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Elucidation of the Cryptic Methyl Group Epimerase Activity of Dehydratase Domains from Modular Polyketide Synthases Using a Tandem Modules Epimerase Assay

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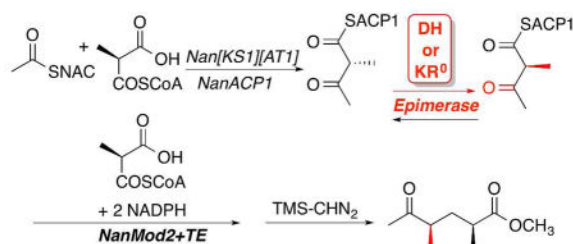
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Abstract

Dehydratase (DH) domains of cryptic function are often found in polyketide synthase (PKS) modules that produce epimerized (2*S*)-2-methyl-3-ketoacyl-ACP intermediates. A combination of tandem equilibrium isotope exchange (EIX) and a newly developed Tandem Modules Epimerase (TME) assay revealed the intrinsic epimerase activity of NanDH1 and NanDH5, from modules 1 and 5, respectively, of the nanchangmycin (**1**) PKS as well of NigDH1, from module 1 of the nigericin (**3**) PKS. Unexpectedly, all three epimerase-active DH domains were also found to possess intrinsic dehydratase activity, while the conventional DH domains, EryDH4, from module 4 of the erythromycin synthase, and NanDH2 from module 2 of the nanchangmycin synthase, were shown to have cryptic epimerase activity.

Graphical abstract



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Notes

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Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/jacs.*****

Sequence alignments and protein structure comparisons, PKS domain boundaries, SDS-PAGE and LC-MS analysis of recombinant proteins, Tandem EIX assay, Tandem Modules Epimerase assay, chiral GC-MS analysis.

Modular polyketide synthases (PKSs) are responsible for the biosynthesis of an enormous range of structurally and stereochemically complex natural products. In a prototypical Type I modular PKS, each module is responsible for a single step of polyketide chain elongation and functional group modification.^{1,2} Each PKS module carries a core set of three individual biosynthetic domains: 1) An acyl carrier protein (ACP) with an attached phosphopantetheinyl prosthetic group to which the growing polyketide chain is covalently tethered; 2) an acyl transferase (AT) domain responsible for loading a specific chain extension unit, most often methylmalonyl-CoA or malonyl-CoA, onto the pantetheinyl side chain of the ACP domain; and 3) a ketosynthase (KS) domain that catalyzes the actual chain elongation reaction, a decarboxylative acylation typically between the malonyl- or methylmalonyl-ACP and either an acetyl or propionyl starter unit or a partially elaborated polyketide chain provided by the proximal upstream PKS module so as to generate a 3-ketoacyl-ACP or (2*R*)-2-methyl-3-ketoacyl-ACP intermediate, respectively.³ Variable combinations of additional ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains then catalyze additional modifications prior to transfer of the growing polyketide chain to the KS domain of the immediately downstream module.

KR domains control the vast majority of stereocenters in the resulting polyketide, with diastereospecific reduction of the common (2*R*)-2-methyl-3-ketoacyl-ACP intermediate, either with or without coupled KR-catalyzed epimerization of the 2-methyl group, to generate one of four specific 2-methyl-3-hydroxyacyl-ACP diastereomers.^{3a,4,5} Alternatively, redox-inactive KR⁰ domains may catalyze epimerization of the 2-methyl group before transfer of the resulting (2*S*)-2-methyl-3-ketoacyl-ACP intermediate to the downstream KS domain.^{3b,6,7} Intriguingly, there are also examples of modules that apparently catalyze epimerization notwithstanding the complete absence of a KR- or KR⁰-like domain.

Several DH domains have been shown to catalyze the net *syn* dehydration of (2*R*,3*R*)-2-methyl-3-hydroxyacyl-ACP substrates to the corresponding (*E*)-2-methyl-2-enoyl-ACP,⁸ while there is also one example of *syn* dehydration of a diastereomeric (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP to (*E*)-2-methyl-2-enoyl-ACP.⁹ The highly conserved structures of functional DH domains display a characteristic double hot dog fold and active site HXXXGXXXXP, GYXYGPXF, and DXXXH(Q) motifs, with the boldface His and Asp residues directly implicated in the dehydration mechanism.^{9,10}

Our attention was drawn to cryptic DH domains found in PKS modules that evidently generate unreduced, epimerized (2*S*)-2-methyl-3-ketoacyl-ACP intermediates, as inferred from the structure and stereochemistry of the derived mature polyketide products. Such DH domains may be paired with a redox-inactive, epimerase-active KR⁰ domain, as is the case for both NanDH1 and NanDH5 from modules 1 and 5, respectively, of the nanchangmycin (1) PKS¹¹ or Mon DH1 from module 1 of the monensin (2) synthase (Figure 1).¹² In other cases, the cryptic DH domain may be present in a module that lacks a KR⁰ domain, as seen for NigDH1 from module 1 of the nigericin (3) PKS.¹³ By contrast, some epimerizing PKS modules harbor only a KR⁰ domain, as in module 7 of the salinomycin (4) PKS.^{3b,14} Such cryptic DH domains have a high degree of overall sequence similarity to canonical dehydrating DH domains and also retain the conserved active site sequence motifs (Figure

S1).^{10a} Moreover, homology-based protein structure models of NanDH1, NanH5, and NigDH1 each show high degrees of structural overlap with the reported 1.85-Å structure of EryDH4 from module 4 of the 6-dEB synthase (Figure S2).^{10a} We now report that NanDH1, NanDH5, and NigDH1 can each catalyze the epimerization of a (2*R*)-2-methyl-3-ketoacyl-ACP to the corresponding (2*S*)-2-methyl-3-ketoacyl-ACP. Each epimerizing DH domain is also shown to have intrinsic dehydratase activity, while canonical active DH domains unexpectedly also possess cryptic epimerase activity.

NanDH1 and NanDH5 were each expressed in *Escherichia coli* as *N*-terminal His₆-tagged proteins from DNA amplified by PCR from the parent nanchangmycin synthase modules using consensus domain boundaries.^{3b} In similar manner, a codon-optimized synthetic gene for NigDH1 was used for expression of the corresponding His₆-tagged protein (Figure S3). Each of these three recombinant DH domains was purified to homogeneity by immobilized metal ion affinity chromatography (Figure S4, Table S1). All three cryptic DH domains displayed intrinsic dehydratase activity, catalyzing the *syn* dehydration of both (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetyl-cysteamine thioester (SNAC) (**5a**) and (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6a**) to the corresponding (*E*)-2-methyl-2-pentenoyl thioesters **7** and **8** (Scheme 1, Figures S5, S7–S10), as previously observed for the canonical dehydratases EryDH4 and NanDH2 (from module 2 of the nanchangmycin PKS).⁸ Consistent with these results, no dehydration was observed when either NanDH1, NanDH5, or NigDH1 was incubated with the diastereomeric substrates, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC (**5b**) or (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6b**) (Figure S6).

To probe the potential epimerase activity of NanDH1, NanDH5, and NigDH1, each of these cryptic DH domains was subjected to tandem equilibrium isotope exchange (EIX) assays, previously used to establish the intrinsic epimerase activity of a variety of KR⁰ domains (Scheme 2).^{3b,6} EryKR6, the redox-active, epimerase-inactive ketoreductase domain from module 6 of the 6-deoxyerythronolide B synthase, was used to reversibly oxidize reduced, configurationally stable [2-²H]-**6b** in the presence of a catalytic quantity of NADP⁺ (0.2 mol % relative to EryKR6), thus generating in situ the deuterated, configurationally labile intermediate [2-²H]-(2*R*)-2-methyl-3-ketopentanoyl-EryACP6 ([2-²H]-**9a**). In the presence of an active epimerase, the transiently generated ([2-²H]-**9a**) undergoes reversible epimerization to (2*S*)-2-methyl-3-ketopentanoyl-EryACP6 (**9b**) with concomitant isotope exchange. EryKR6-catalyzed reduction of the resultant unlabeled **9a** in the presence of the transiently generated NADPH regenerates non-deuterated **6b**. The net exchange of deuterium from **6b** is directly monitored by periodic withdrawal of aliquots over the course of the 60-min incubation and LC-ESI-MS-MS analysis of the derived pantetheinate ejection fragments (**10**) at *m/z* 376 (*d*₁) and *m/z* 375 (*d*₀), as previously described (Scheme 2B).^{3b,6,15} Individual incubations of NanDH1, NanDH5, and NigDH1 each resulted in first-order, time-dependent washout of deuterium from [2-²H]-**6b**, comparable to the results previously reported for a variety of redox-inactive, epimerase-active KR⁰ domains, including NanKR1⁰ and NanKR5⁰ (Tables S2–S3, Figure S11).^{3b,16} In the negative control with EryKR6 in the absence of added DH domain, no more than 5–6% of isotope exchange was observed over a 1-h incubation, due to slow buffer-catalyzed exchange of the transiently-

generated labile intermediate [2-²H]-**9a**.^{3b} Intriguingly, the functional dehydratase domains EryDH4 and NanDH2 also displayed a comparable level of isotope washout when subjected to tandem EIX assay (Table S3, Figure S11).

Although the tandem EIX assay provides strong circumstantial evidence for the epimerase activity of either DH or KR⁰ domains, the observed washout of deuterium is in fact a necessary but not sufficient characteristic of epimerization. Strictly speaking, the loss of deuterium label does not require transient formation of the epimerized product **9b**, only that the transiently generated [2-²H]-**9a** undergo reversible enzyme-catalyzed deprotonation followed by reprotonation on either face of the intermediate enol or enolate. We have therefore developed a new and broadly useful enzyme-coupled analytical tool, termed the Tandem Modules Epimerase (TME) assay, which provides direct evidence of functional epimerase activity.

Incubation of recombinant Nan[KS1][AT1] and NanACP1 with acetyl-SNAC and methylmalonyl-CoA generates (2*R*)-2-methylbutyryl-ACP1 (**11a**) (Scheme 3).^{3b} The TME assay mixture also contains the previously described NANS module 2+TE, which we have shown to be strictly specific for chain elongation of only the (2*S*)-2-methylbutyryl-ACP1 (**11b**) diastereomer in the presence of methylmalonyl-CoA and NADPH, thus resulting in the exclusive formation of (2*S*,4*R*)-2,4-dimethyl-5-ketohexanoic acid (**12**).^{8b,16} The two components of the TME assay can be functionally coupled, however, only by the addition of a suitable epimerase activity capable of converting the initially formed (2*R*)-**11a** intermediate to the requisite (2*S*)-**11b** substrate for NanKS2. Significantly, addition of NanDH1 to the TME assay mixture and analysis of the derived triketide methyl esters by chiral GC-MS, obtained after TME incubation times of 15, 30, and 60 min, confirmed the exclusive time-dependent formation of methyl (2*S*,4*R*)-2,4-dimethyl-5-ketohexanoate (**12-Me**) (Figures 2 and S13). In the absence of added epimerase, neither **12-Me** nor any of its diastereomers could be detected. The formation of **12-Me** displayed a small but reproducible lag over the first 15-min interval compared to the 30-min and 60 min time points as the system achieved steady state. Essentially identical TME assay results were also obtained by the addition of either NanDH5 or NigDH1 in place of NanDH1. Alternatively, supplementation of the TME assay mixture with either of the known redox-inactive, epimerase-active ketoreductases, NanKR1⁰, from nanchangmycin synthase module 1,^{3b} or PicKR3⁰, from picromycin module 3,^{6a,7} respectively, also resulted in time-dependent formation of **12-Me** (Figures S12 and S15). Remarkably, essentially identical results were also obtained using the canonical PKS dehydratases EryDH4 or NanDH2, which were thus unexpectedly found also to have intrinsic epimerase activity (Figures S14 and S16).^{18,19} Importantly, in a set of negative control experiments, neither of the previously described^{6b} epimerase-inactive double mutants – PicKR3⁰-S385A/Y398F and EryKR3⁰-S349A/Y362F – showed *any* detectable epimerase activity in the coupled TME assay, even after 1 h incubation (Figure S17).

The dimethyltetrahydropyranyl rings of nanchangmycin (**1**), monensin (**2**), and nigericin (**3**) are a common structural feature of numerous polyether natural products, including laidlomycin, lenoremycin, grisorixin, mutalomycin, septamycin, and carriomycin.²⁰ All are evidently derived from the common (2*S*)-2-methylbutyryl-ACP diketide (**11b**) whose

formation requires the active participation of an epimerase domain in module 1 of the responsible PKS. It is therefore striking that this epimerase activity can be contributed by a DH domain alone (NigDH1 in nigericin biosynthesis [Figure 1] or presumptively by LadDH1 in laidlomycin biosynthesis [GenBank protein ID: AFL48525.1]) or by a paired DH domain and redox-inactive KR⁰ domain (Nan[DH1][KR1⁰] in nanchangmycin biosynthesis and Mon[DH1][KR1⁰] in monensin biosynthesis). By contrast, in other PKS modules the requisite epimerase activity is provided exclusively by a redox-inactive KR⁰ domain (SalKR7⁰ from module 7 of the salinomycin (**4**) PKS and apparently NigKR9 from module 9 of the nigericin PKS) (Figure 1). Thus the presence of either a DH domain *or* a KR⁰ domain in a given PKS module is sufficient to support epimerase activity, with neither domain being essential, provided that at least one or the other be present. It is unclear why some modules should utilize a catalytically redundant DH and KR⁰ pair to support the required epimerization.²¹ Whichever domain is responsible for epimerization, the KS domain of the proximal downstream module effects a kinetic resolution of the dynamic mixture of the original (*2R*)- and the epimerized (*2S*)-2-methyl-3-ketoacyl-ACP intermediates, selecting only the (*2S*) diastereomer for chain elongation and further processing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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18. In the TME assay, neither of the integrated NanDH2 and NanKR2 domains of nanchangmycin module 2 act independently on either of the diffusible intermediates (2*R*)- or (2*S*)-2-methyl-3-ketobutyryl-SNanACP1 ((2*R*)-**11** and (2*S*)-**11**), the latter only being processed by the integrated NanKS2.
19. The rate-limiting step in the coupled TME assay is dominated by the k_{cat} values for the Nan[KS1] and Nan[KS2] domains, which are typically 50–100 times slower ($1\text{--}5\text{ min}^{-1}$) than those of β -carbon-processing KR or DH (and probably ER) domains (typically $1\text{--}10\text{ s}^{-1}$). Differences in the absolute rate of DH- or KR⁰-catalyzed epimerization will therefore have an insignificant effect on the observed overall rate of formation of triketide **12** in the TME assay.
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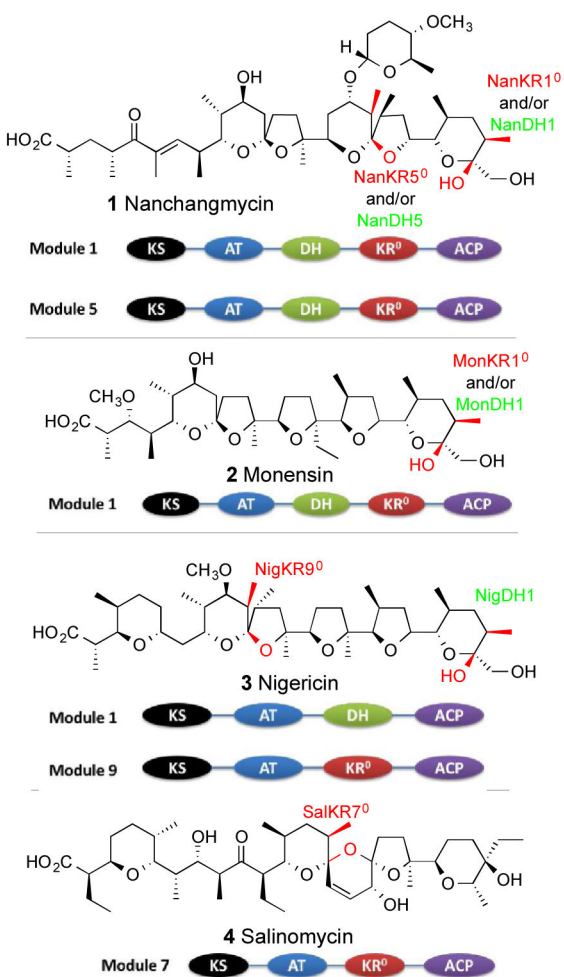


Figure 1.
Representative polyether polyketides and domain organization of the PKS modules that generate epimerized α -methyl ketones.

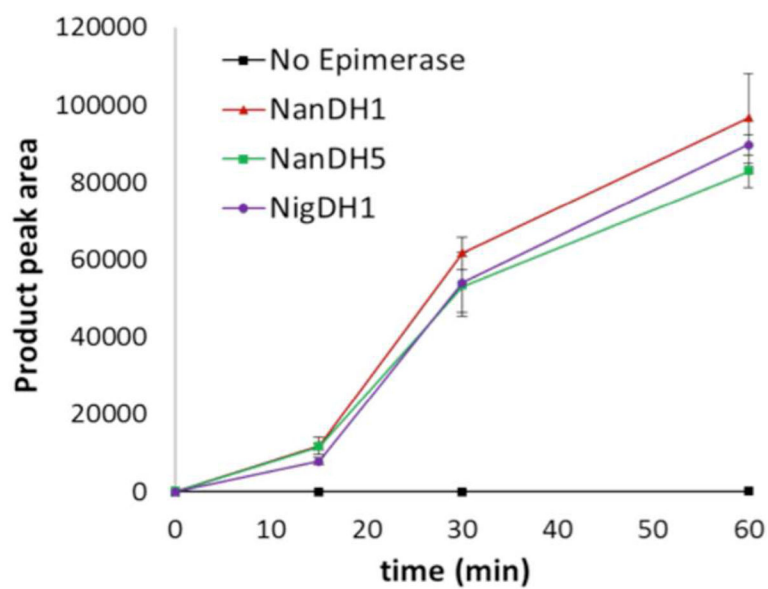
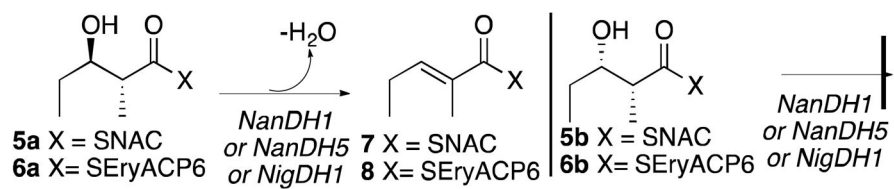
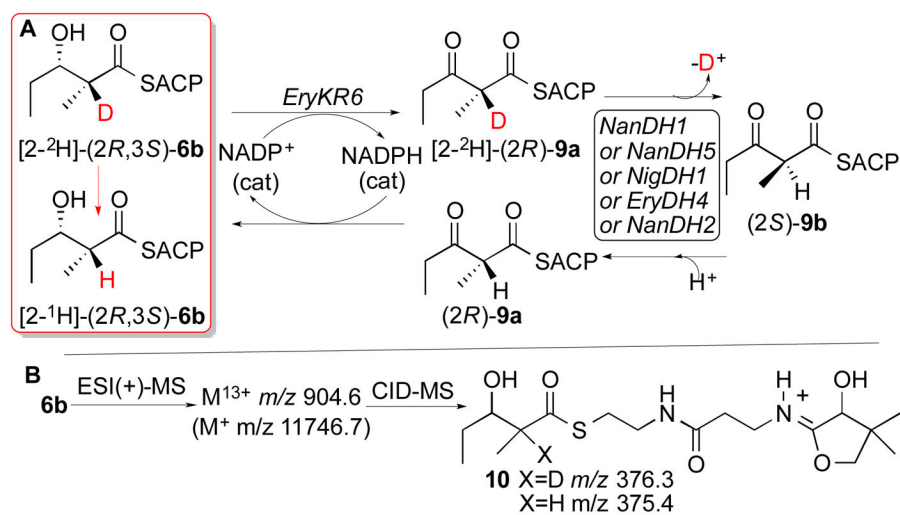


Figure 2. TME Assay

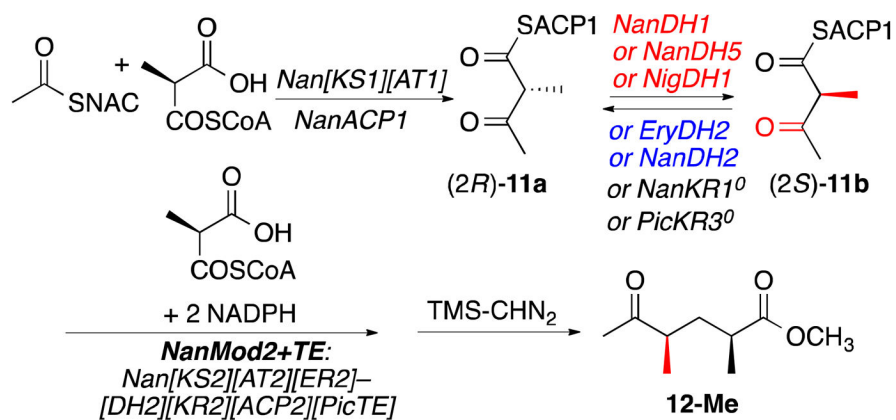
12-Me was generated by incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and epimerase-active NanDH1, NanDH5, or NigDH1, with GC-MS quantitation of the derived **12-Me** (XIC, *m/z* 88).



Scheme 1.



Scheme 2.
Tandem EIX Assay of DH Domains.



Scheme 3.
Tandem Modules Epimerase Assay