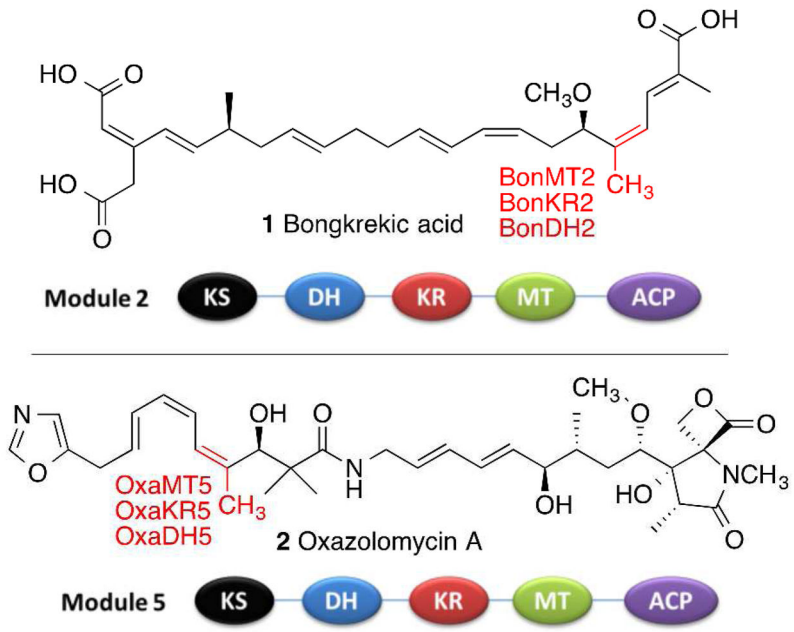


Figure 1.

Domain organization of the *trans*-AT polyketide synthase modules that generate (*Z*)-trisubstituted double bonds in bongkrekic acid (**1**) and oxazolomycin (**2**). The indicated methyl groups are introduced by *C*-methyl transferase (MT) domains.

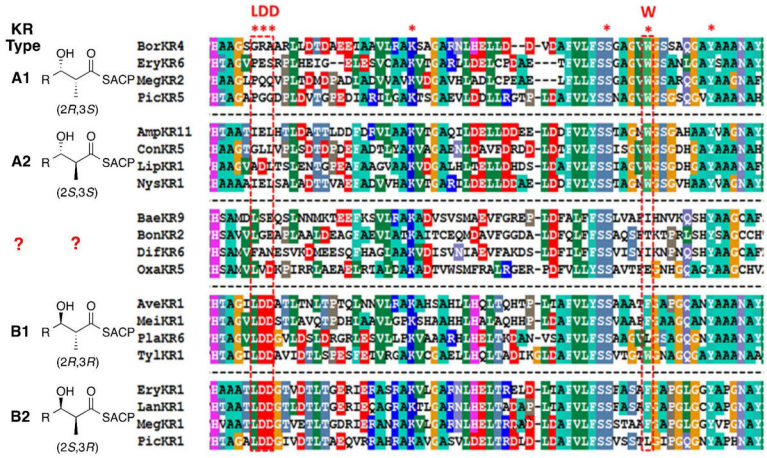
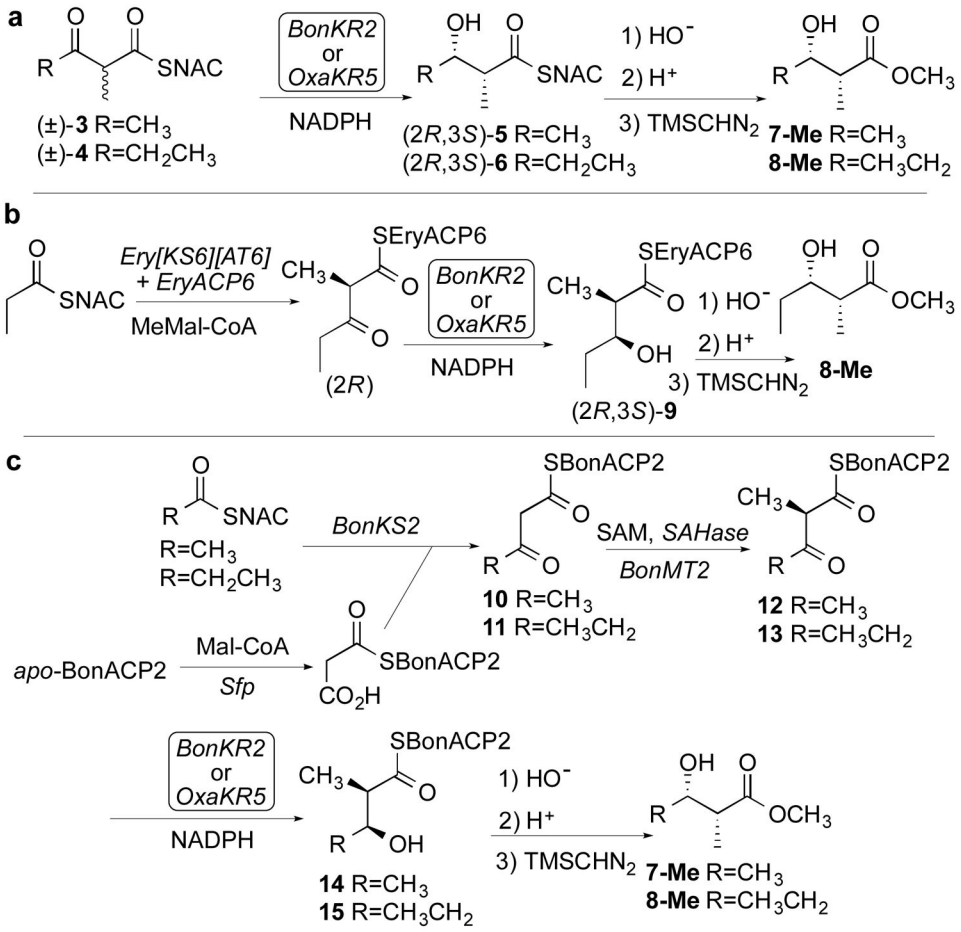


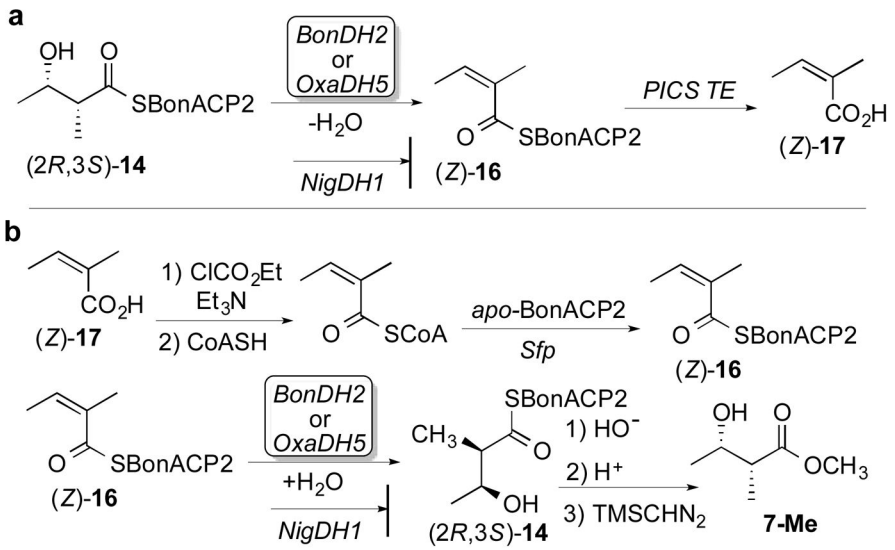
Figure 2.

Mega3.0 (http://www.megasoftware.net) sequence alignment of PKS ketoreductase domains that reduce 2-methyl-3-ketoacyl-ACP intermediates, showing the four different stereochemical classes of KR domains. Conserved K, S, and Y residues constitute the canonical active site catalytic triad of SDR proteins. The BaeKR9, BonKR2, DifKR6, and OxaKR5 domains lack the conserved sequence motifs diagnostic of established KR Types. PKS source: Amp, amphotericin; Ave, avermectin; Bae, bacillaene; Bon, bongkrekic acid; bor, borrelidin; Con, concanamycin A; Dif, difficidin; Ery, erythromycin; Lan, lankamycin; Lip, lipomycin; Meg, megalomicin; Mei, meilingmycin; Nys, nystatin; Oxa, oxazolomycin; Pic, picromycin; Pla, pladienolide; Tyl, tylactone.



Scheme 1.

Stereochemistry of BonKR2- and OxaKR5-Catalyzed Reduction of 2-Methyl-3-ketoacyl Thioesters.



Scheme 2.

Stereochemistry of BonDH2- and OxaDH5-Catalyzed Dehydration/Hydration Reactions