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Bioorganic & Medicinal Chemistry Letters 16 (2006) 213–216
Bioorganic & Medicinal Chemistry Letters

Modular polyketide synthases: Investigating intermodular communication using 6 deoxyerythronolide B synthase module 2

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Received 20 August 2005; revised 1 September 2005; accepted 6 September 2005
Available online 5 October 2005

Abstract—A novel variant of 6-deoxyerythronolide B synthase (DEBS) module 2 was constructed to explore the balance between protein–protein-mediated intermodular channeling and intrinsic substrate specificity within DEBS. This construct, termed (N3)Mod2 + TE, was co-incubated with a complementary, donor form of the same module, (N5)Mod2(C2), as well as with a mutant of (N5)Mod2(C2) with an inactive ketosynthase domain, in order to determine the extent of intermediate channeling versus substrate diffusion into the downstream module.

Polyketide synthases (PKSs) are large modular enzymes responsible for the biosynthesis of numerous medically important polyketides. The most extensively studied PKS, 6-deoxyerythronolide B synthase (DEBS), transforms one propionyl CoA starter unit and six methylmalonyl CoA extender units into 6-deoxyerythronolide B, the heptaketide precursor of erythromycin.[1] DEBS is comprised of three polypeptide chains with each chain consisting of two distinct modules, each of which possesses all the catalytic machinery necessary for a single round of polyketide chain elongation and modification. The growing polyketide chain is passed from module to module in an assembly line format. This transfer of the polyketide intermediate from one module to the next not only requires some means of intermodular recognition, but is also sensitive to the inherent substrate specificity of the downstream module.[2,3] Modules recognize their correct pairing partner through the use of short linker peptides, also termed docking domains, of ~35–100 amino acids located at the C- and N-termini of the donor and acceptor module, respectively.[2,4–7] These docking domains may be genetically fused to new PKS modules to force new module pairings.[2,4]

Several DEBS module constructs have been previously studied individually and in combination. DEBS module 2, bearing the N-terminal 35-aa docking domain of DEBS module 5 (N5) and a C-terminal thioesterase (TE) domain, which promotes lactonization and release of the final polyketide product,[2] produces triketide lactone **2** when incubated with methylmalonyl-CoA, NADPH, and **1**, the *N*-acetylcysteamine (SNAC) thioester analog of the natural diketide product of DEBS module 1 (Scheme 1).[2]

Previously, we have shown that incubation of (N5)Mod2(C2), which lacks the thioesterase domain, with methylmalonyl-CoA, NADPH, and **1** also results in the production of triketide lactone **2**, albeit at a rate approximately 10-fold slower than (N5)Mod2 + TE, due to the absence of TE-catalyzed release of the enzyme-bound acyclic triketide product. On the other hand, incubation of (N5)Mod2(C2) and its downstream modular pairing partner, (N3)Mod3 + TE, with **1**, methylmalonyl-CoA, and NADPH, yielded almost exclusively the tetraketide ketolactone **3** (Scheme 1). The generation of the tetraketide **3** with concomitant suppression of triketide **2** formation by this bimodular mixture is the result of a vectorial transfer of the acyclic triketide product of module 2 to the ketosynthase (KS) domain of the paired module 3, facilitated by the complementary pair of docking domains (C2)/(N3), followed by an additional round of polyketide chain elongation and product release.

Keywords: Biosynthesis; Polyketides; Molecular recognition.
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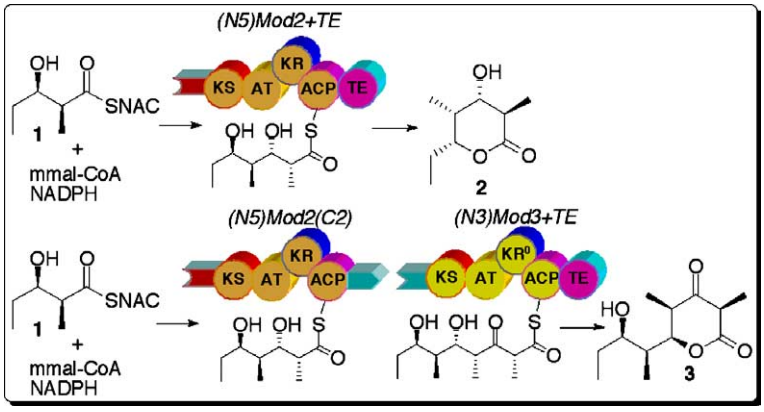
doi:10.1016/j.bmcl.2005.09.017



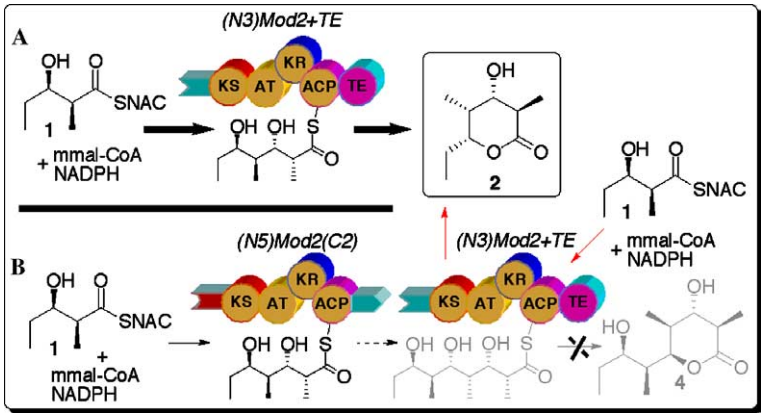
Scheme 1. Intermodular transfer of polyketide intermediates.

We now report that (1) productive intermodular vectorial transfer depends not only on complementary module–module recognition, but also on the intrinsic substrate specificity of the acceptor module for the product of the donor module and (2) illegitimate transfer can interfere with the normal catalytic efficiency of the target downstream module.

To explore the balance between intermodular communication and inherent substrate specificity, we first constructed a variant of DEBS module 2 with an N-terminal docking domain derived from DEBS module 3 (N3).[8] Incubation of the resultant (N3)Mod2 + TE with diketide **1**, methylmalonyl-CoA, and NADPH produced the predicted triketide lactone **2** with a K_m of 8.1 mM and k_{cat} of 0.9 min^{-1}, values similar to those exhibited by (N5)Mod2 + TE (Scheme 2A).[9] This result confirmed that the replacement of the N-terminal docking domain had no significant effect on the measured catalytic activity of (N3)Mod2 + TE.

Co-incubation of the new donor–acceptor module pair, (N5)Mod2(C2) and (N3)Mod2 + TE, carrying complementary docking domains, was expected to generate either the tetraketide lactone **4**, resulting from vectorial transfer of the intermediate triketide from (N5)Mod2(C2) to (N3)Mod2 + TE, or cyclized triketide **2**, resulting from processing of the diketide substrate by either module. Because both engineered modules are derived from the same DEBS module 2, both have identical ketosynthase domains and, therefore, the same inherent specificity and affinity for the diketide substrate **1**. In fact, when a 1:1 mixture of (N5)Mod2(C2) and (N3)Mod2 + TE (1 μM each) was incubated with **1**, 2-[methyl-^{14}C]methylmalonyl-CoA, and NADPH for 3 h at 30 °C, the sole product obtained was the triketide lactone **2**, with no detectable formation of tetraketide lactone **4** as monitored by TLC-phosphorimaging (Scheme 2B). Surprisingly, however, the yield of triketide lactone **2** was reduced by 50% compared to the yield of **2** observed under identical incubation conditions using (N3)Mod2 + TE alone (Fig. 1, blue). Furthermore, increasing concentrations of the presumptive donor module (N5)Mod2(C2) progressively decreased the observed k_{cat} for formation of **2** (Fig. 1, blue).[10] At a 7:1 ratio of (N5)Mod2(C2) to (N3)Mod2 + TE the yield of triketide **2** was only 20% that obtained with (N3)Mod2 + TE.

To investigate the molecular basis for this unexpected inhibition of diketide processing, we next examined the acylation of the donor and acceptor modules by labeled diketide **1**. Incubation of either (N5)Mod2(C2) or (N3)Mod2 + TE with [1-^{14}C]diketide **1** in the absence of NADPH and methylmalonyl-CoA (non-turnover conditions) stoichiometrically labeled each protein, due to the expected acylation of the ketosynthase domain, as evidenced by the detection of labeled protein by



Scheme 2. Formation of triketide **2** by complementary module 2 pairs.

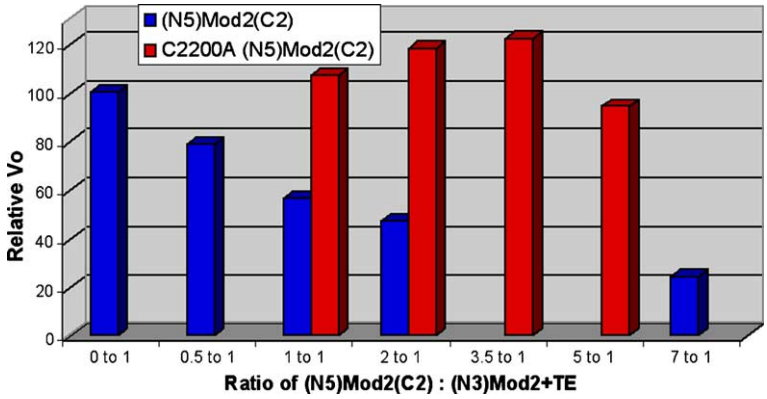


Figure 1. Inhibition of the formation of triketide lactone **2** by 1 μM (N3)Mod2 + TE as a function of the proportion of added (N5)Mod2(C2) (blue) or (N5)Mod2(C2)/C2200A (red).

SDS–PAGE/phosphorimaging (Fig. 2A, and Supplementary data). When the incubation with [1-^{14}C]diketide **1** was carried out on a 1:1 mixture of the two modules, each module underwent the normal level of acylation.

When the analogous incubation was performed with [1-^{14}C]diketide **1** in the presence of the co-substrates methylmalonyl-CoA and NADPH, the recovered (N3)Mod2 + TE protein retained only relatively minor amounts of covalently attached radiolabel, whether or not (N5)Mod2(C2) was present (Fig. 2B). This result was not unexpected, since the presence of the TE domain promotes release of the derived product, triketide **2**. By contrast, the presumptive donor module, (N5)Mod2(C2), still retained the same amount of radiolabel, independent of the presence or absence of acceptor module (N3)Mod2 + TE. These results suggest that any labeled triketide that might be generated by (N5)Mod2(C2) does not acylate the KS domain of the downstream module, (N3)Mod2 + TE. Alternatively, if such acylation were to occur, the resultant KS-bound triketide may be too unstable to be detected by SDS–PAGE analysis.

In principle, the observed attenuation of triketide lactone formation when both modules are present could result from two distinct types of interaction: (1) protein–protein association of (N5)Mod2(C2) and (N3)Mod2 + TE mediated by their complementary docking domains could prevent access of free diketide **1** to the active site of the downstream module, (N3)Mod2 + TE; or (2) the enzyme-bound, acyclic triketide product of the upstream (N5)Mod2(C2) module might act as a non-covalent (or unstable covalent) inhibitor of the downstream (N3)Mod2 + TE. To distinguish between these two possibilities, we used site-directed mutagenesis to construct the inactive C2200A mutant of (N5)Mod2(C2) in which the active site cysteine of KS2 was replaced by alanine.[11] As expected, this KS^0 mutant underwent acylation by 2-[methyl-^{14}C]methylmalonyl-CoA but not by [1-^{14}C]diketide **1** (data not shown). When this C2200A (N5)Mod2(C2) mutant was co-incubated with (N3)Mod2 + TE in ratios from 1:1 to 5:1, there was no significant decrease in the net rate of formation of triketide lactone **2** (Fig. 1, red). The ability of (N5)Mod2(C2) to inhibit the formation of triketide lactone **2** by (N3)Mod2 + TE therefore is shown to require the generation of acyclic triketide by the upstream module.

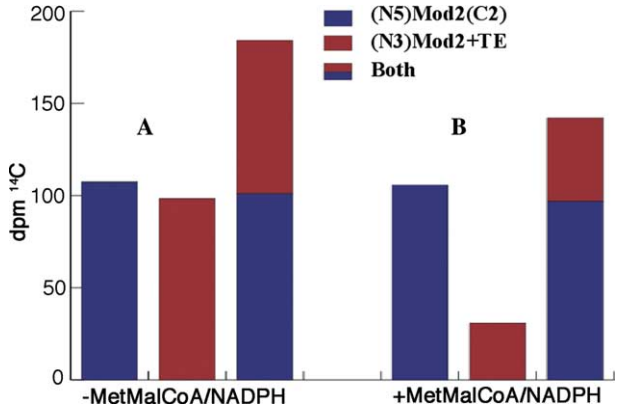


Figure 2. Acylation of module 2 by diketide **1**. Proteins were incubated with [1-^{14}C]-**1** in the absence (A) or the presence (B) of methylmalonyl-CoA and NADPH before analysis by SDS–PAGE/phosphorimaging. Lanes 1 and 4, (N5)Mod2(C2); lanes 2 and 5, (N3)Mod2 + TE; lanes 3 and 6, 1:1 (N5)Mod2(C2) + (N3)Mod2 + TE.

The failure of the combined (N5)Mod2(C2) and (N3)Mod2 + TE module pair to generate tetraketide lactone **4** is presumably due to the inability of the downstream module to process efficiently the ACP-bound acyclic triketide generated by the upstream module. At the same time, the observed suppression of (N3)Mod2 + TE activity by active (N5)Mod2(C2) is apparently due to inhibition of the downstream module by this same acyclic triketide, thereby preventing access of free diketide to the ketosynthase active site in (N3)Mod2 + TE. It is conceivable that the triketide intermediate itself may synergistically enhance the intrinsic affinity of the complementary (C2)- and (N3)-terminal docking domains. Notably, while the free SNAC derivative of the ACP-bound (2*R*,3*S*,4*S*,5*R*)-triketide has never been directly used as an in vitro substrate for PKS modules, due to its propensity to cyclize rapidly in the absence of enzyme, the stereochemically related *syn*-(2*R*,3*S*)-2-methyl-3-hydroxyl diketide-SNAC, the enantiomer of the natural diketide, (2*S*,3*R*)-**1**, has a k_{cat}/K_m for DEBS module 2 that is only 1% that of diketide **1**.[12] These results reinforce earlier

findings that the successful engineering of novel PKS module–module combinations requires both functional communication between donor and acceptor modules and the transfer of polyketide chain elongation intermediates that are compatible with the intrinsic substrate specificity of the downstream, acceptor module.[13] Recently, Menzella et al. have reported that DEBS module 2 can transfer (2*R*,3*S*)-diketide in vivo to a paired DEBS module 2 acceptor but with an efficiency that is <2% that for transfer and processing of the natural (2*S*,3*R*)-diketide substrate of module 2.[14] The observed intermediate-mediated inhibition of the downstream module may explain why in precursor-directed biosynthesis experiments, exogenously added substrates enter the polyketide assembly line only at the most upstream functional module, and are not taken up and processed by any of the available downstream modules.[13c,15]

Acknowledgments

This research was supported by NIH Grants GM22172 (D.E.C) and CA66736 (C.K.). D. A. Moffet is the recipient of an NRSA Postdoctoral Fellowship from the NIH, F32 GM067376.

Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.017. Detailed materials and methods, kinetic data, and phosphorimaging data are available as supporting information and may be accessed free from the Internet.

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8. Plasmid p28N3M2TE was prepared as follows: the mod 2 gene was excised from pPK22 [Ref. 4] using the restriction enzymes BsaBI and SpeI. The gene was ligated into the pUC18-based vector pST117 similarly digested. The resulting construct (pUCN3M2TE) was digested with NdeI and EcoRI and the gene insert was ligated into pET28A to yield p28N3M2TE.
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11. The inactive C2200A mutant of (N5)Mod2(C2) was prepared using the Stratagene QuikChange mutagenesis kit with plasmid pPK22 as template. Forward primer: GCCGGACGGCCGGGCAAAGCCCTTCTCGGAC; reverse primer: GTCCGAGAAGGGCTTTGCCCGGC CGTCCGGC. The resulting plasmid was digested with NdeI and ScaI, and the excised DNA insert was ligated into a similarly digested wt pPK22 and the sequence of the mutant was verified by dideoxy sequencing.
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