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Mechanism and Stereospecificity of a Fully Saturating Polyketide Synthase Module: Nanchangmycin Synthase Module 2 and Its Dehydratase Domain

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Abstract: Recombinant nanchangmycin synthase module 2 (NANS module 2), with the thioesterase domain from the 6-deoxyerythronolide B synthase (DEBS TE) appended to the C-terminus, was cloned and expressed in *Escherichia coli*. Incubation of NANS module 2+TE with (±)-2-methyl-3-keto-butyryl-*N*-acetylcysteamine thioester (**1**), the SNAC analog of the natural ACP-bound substrate, with methylmalonyl-CoA (MM-CoA) in the absence of NADPH gave 3,5,6-trimethyl-4-hydroxypyrone (**2**), identified by direct comparison with synthetic **2** by radio-TLC-phosphorimaging and LC-ESI(+)-MS-MS. The reaction showed k_{cat} 0.5 ± 0.1 min^{-1} and K_m(**1**) 19 ± 5 mM at 0.5 mM MM-CoA and k_{cat}(app) 0.26 ± 0.02 min^{-1} and K_m(MM-CoA) 0.11 ± 0.02 mM at 8 mM **1**. Incubation in the presence of NADPH generated the fully saturated triketide chain elongation product as a 5:3 mixture of (2*S*,4*R*)-*2*,4-dimethyl-5-ketohexanoic acid (**3a**) and the diastereomeric (2*S*,4*S*)-**3b**. The structure and stereochemistry of each product was established by comparison with synthetic **3a** and **3b** by a combination of radio-TLC-phosphorimaging and LC-ESI(−)-MS-MS, as well as chiral capillary GC-MS analysis of the corresponding methyl esters **3a-Me** and **3b-Me**. The recombinant dehydratase domain from NANS module 2, NANS DH2, was shown to catalyze the formation of an (*E*)-double bond by *syn*-dehydration of the ACP-bound substrate *anti*-(2*R*,3*R*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoyl-ACP6 (**4**), generated *in situ* by incubation of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**5**), methylmalonyl-CoA, and NADPH with the recombinant [KS6][AT6] didomain and ACP6 from DEBS module 6 along with the ketoreductase from the tylactone synthase module 1 (TYLS KR1). These results also indirectly establish the stereochemistry of the reactions catalyzed by the KR and enoylreductase (ER) domains of NANS module 2.

Modular polyketide synthases (PKSs) are exceptionally large, multifunctional proteins responsible for the elaboration of an enormous variety of complex, branched-chain, polyoxygenated polyketides of microbial origin, many of which play important roles in human and veterinary medicine as antibiotics, immunosuppressants, or antitumor, antifungal, or antiparasitic agents.[1] Each PKS protein is composed of one or more modules, assemblages of three or more catalytic centers, termed domains, that together are responsible for a single round of polyketide chain elongation and functional group modification. In the assembly line biosynthesis catalyzed by a modular PKS, the intermediates of the individual

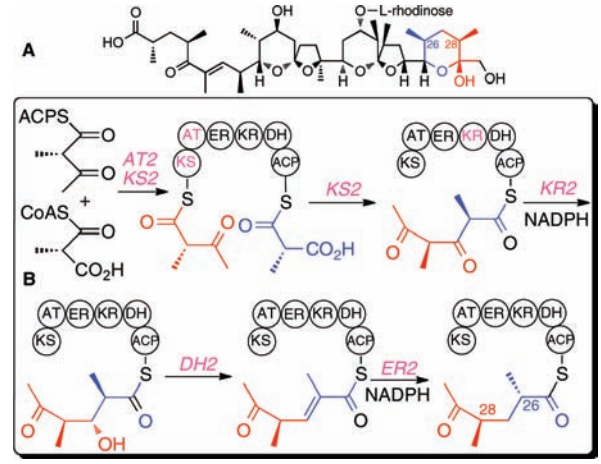

Figure 1. (A) Nanchangmycin. (B) Chain elongation, ketoreduction, dehydration, and enoyl reduction catalyzed by NANS module 2.

biochemical reactions remain covalently tethered to acyl carrier protein domains throughout the entire cycle, with the end product of each module being passed to the appropriate downstream module for the next round of chain elongation and modification. Since none of the intermediates are released from the modular PKS prior to the final thioesterase (TE)-catalyzed hydrolysis or lactonization reaction, the structure and stereochemistry of the product generated by each module are normally deduced from the structure of the corresponding segment of the ultimate, full-length polyketide. By contrast, the structure and stereochemistry of the specific intermediates of a single round of chain elongation and modification, which may not be evident in the structure of the eventually formed full-length polyketide, are usually inferred from the specific domain composition of the responsible module, by analogy to the well-established mode of action of the individual chain modifying proteins of a Type II fatty acid synthase, according to which a ketosynthase (KS) domain is expected to generate a β-ketoacyl-ACP intermediate, a ketoreductase (KR) domain, a β-hydroxyacyl-ACP intermediate, a dehydratase (DH) domain, a 2,3-enoyl-ACP intermediate, and an enoylreductase (ER) domain, the fully saturated acyl-ACP chain elongation product.[1b] These models have been validated by targeted deletion of one or more catalytic domains of the PKS and the determination of the structure of the resulting modified polyketide product.[2]

An exceptionally powerful tool for directly probing the mechanism of polyketide chain elongation and for determination of the structures of polyketide chain elongation intermediates has been to utilize recombinant forms of individual PKS modules as well as their constituent KS, AT, KR, DH, and ACP domains.[3,4] Unfortunately, no PKS module containing the full complement of active

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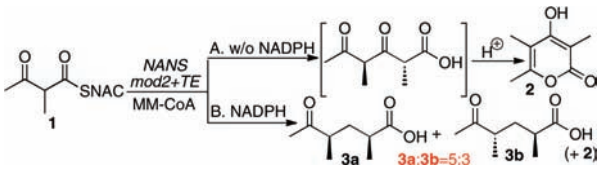
KS, KR, DH, and ER domains has yet been expressed *in vitro* as an active single modular protein. To address this problem, we chose to examine the nanchangmycin synthase (NANS) which has been shown to be responsible for the multistep biosynthesis of the polyketide backbone of the anticoccidial polyether nanchangmycin (Figure 1A).[5] Within this PKS, the monomodular protein NANS module 2, which harbors the full complement of β-carbon-processing KR, DH, and ER domains, is predicted to catalyze the second round of polyketide chain elongation, resulting in the formation of a saturated (2*R*,4*S*)-*2*,4-dimethyl-5-ketohexanoyl-ACP triketide intermediate (Figure 1B).[5,6] We therefore cloned and expressed recombinant NANS module 2, appending the TE domain of the 6-deoxyerythronolide B synthase (DEBS) to the C-terminus so as to promote release of the anticipated NANS ACP2-bound triketide products and to thus allow multiple catalytic turnovers.

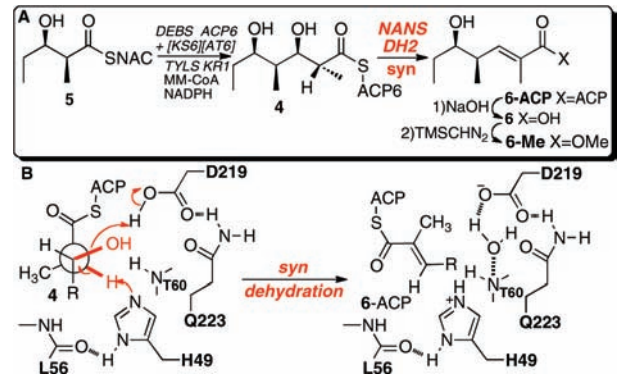
Incubation of recombinant NANS module 2+TE with the *N*-acetylcysteamine thioester of (±)-2-methyl-3-ketobutyric acid (**1**), the SNAC analog of the natural (2*S*)-2-methyl-3-ketobutyryl-ACP1 diketide produced by NANS module 1, with methylmalonyl-CoA (MM-CoA) in the absence of NADPH, afforded 3,5,6-trimethyl-4-hydroxypyrone (**2**), derived by TE- or buffer-catalyzed cyclization of the presumed unreduced (2*R*,4*S*)-2,4-dimethyl-3,5-diketohexanoyl-ACP2 intermediate, as established by direct comparison of the enzymatically generated product with authentic synthetic **2**[7a] by both radio-TLC-phosphorimaging and LC-ESI(+)-MS-MS (Scheme 1, reaction A). The reaction exhibited k_{cat} 0.5 ± 0.1 min^{-1} and K_m(**1**) 19 ± 5 mM at 0.5 mM MM-CoA and k_{cat}(app) 0.26 ± 0.02 min^{-1} and K_m(MM-CoA) 0.11 ± 0.02 mM at 8 mM **1**.[8] When the incubation was carried out in the presence of NADPH, the resulting product consisted instead of a 5:3 mixture of the natural reduced (2*S*,4*R*)-*2*,4-dimethyl-5-ketohexanoic acid (**3a**) and the diastereomeric (2*S*,4*S*)-**3b** accompanied by trace amounts of pyrone **2** (Scheme 1, reaction B), as established by comparison with authentic synthetic standards (Supporting Information)[7] by a combination of TLC-phosphorimaging and LC-ESI(−)-MS-MS. The diastereomeric composition of the ketotriketide acid product **3a** and **3b** was unambiguously established by chiral capillary GC-EI(+)-MS analysis of the derived methyl esters **3a**-**Me** and **3b**-**Me** and direct comparison with a synthetic mixture of established absolute configuration. The formation of both the (4*R*)- and (4*S*)-methyl diastereomers of **3** indicates that recombinant NANS module 2 does not cleanly discriminate between the two enantiomers of the SNAC analogue of its natural substrate. This apparent lack of substrate stereoselectivity would not be critical to the natural assembly line biosynthesis in the intact nanchangmycin PKS since only the correct stereoisomer of the diketide intermediate, (2*S*)-**1**-ACP1, will be delivered by NANS module 1 to downstream NANS KS2.

Scheme 1. Incubation of NANS Module 2+TE with **1** and Methylmalonyl-CoA (A) without NADPH and (B) with NADPH



To establish the structure and stereochemistry of the individual triketide intermediates generated by NANS module 2, we used a synthetic gene with codons optimized for expression in *E. coli* to express NANS DH2 domain as a recombinant standalone protein corresponding to the region from G929 to G1241 of NANS module 2 which is flanked by the corresponding AT2 and KR2 domains.[4d,5]

Scheme 2. (A) Stereochemistry of NANS DH2-Catalyzed Dehydration and (B) NANS DH2 *Syn*-Dehydration Mechanism



The requisite *anti*-(2*R*,3*R*)-2-methyl-3-hydroxyacyl-ACP substrate **4** was generated, as previously described, by incubation of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**5**), methylmalonyl-CoA, and NADPH with the recombinant [KS6][AT6] didomain and ACP6 from DEBS module 6 along with the KR1 reductase from the tylactone synthase (TYLS) module 1 (Scheme 2A).[4c,e,9] Coincubation with NANS DH2 resulted in formation, after basic hydrolysis and acidification, of (4*R*,5*R*)-(*E*)-2,4-dimethyl-5-hydroxy-2-heptenoic acid (**6**), as established by direct comparison with synthetic **6** by TLC-phosphorimaging and chiral GC-EI(+)-MS analysis of the derived methyl ester (**6**-**Me**). A control incubation (Supporting Information) in which DEBS KR6 was substituted for TYLS KR1 so as to generate the corresponding *syn*-(2*R*,3*S*)-2-methyl-3-hydroxyacyl-ACP6 stereoisomer[9a] did not yield any dehydration product, generating only the derived triketide lactone, as expected.[9a] Taken together these results firmly establish that the NANS DH2 domain (a) utilizes an ACP-bound *anti*-(2*R*,3*R*)-2-methyl-3-hydroxyacyl substrate and (b) produces an (*E*)-2-methylenoyl-ACP product by a (c) net *syn* dehydration.[10] NANS DH2 contains the universally conserved active site motifs **H**XXXDXXXXP and **D**XXX**Q**, in which H49 (H977 of full-length NANS module 2) is thought to serve as the active site base, with D219, acting as the general acid to donate a proton to the 3-hydroxyl leaving group, resulting in a net *syn* elimination of water (Scheme 2B).[4d,11] We have also established the same specificity for *anti*-(2*R*,3*R*)-2-methyl-3-hydroxyacyl-ACP substrates and *syn* dehydration stereochemistry for both the DEBS DH4 and TYLS DH2 domains.[9b,c] It can therefore be predicted with confidence that NANS KR2, the natural partner of NANS DH2, will generate the *anti*-(2*R*,3*R*)-2-methyl-3-hydroxy-acyl-ACP2 triketide intermediate from the corresponding KS2-generated (2*R*)-2-methyl-3-ketoacyl-ACP2 substrate, while the downstream NANS ER2 domain will reduce the (*E*)-2-methylenoyl-ACP2 unsaturated triketide, with capture of a proton on the 2*re*-face of the double bond, to give a reduced product with the observed (2*S*)-methyl configuration. We have thus established the stereochemistry of every step of the NANS module 2-catalyzed chain elongation and saturation process, with the exception of the still cryptic stereochemistry of ER2-catalyzed hydride addition to C-3 of the unsaturated triketide. Experiments are in progress to characterize directly NANS KR2, whose structural and catalytic subdomains flank the ER2 domain.

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Supporting Information Available: Experimental procedures, phosphorimaging, GC-MS, and LC-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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