JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Substrate Controlled Divergence in Polyketide Synthase Catalysis

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Supporting Information

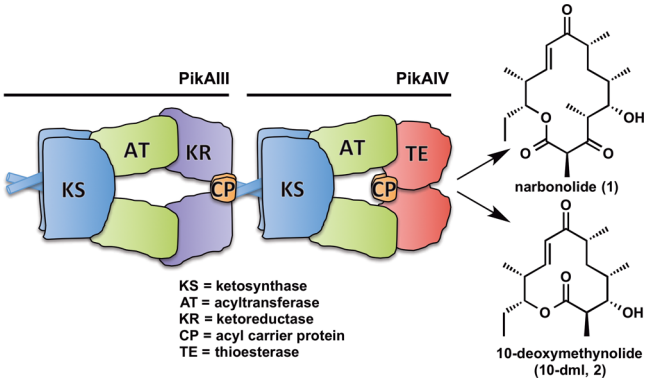
ABSTRACT: Biochemical characterization of polyketide synthases (PKSs) has relied on synthetic substrates functionalized as electrophilic esters to acylate the enzyme and initiate the catalytic cycle. In these efforts, *N*-acetylcysteamine thioesters have typically been employed for in vitro studies of full PKS modules as well as excised domains. However, substrate engineering approaches to control the catalytic cycle of a full PKS module harboring multiple domains remain underexplored. This study examines a series of alternatively activated native hexaketide substrates on the catalytic outcome of PikAIV, the sixth and final module of the pikromycin (Pik) pathway. We demonstrate the ability to control product formation with greater than 10:1 selectivity for either full module catalysis, leading to a 14-membered macrolactone, or direct cyclization to a 12-membered ring. This outcome was achieved through modifying the type of hexaketide ester employed, demonstrating the utility of substrate engineering in PKS functional studies and biocatalysis.

Modular type I polyketide synthases (PKSs) are complex bacterial proteins comprised of multiple catalytic domains. In vivo, PKS modules form multienzyme complexes leading to the production of numerous therapeutic agents.[1] Typically, PKS modules act successively whereby a two-carbon extension of a growing polyketide chain requires a minimum of three domains: an acyltransferase (AT) that accepts an acyl-coenzyme A extender unit and passes it to the acyl carrier protein (ACP), and a ketosynthase (KS) that accepts a growing chain from the ACP of the previous module and catalyzes decarboxylative Claisen condensation to extend the polyketide.[1a] In addition to KS, AT, and ACP, modules commonly contain up to three additional domains that tailor the β-keto functionality prior to the next round of chain extension. Finally, the terminal module typically contains a thioesterase (TE) domain located at the C-terminus that is responsible for polyketide chain release.[2] PikAIV is the final module in the pikromycin (Pik) biosynthetic pathway, containing the core KS, AT, and ACP domains, as well as a terminal TE domain responsible for macrolactonization to form either 12-membered 10-deoxymethynolide (10-dml, **2**) or the 14-membered macrocycle narbonolide (**1**, Scheme 1).[3]

In order to study isolated PKS modules in vitro, investigations have relied upon electrophilic thioesters for diffusive KS domain loading in lieu of transfer from an upstream ACP. Historically, *N*-acetylcysteamine[4] (NAC) has been the thioester of choice, as it mimics the terminal portion of the phosphopantetheine arm that tethers a growing polyketide chain.[5] In vitro studies of PikAIV with its native substrate have highlighted a key observation: Specifically, when incubated directly with *N*-acetylcysteamine Pik hexaketide **4**, PikAIV afforded a 4:1 ratio of macrolactones 10-dml (**2**) and narbonolide (**1**).[6] However, reaction schemes pairing PikAIII/PikAIV[3,7,8] with Pik pentaketide **3** where PikAIII performs an extension and delivers the hexaketide to PikAIV via an ACP_S thioester favor narbonolide as the major product. Additionally, optimization of PikAIII (as an unnatural TE fusion[9] or when paired with the final module, PikAIV) demonstrated improved catalysis with thiophenol thioesters[10] over *N*-acetylcysteamine thioesters.[7] These results suggest that the traditionally employed *N*-acetylcysteamine thioester might be a poor choice for loading of the KS domain and motivated exploratory substrate engineering approaches with PikAIV. However, the previously observed instability of the native Pik hexaketide **4** imposes considerable experimental challenges[7,11] and required a practical solution prior to downstream studies (Figure 1).

While mature natural products typically possess adequate stability to survive isolation and purification from biological sources, polyketide intermediates often degrade rapidly through intramolecular hemiketalization and dehydration pathways presenting experimental bottlenecks in terms of synthetic accessibility and limited shelf life.[7,11,12] Although the structural basis remains unclear, polyketide elongation intermediates that are covalently attached to the ACP domain during biosynthesis are likely stabilized through sequestration within the PKS polypeptide scaffold.[13]

Scheme 1. PikAIII and PikAIV, the Final PKS Modules from the Pikromycin Pathway



Received: November 14, 2014
Published: March 2, 2015

DOI: 10.1021/ja511743n
J. Am. Chem. Soc. 2015, 137, 3735−3738

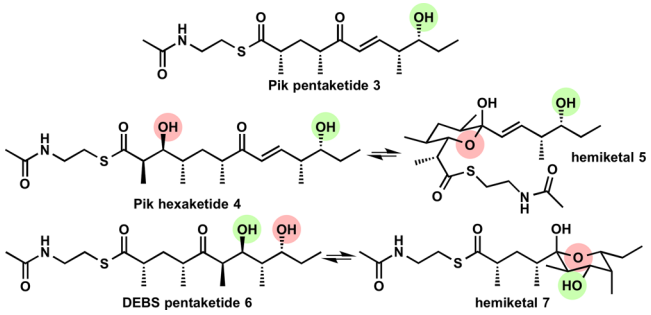


Figure 1. Examples of previously studied native chain elongation intermediates.[7,11,12a] Hydroxyl groups highlighted in red form hemiketals, while those highlighted in green are unreactive.

Thus, to protect the pikromycin hexaketide for enhanced stability to enable downstream biochemical studies, we considered two distinct stabilization strategies: (i) a sterically undemanding protecting group that would remain attached throughout the catalytic cycle, and (ii) a protecting group that could be removed in a controlled manner to provide the native hexaketide immediately before use in enzymatic reactions. Ultimately, a methyl ether protecting group was chosen to satisfy (i) and a photocleavable 2-nitrobenzyloxymethyl ether moiety (NBOM)[14] was explored to address objective (ii).

An engineered strain of *Streptomyces venezuelae* ATCC 15439 provided 10-deoxymethynolide (**2**) from which seco-acids could be generated (Scheme 2). Methylation of the C3-hydroxyl group under commonly employed etherification conditions for sterically demanding alcohols proved challenging. Only trace etherification of the hindered C3-hydroxyl group was detected in neat MeI/Ag_2O, and decomposition to a complex mixture of products was observed with excess Me_3OBF_4/1,8-bis(dimethylamino)naphthalene in CH_2Cl_2.[15] Ultimately, 1.2 equiv each of MeOTf and 2,6-di-*tert*-butylpyridine in CH_2Cl_2/PhMe (2:1, 2 M) at 4 °C furnished the desired product **9**, albeit at an extended reaction time (72 h). Concurrently, a 2-nitrobenzyloxymethyl (NBOM)[14] group was appended to **2** by employing **8** with stoichiometric $CuBr_2$.[16,17] Attention was then focused on opening the macrolactone ring known to be particularly recalcitrant toward hydrolysis.[6,7] We reasoned that a two-step global reduction and selective oxidation would neatly side-step problematic hydrolysis procedures. Excess $LiAlH_4$ in THF proved sluggish and gave a mixture of diastereomers, whereas reduction with DIBAL-H proceeded smoothly to provide single stereoisomers. Selective oxidation of triols **11** and **12** with TEMPO/PIDA[18] adjusted the oxidation state of the primary hydroxyl group to the carboxylic acid, and the allylic alcohol to the desired α,β-unsaturated ketone without oxidizing the homoallylic hydroxyl group at C-11. With desired seco-acids **13** and **14** in hand, we synthesized a series of hexaketides with a variety of thio- and oxoesters. Next, the efficiency of PikAIV loading was evaluated with each substrate in vitro using methylmalonyl *N*-acetylcysteamine (MM-NAC)[10,19] as the extender unit (Table 1).

Scheme 2. Second-Generation[7] Route to Stable Seco-acids 13 and 14 Derived from 10-dml (2)

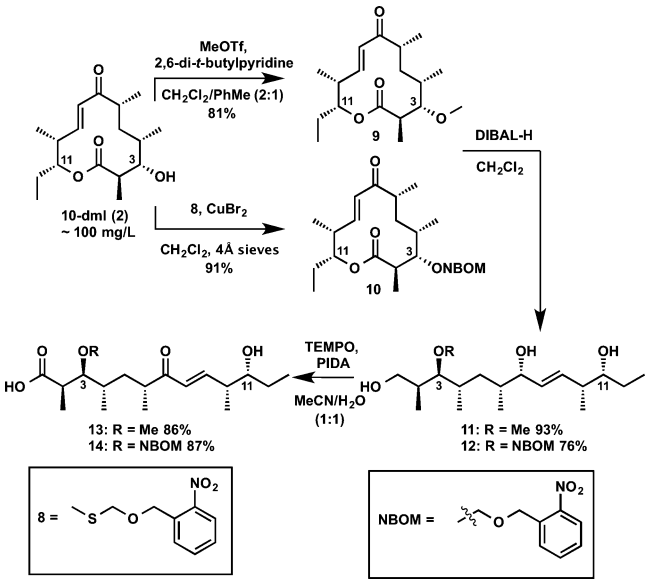
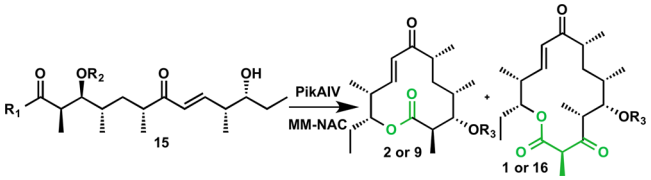
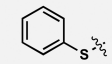
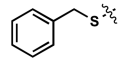
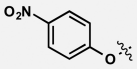
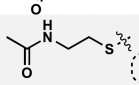


Table 1. Evaluation of Stabilized Pik Hexaketides 15 with PikAIV and MM-NAC Extender Unit[a]



Entry	R_1	R_2	$h\nu$	Conv to 2 or 9 (%)		Conv to 1 or 16 (%)
1		Me	-	4 ± 0.1	:	6 ± 0.2
2		NBOM	+	3 ± 0.3	:	41 ± 0.5
3		Me	-	trace	:	ND
4		NBOM	+	trace	:	trace
5		Me	-	4 ± 0.2	:	ND
6		NBOM	+	trace	:	15 ± 0.6
7		Me	-	18 ± 3.3	:	3 ± 0.4
8		NBOM	+	3 ± 1.3	:	trace
9		Me	-	28 ± 2.1	:	ND
10		NBOM	+	52 ± 2.9	:	4 ± 0.3

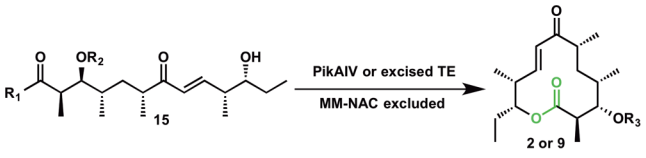
[a]Enzymatic reaction conditions: 1 mM Pik hexaketide, 20 mM MM-NAC, 8 mM 2-vinylpyridine, 1 mM sodium metabisulfite and 25 mM ascorbic acid with NBOM photolysis, 0.25 mol % PikAIV (2.5 μM), 4 h, rt. Conversion to **2** (R_3 = H) or **9** (R_3 = Me) and **1** (R_3 = H) or **16** (R_3 = Me) was monitored (HPLC) with data represented as the mean ± standard deviation where n = 3. ND = not detected.

For NBOM protected substrates, 4-nitrophenol (entry 6) and *N*-hydroxysuccinimide (entry 8) substrates decomposed rapidly upon photolysis and subsequently gave low conversion to macrolactones. In contrast, the corresponding hexaketide thiophenol, benzyl mercaptan, and *N*-acetylcysteamine thioesters photolyzed smoothly (entries 2, 4, and 10, respectively) though benzyl mercaptan thioesters (entry 4) gave lower overall conversion to either macrolactone. Remarkably, we observed significant selectivity in product formation depending on the type of ester employed, where the hexaketide thiophenol thioester (entry 2) demonstrated greater than 10:1 selectivity for generation of narbonolide (**1**), while the hexaketide *N*-acetylcysteamine thioester (entry 10) showed greater than 10:1 selectivity for 10-dml (**2**) production.

DOI: 10.1021/ja511743n
J. Am. Chem. Soc. 2015, 137, 3735−3738

In parallel experiments (Table 1), methylated substrates were converted to methyl protected 10-dml (**9**) or methyl protected narbonolide (**16**) albeit with selectivity shifted toward methyl 10-dml (**9**) and reduced overall conversions relative to native substrates generated through initial NBOM photolysis. To further establish the basis for macrolactone product distribution imparted by the thio- or oxoester employed, we altered the reaction conditions by excluding MM-NAC in PikAIV reactions,[6] and also by examining the excised Pik TE domain,[2,11] eliminating the possibility of generating narbonolide (**1**) or methyl protected narbonolide (**19**) (Table 2).

Table 2. Evaluation of Stabilized Pik Hexaketides 15 with PikAIV and Pik TE without MM-NAC Extender Unit Present[a]



Entry	R_1	R_2	$h\nu$	Enzyme	Conv to 2 or 9 (%)
1		Me	-	PikAIV	4 ± 0.2,* 6 ± 0.15**
2		Me	-	TE	10 ± 0.6
3		NBOM	+	PikAIV	17 ± 0.8,* 29 ± 3.5**
4		NBOM	+	TE	36 ± 0.8
5		Me	-	PikAIV	trace,* 3 ± 0.5**
6		Me	-	TE	4 ± 0.3
7		NBOM	+	PikAIV	5 ± 0.5,* 4 ± 0.5**
8		NBOM	+	TE	14 ± 0.5
9		Me	-	PikAIV	4 ± 0.2,* 12 ± 0.7**
10		Me	-	TE	11 ± 0.3
11		NBOM	+	PikAIV	15 ± 0.5,* 11 ± 0.6**
12		NBOM	+	TE	16 ± 0.6
13		Me	-	PikAIV	22 ± 4.2,* 35 ± 1.3**
14		Me	-	TE	55 ± 6.2
15		NBOM	+	PikAIV	5 ± 0.5,* 4 ± 0.7**
16		NBOM	+	TE	6 ± 0.18
17		Me	-	PikAIV	29 ± 2.7,* 66 ± 4.7**
18		Me	-	TE	66 ± 4.7
19		NBOM	+	PikAIV	61 ± 5.6,* 90 ± 2.1**
20		NBOM	+	TE	90 ± 1.9

[a]Enzymatic reaction conditions: 1 mM Pik hexaketide, 8 mM 2-vinylpyridine, 1 mM sodium metabisulfite and 25 mM ascorbic acid with NBOM photolysis, 0.25 mol % PikAIV (2.5 μM) (*) or 1 mol % PikAIV (10 μM) (**) or 1 % TE (10 μM), 4 h, rt. Conversion to **2** (R_3 = H) or **9** (R_3 = Me) was monitored (HPLC) with data represented as the mean ± standard deviation where n = 3.

Incubation of hexaketides with PikAIV in the absence of MM-NAC or with the excised TE domain demonstrated variation in macrolactonization efficiency to 10-dml (**2**) or methyl 10-dml (**9**) dictated by the ester employed (Table 2). Consistent with PikAIV reactions where MM-NAC was present, the *N*-acetylcysteamine thioester (Table 2, entries 17−20) gave the highest conversion to 10-dml (**2**) or methyl protected 10-dml (**9**) under all conditions tested, with *N*-hydoxysuccinimide esters providing moderate conversion to methyl protected 10-dml (**9**) (Table 2, entries 13−14). These experiments demonstrate that thiophenol thioesters are inefficient for direct macrolactonization utilizing either PikAIV or the excised TE domain (Table 2, entries 1−4).

A series of reactions to explore substrate flexibility were conducted with NBOM protected hexaketides and PikAIV (excluding MM-NAC) or Pik TE without photolysis, and yielded surprising conversion to NBOM protected 10-dml (**10**; see Supporting Information Table S1). The same general trends were observed with *N*-acetylcysteamine giving the highest levels of conversion, followed by *N*-hydroxysuccinimide, with aryl and benzyl thio- and oxoesters giving uniformly low levels of product formation. Further exploration with PikAIV (and MM-NAC included) failed to generate NBOM protected narbonolide, and heat inactivated enzymes also failed to produce either NBOM protected macrolactone.

Methyl ether mediated stabilization proved to be a viable strategy for reactions with the excised Pik TE, though suboptimal for full module catalysis. The NBOM protecting group proved to effectively stabilize the Pik hexaketide prior to in vitro reactions and should offer ready stabilization of a range of advanced polyketide chain elongation substrates for PKS functional studies. Moreover, its ability to undergo rapid and efficient photoinduced deprotection demonstrates its role as an effective tool for in vitro studies of PKS modules.

The work described herein provides further insight into substrate loading parameters in modular PKS catalysis using thiophenol thioesters,[10] indicating that product formation can be influenced by the type of substrate ester employed. While reactions with the native, upstream acyl-ACP likely offer the highest level of biosynthetic fidelity through docking domain mediated chain transfer,[20] thiophenol thioesters appear to be highly effective for achieving full module catalysis. We propose that substrate control can be explained, at least in part, through the observation that thiophenol thioesters suffer diminished conversion when incubated with the excised Pik TE domain (Table 2, entries 2, 4) relative to the corresponding NAC thioesters (Table 2, entries 18, 20). Moreover, we[11] and others[21] have shown previously that PKS TE domains function as flexible hydrolases when substrate macrolactonization cannot be achieved. PikAIV is unique among terminal PKS modules as the TE domain is able to form both 12- and 14-membered rings. However, the likely result of errant TE acylation in typical PKS modules would be substrate hydrolysis and decreased conversion to the desired macrocyclic product. As such, optimizing PKS substrates through substrate engineering may minimize hydrolysis and other undesired side reactions.

Further exploration of these strategies in other type I PKS pathways are underway with the expectation that substrate engineering will serve to better emulate natural PKS function when studying these complex enzymes in vitro, and for chemoenzymatic synthesis of secondary metabolites.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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DOI: 10.1021/ja511743n
J. Am. Chem. Soc. 2015, 137, 3735−3738

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by Rackham Merit Predoctoral Fellowships (D.A.H. and A.A.K.), American Foundation for Pharmaceutical Education Predoctoral Fellowship (D.A.H.), NIH T-32-CA009676 (A.A.K.), NIH Grant GM076477, and the Hans W. Vahlteich Professorship (D.H.S.). We are grateful to Dr. J. W. Kampf for small molecule X-ray crystallography.

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DOI: 10.1021/ja511743n
J. Am. Chem. Soc. 2015, 137, 3735−3738