LETTER

Structural rearrangements of a polyketide synthase module during its catalytic cycle

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The polyketide synthase (PKS) mega-enzyme assembly line uses a modular architecture to synthesize diverse and bioactive natural products that often constitute the core structures or complete chemical entities for many clinically approved therapeutic agents1. The architecture of a full-length PKS module from the pikromycin pathway of Streptomyces venezuelae creates a reaction chamber for the intramodule acyl carrier protein (ACP) domain that carries building blocks and intermediates between acyltransferase, ketosynthase and keto-reductase active sites (see accompanying paper2). Here we determine electron cryo-microscopy structures of a full-length pikromycin PKS module in three key biochemical states of its catalytic cycle. Each biochemical state was confirmed by bottom-up liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry. The ACP domain is differentially and precisely positioned after polyketide chain substrate loading on the active site of the ketosynthase, after extension to the β-keto intermediate, and after β-hydroxy product generation. The structures reveal the ACP dynamics for sequential interactions with catalytic domains within the reaction chamber, and for transferring the elongated and processed polyketide substrate to the next module in the PKS pathway. During the enzymatic cycle the ketoreductase domain undergoes dramatic conformational rearrangements that enable optimal positioning for reductive processing of the ACP-bound polyketide chain elongation intermediate. These findings have crucial implications for the design of functional PKS modules, and for the engineering of pathways to generate pharmacologically relevant molecules.

Modular PKSs produce chemically diverse polyketide natural products that account for a large number of pharmaceutical compounds1. The modular organization of these enzyme assembly lines provides important opportunities for synthetic biology applications and bioengineering3–5.

Figure 1 | Cryo-EM structure of pentaketide–KS5–PikAIII. a, Solid rendering (left) and transparent representation with modelled structures (right) of the pentaketide–KS5–PikAIII cryo-EM map. b, Interface of AT12 and ACP14 docked within the cryo-EM density. ACP helix 2 (residues 1439–1452) contacts the two AT lid helices (residues 701–709 and 732–744). The phosphopantetheinyalted Ser 1438 of ACP is 35 Å from the AT active site.

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However, many combinatorial biosynthesis efforts have been beset by problems of inefficiency and a limited ability to display ‘plug and play’ behaviour4–6. Thus, there is a critical need to dissect and understand the bacterial type I PKS module architecture and its conformational dynamics during polyketide chain elongation and processing.

Each module in the bacterial type I PKS catalyses the elongation of an intermediate by two carbon atoms in a linear sequence, and may also modify the intermediate by reductive processing or β-branching5. An ACP domain within each module shuttles extender units and polyketide chain elongation intermediates between the catalytic domains. In an accompanying paper2, we describe the overall architecture of PKS module 5 (PikAIII) from the pikromycin pathway (Pik)10. This PKS module is organized around a dimeric ketosynthase (KS) domain and a central chamber for the ACP, with the active sites of catalytic domains facing the chamber. Here we examined the dynamics of the PikAIII module 5 ACP (ACP5) within the reaction chamber of PikAIII to probe its localization and underlying protein–protein interactions during the catalytic cycle. Within PikAIII, the acyltransferase (AT5) loads ACP5 with a methylmalonol (MM) extender unit, and the KS (KS5) catalyses a decarboxylative condensation reaction between the MM group and the pentaketide delivered by the module 4 ACP to produce a β-ketohexaketide intermediate. The β-hydroxyhexaketide product is subsequently generated through NADPH-dependent reduction by the ketoreductase (KR5) domain of PikAIII.

Incubation of full-length holo-PikAIII with thiophenol-activated pentaketide13 generated pentaketide–PikAIII, in which this natural substrate was bound to the catalytic Cys 209 of KS5 (Extended Data Fig. 1a–c). The three-dimensional electron cryo-microscopy (cryo-EM) map of pentaketide–KS5–PikAIII was calculated at a resolution of 7.9 Å (Extended Data Fig. 2a, d), enabling precise docking of the structures of 6-deoxyerythronolide B synthase (DEBS) module 5 KS and AT12, DEBS module 1 KR13, and DEBS module 2 ACP14. This EM reconstruction revealed several striking conformational changes compared to the structure of holo-PikAIII (Fig. 1a)2. The AT5 moves towards KS5 by ~2 Å and partially occludes the side entrance to the KS5 active site (Extended Data Fig. 3a), which may sequester the labile polyketide intermediate until the extension reaction occurs. In addition, several loops on the KS surface appear to have changed position and additional densities appear at the bottom of the KS, below the catalytic Cys 209 (Extended Data Fig. 3a, b). These KS conformational changes upon pentaketide loading on Cys 209 may be allosterically sensed by the AT domains, leading to the AT5 shift. Pentaketide-induced conformational changes are also evident in the position of ACP5, which shifts 10 Å and 30 Å towards AT5 from its positions in the two holo-PikAIII conformers2. In this position ACP5 appears to insert helix 2 between the two helices of the AT5 lid subdomain (Fig. 1b). The interaction requires small conformational changes to one or both domains, although the details are not discernable in the EM maps. The reactive Ser 1438 of ACP5 is directed towards the KS5 active site, presumably poising the ACP for loading with the MM extender unit by the AT domain. We probed the ACP–AT interaction with single alanine substitutions in AT5 of the PikAIII–TE protein used for functional assays15,16 (PikAIII fused to PikAIV TE, the module 6 thioesterase domain). AT5 Glu 735 is in the ACP5 contact zone, while Arg 747, approximately 20 Å from the AT5 active site, was predicted to reside in the ACP5 docking site of a DEBS AT17. Consistent with the cryo-EM results, the E735A or the R747A PikAIII–TE mutants exhibited tenfold and fivefold reduced formation of the 10-deoxymethylenolide (10-dml) macrolactone product, respectively (Extended Data Fig. 4).

The most surprising feature of the pentaketide–KS5–PikAIII structure is that KR5 has undergone an end-to-end flip compared to the holo-PikAIII reconstruction (Fig. 1c). This finding suggests that the pentaketide-induced repositioning of AT5 and ACP5 also affects the AT5–KR5 linker, causing the large rotation of KR5. The end-to-end flip does not tangle the PikAIII topology, as the linker connections (residues 916 and 1360) are adjacent in KR5 and reside at the junction of the structural (KR5struc)
and catalytic (KRcat) subdomains. The ~180° KR5 rotation positions the catalytic domain proximal to AT5, with the AT7–KR5 interface formed by an AT5 helix and two KRcat helices. Specifically, KR5 Arg 1133 and His 1137 are positioned to interact with AT5 Glu 766 or Glu 768. We confirmed the importance of the KRcat–AT5 interface for catalytic function with R1133E, H1137E, E766R and E768R substitutions, which had 2- to 15-fold reduced activity relative to the wild-type PikAIII–TE (Extended Data Fig. 4). By contrast, substitution of a KRstruc residue at the KRstruc–AT5 interface of holo-PikAIII had no impact on production of 10-dml2. The end-to-end KR5 flip, together with the unrelated repositioning of KR5 observed upon ACP5 deletion2, indicates that its orientation depends on the state of other PikAIII domains.

To characterize the architecture of PikAIII, in which the β-ketohexaketide chain elongation intermediate is tethered to ACP5, we incubated holo-PikAIII with both MM–coenzyme A (CoA) and thiophenol-activated pentaketide (Extended Data Fig. 1d–i). The three-dimensional cryo-EM structure of β-ketohexaketide–PikAIII at 7.8 Å resolution (Extended Data Fig. 2b, d) reveals the same overall PikAIII conformation of the catalytic domains as observed for pentaketide–KS–PikAIII, with the KR5 catalytic domain proximal to AT5 (Fig. 2a). Furthermore, in the β-ketohexaketide–PikAIII structure, AT5 is shifted towards KS5 by 8 Å compared with the holo state and occludes the KS5 side entrance (Fig. 2b), probably preventing intermediate transfer from the upstream PikAIII ACP5 to the KS5 active site.

KR domains catalyse the NADPH-dependent reduction of the β-keto group to form either a D- (B-type KR) or L- (A-type KR) hydroxyl group. To form the two hydroxyl group stereochemistries, A- and B-type KRs are proposed to guide substrates into their active sites from opposite directions18,19 through the differential ordering of the KR 'lid loop' and 'lid helix' upon cofactor and substrate binding20. The β-ketohexaketide ACP5 docks with the 'lid helix' and 'lid loop' of the A-type KR5, with ACP5 Ser 1438 pointing towards the KR5 active site at a distance of 19 Å (Fig. 2c). Given the long linker between KR and ACP (43 amino acids), it is unclear whether ACP5 interacts with the KR5 of the same or the opposite monomer. An R1308E substitution in the KR5 lid helix reduced activity tenfold compared with wild type, validating the observed docking site (Extended Data Fig. 4a, b). From this position, a β-keto ACP substrate can enter the KR active site from either direction, facilitating formation of β-hydroxy products of KR-specified chirality19. Thus, the observed ACP docking site on the KR is a general solution for PKS modules with either A- or B-type KRs.

To form β-hydroxyhexaketide–PikAIII, we incubated holo-PikAIII with MM–CoA, thiophenol-activated pentaketide and NADPH (Extended Data Fig. 1j), and applied cryo-EM to obtain its structure. During initial three-dimensional reconstructions we observed three different positions for ACP5 and applied multiple-reference-supervised classification to calculate three independent three-dimensional reconstructions at resolutions of ~11 Å (Fig. 3a and Extended Data Fig. 2c, d)21,22. In all three conformers the catalytic domains are identically positioned, with the KS5 side entrance occluded and the KR5 domain oriented with its active site proximal to AT5, as is the case with the pentaketide–PikAIII and β-ketohexaketide–PikAIII structures. In each conformer the ACP5 domains are below the KSstruc and completely outside the catalytic chamber (Fig. 3b). The ACP structure can be precisely docked within each corresponding density in all three states, indicating that the three positions are highly populated and that ACP5 is not particularly flexible under these conditions (Fig. 3b, c). In these configurations, Ser 1438 of ACP5 points away from PikAIII, which is fully compatible with ACP5 docking.

Figure 3 | Cryo-EM structures of β-hydroxyhexaketide–PikAIII. a, Solid rendering of cryo-EM maps for the three conformers (i, ii and iii) of β-hydroxyhexaketide–PikAIII. The ACP density is differentially coloured with magenta, yellow and cyan. Right, a transparent representation with modelled structures of the adjacent β-hydroxyhexaketide–PikAIII conformer. b, Docking of the ACP structure24 in the corresponding density map of each

β-hydroxyhexaketide–PikAIII conformer shows that Ser 1438 invariably points away from the module. The ACP structures are coloured according to the conformers in a, c. Bottom view of overlay of the three cryo-EM maps of β-hydroxyhexaketide–PikAIII. The three different ACP positions cover a surface with a radius of 15 Å.
to the KS₈ side entrance of PikAIV for substrate transfer. This demonstrates the mechanism to maintain the directionality of PKS pathways, because only when the substrate is completely modified is the ACP ejected from the reaction chamber and positioned for transfer to the next module (Fig. 4). Consistent with this mechanism, when NADPH was excluded from a coupled PikAIII/PikAIV assay⁴,⁵, thus preventing β-hydroxyhexaketide formation, substrate consumption was halved, indicating that intermediate transfer from PikAIII to PikAIV is impaired when ACP is sequestered within the reaction chamber (Extended Data Fig. 5).

Our studies capture the travels of PikAIII ACP₅ in the reaction chamber of a complete PKS module and reveal a stunning ACP localization dependent on the identity of the phosphopantetheine-linked polyketide intermediate or extender unit (Fig. 4). The cryo-EM structures identified interaction surfaces for ACP₅ on each of the catalytic domains, differing in all cases from predicted sites, and confirmed by site-directed mutagenesis. The interacting surfaces of ACP₅ are generally charge-complementary with partner catalytic domains, and similar to those recently observed in other ACP–enzyme interactions.²³–²⁵ Interestingly, ACP₅ does not engage in specific protein–protein interactions with catalytic domains unless the appropriate substrate is loaded, indicating that tethered substrate is a primary determinant of ACP₅ localization. However, the high level of ordering observed for ACP does not apply to the post-ACP elements. The post-ACP helices, while essential for PikAIII dimerization, do not appear to be in a fixed position relative to the other PikAIII domains, suggesting that docked or fused downstream modules would also not assume a fixed position relative to the upstream module. Remarkably, the cryo-EM structures also demonstrate the existence of interdomain cross-talk within the module, evidenced both by the end-to-end flip of the KR₅ that positions its active site to interact with the β-ketohexaketide-loaded ACP and by movements of AT₃ that appear to limit access to the KS₈ active-site side entrance. Both structural transitions occur when the module contains pentaketide or hexaketide intermediates, indicating that the operation of the assembly line sets the conformational state of the PKS module and positions the domains for the next catalytic step. This clever design of the PKS module leads to optimal throughput, in which substrate availability and the rates of catalysis, rather than transfer rates, are probably the limiting steps in the catalytic cycle of type I modular megasynthases. The striking influence of substrate in the positioning and orientation of ACP, KR and AT has profound implications for the design of PKS modules. Although considerable optimization may be needed in adapting modules to unnatural substrates or in developing effective combinatorial biosynthesis approaches, the detailed insights revealed in this study provide a new structural framework from which to pursue these efforts.

**METHODS SUMMARY**

PikAIII for cryo-EM analysis was purified by Ni-affinity chromatography followed by duplicate gel filtration steps. To trap PikAIII in different states, the purified proteins were incubated with substrate, and adsorbed on holey carbon grids followed by blotting and vitrification. Bottom-up liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC/FT-ICR MS) confirmed substrate loading onto the PikAIII variants. All specimens were imaged on a Tecnai F20 transmission electron microscope (FEI) equipped with a field emission gun operated at 120 kV. Images were recorded at a magnification of ×66,964 on a Gatan US4000 CCD camera and defocus values ranging from −1.5 to −3.5 μm. EMAN routines were used for particle picking, generation of an initial model, and model refinement. Crystal structures of domains were independently fit in the EM maps as rigid bodies. Functional assays that require intermediate processing by PikAIII detected TE-offloaded products from PikAIII–TE or PikAIII/PikAIV⁶–⁸.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 7 May 2013; accepted 28 April 2014.

**Published online 18 June 2014.**

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METHODS
Design of expression constructs. Construction of expression plasmids for PikAIII (pPikAIII) and PikAIV (pPikAIV)19 and for PikAIII fused to PikAIV TE (pPikAIII–TE)20 was described previously. Mutations of PikAIII–TE were made with site-directed mutagenesis using the QuikChange Lightning multi-site kit (Strategene). All DNA constructs were confirmed with sequencing. Primers used for site-directed mutagenesis are as follows (underlined text indicates mutated base pairs in the site-directed mutagenesis primers): PikAIII1371E, CGCCGAGCCGGAACGCCGACACCTC; PikAIII1938E, GACGACGGCTAGCCGAGGCGGATCGCTCC; PikAIII2760E, AAAGCAGGTGACCCGACCCGTTGCT; PikAIII3133E, GGCGACCCGACCCAGCGGAACTCGCCCGCACCTGC; PikAIIIE735A, GGCGACCCGACCCAGCGGAACTCGCCCGCACCTGC; PikAIIIE766R, CGGCAGGTCGAGACGGGCTGGCCGAGGTC; PikAIIIE768R, GTCGAGATCATCGCTGCGCGGATCATCCCGGTC; PikAIIIR1133E, CTCGGCAGCCAGAGCATGAGCGCGGCATCCGTCC; PikAIIIR1308E, GACGACGGCTAGCCGAGGCGGATCGCTCC; PikAIIIR1333E, CTGGCAGAGCGCGGCATCCGTCC.

Expression and purification. All expression plasmids were used in E. coli Bap1 (ref. 28) cells to produce holo ACP. Transformed bacteria were cultured at 37 °C to an OD600 nm of 0.5 in 1 l of TB media with 50 μg ml–1 kanamycin. After incubation at 20 °C for 1 h, cells were induced with 200 μM isopropyl-β-D-thiogalactoside (IPTG) and allowed to express for approximately 18 h.

Cell pellets were re-suspended in 50 mM HEPES pH 7.4 (buffer A), 300 mM NaCl, 10% glycerol containing 0.1 mg ml–1 lysozyme, 0.05 mg ml–1 DNase, 2 mM MgCl2, and 20 mM imidazole. Cells were lysed by sonication, centrifuged, and the supernatant was loaded onto a 5 ml HisTrap column (GE Healthcare). A gradient of 15–300 mM imidazole in buffer A over 10 column volumes was used to elute the proteins.

For the PikAIII, PikAIV and PikAIII–TE proteins used in activity assays, the peak fractions from the HisTrap column were dialysed overnight into buffer A to remove imidazole and frozen. For PikAIII proteins analysed with EM, peak fractions from the His column were collected and further purified with a HiPrep 16/60 Sephacryl S300 HR column in buffer A. The peak fractions from the first gel filtration column were collected and further purified on a second HiPrep 16/60 Sephacryl S300 HR column.

Substrate loading of PikAIII constructs. All proteins were dialysed into 50 mM HEPES pH 7.4, 100 mM NaCl before incubation with substrates. For the pentaketide–PikAIII, PikAIII state, 1 μM holopikAIII was incubated with 1 mM thiopeinol-activated pentaketide21 for 30 min at room temperature. For the β-hydroxyhexaketide–PikAIII state, 1 μM holopikAIII was incubated with 500 μM MM–CoA and 1 mM thiopeinol-activated pentaketide for 30 min at room temperature. For the β-hydroxyhexaketide–PikAIII state, 1 μM holopikAIII was incubated with 1 mM thiopeinol-activated pentaketide for 30 min at room temperature. The peak fractions from the first gel filtration column were collected and further purified on a second HiPrep 16/60 Sephacryl S300 HR column.

Mass spectrometric analysis of active site occupancy. Bottom-up liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC/FT-ICR MS) was used to confirm the presence or absence of substrate in each domain of pentaketide–PikAIII, β-ketoheptadecate–PikAIII and β-hydroxyhexaketide–PikAIII. Loading reactions were as described earlier except 2 μM holopikAIII was used and substrate concentrations were increased accordingly. Twenty-five microlitres of each loading reaction were diluted with 20 μl 20 mM MM–CoA and 1 mM thiophenol-activated pentaketide for 30 min at room temperature followed by incubation with 500 μM MM–CoA and 1 mM thiopeinol-activated pentaketide for 30 min at room temperature.

Enzyme assays. Enzyme activities were determined using a 1×-buffer containing 25 mM HEPES pH 7.4, 100 mM NaCl, 1 mM thiophenol, and 2 mM MgCl2. The reaction mixture containing 0.5 mM NADP+, 0.5 U ml–1 glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate in 400 mM sodium phosphate pH 7.2, 20% glycerol, 5 mM NaCl. This mixture was incubated for 10 min at room temperature. The reaction was quenched by addition of a threefold excess of methanol, vortexed, incubated for 15 min at –20 °C, and centrifuged. The supernatant was analysed by reverse-phase HPLC on a Luna...
C18(2) (5 μm, 250 × 4.6 mm) column (Phenomenex) with a flow rate of 1.5 ml min\(^{-1}\) and by following this protocol: 5% solvent B (acetonitrile with 0.1% formic acid) for 1 min, 5–100% solvent B for 10 min, 100% solvent B for 4 min, and 5% solvent B for 2.5 min. Solvent A was water with 0.1% formic acid. Authentic standards confirmed the elution time of 10-dml and narbonolide (nbl). For the PikAIII–TE mutants, peak areas of 10-dml normalized to the values for wild-type PikAIII–TE were used to assess activity. For the PikAIII/PikAIV assays lacking NADPH, the peak areas of starting material were normalized to values for a reaction that lacked enzyme and compared to a reaction that contained NADPH. All reactions were completed in triplicate and the data are presented as average ± standard deviation.

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Extended Data Figure 1 | Partial mass spectra of active site peptides from LC/FT-ICR MS of trypsin-digested PikAIII. a–c, KS₅ peptides observed after incubation with thiophenol-activated pentaketide. Pentaketide was detected on KS₅ Cys 209. The pentaketide thioester at KS₅ Cys 209 is labile, and substantial quantities of unloaded KS₅ Cys 209 are expected to form during trypsin digestion and handling for MS analysis. d–i, Example mass spectra of active site KS₅, AT₅ and ACP₅ peptides after incubation with both MM–CoA and thiophenol-activated pentaketide. Pentaketide was detected on KS Cys 209, MM was detected on the AT Ser 655, and β-ketohexaketide was detected on the ACP Ser 1438. j, Active site ACP₅ peptide after incubation with MM–CoA and thiophenol-activated pentaketide (top), and MM–CoA, thiophenol-activated pentaketide and buffered NADPH (bottom). After addition of NADPH, β-hydroxyhexaketide was detected on the ACP Ser 1438.
Extended Data Figure 2 | Estimation of cryo-EM map resolution by Fourier shell correlation and assessment of over-refinement. 
a, b, Conventional Fourier shell correlation (FSC) curves (blue), gold-standard FSC (red) and high-resolution phase randomization test (green) for pentaketide–KS₅–PikAIII (a) and pentaketide–KS₅–PikAIII (b) three-dimensional reconstructions. The indicated resolution at FSC = 0.5 of the conventional FSC is in agreement with FSC = 0.143 of the gold-standard FSC. FSC calculations with phase randomized data show a sharp drop-off at the expected resolution level (10 Å). 
c, Conventional FSC curves for each conformer of β-hydroxyhexaketide–PikAIII showing a resolution between 10.7 Å and 11.6 Å at FSC = 0.5. 
d, Summary of conventional and gold-standard FSC resolution indications. Additionally provided is the agreement by FSC (at 0.5) between the average map of the two gold-standard half-maps and the conventional map (full data set), showing consistency with conventional and gold-standard FSC calculations. The right column of the table lists the cross-correlation values between each PikAIII three-dimensional reconstruction and the produced pseudo-atomic resolution model of the docked crystal structures at the indicated resolution range.
Extended Data Figure 3 | Stereo views of the pentaketide–PikAIII three-dimensional map fit with the DEBS module 5 KS (blue) and AT (green) crystal structures. a, A view looking towards the side of the KS. Upon formation of the pentaketide–KS intermediate, the side entrance to the KS active site (red star) is partially occluded and certain loop regions on the top of KS (indicated with an arrow) appear to move as indicated by differences between the EM map and KS crystal structure. b, Cross-section of the view looking towards the bottom entrance to the KS active site. Upon KS–pentaketide formation, new density (blue) appears around the KS bottom entrance that is not accounted for by the KS crystal structure.
Extended Data Figure 4 | PikAIII–TE functional assays. a, Example high-performance liquid chromatography traces of PikAIII–TE assay. The levels of 10-dml produced by wild type PikAIII–TE (red trace), R747A PikAIII–TE (green), R1133E PikAIII–TE (orange), H1137E PikAIII–TE (blue), R1308 PikAIII–TE (brown) and a no enzyme control (yellow) are shown. b, Activity of PikAIII–TE mutants. All reactions were completed in triplicate and the data are presented as average ± standard deviation.
**Extended Data Figure 5** | PikAIII/PikAIV functional assays. 

**a**, Example high-performance liquid chromatography (HPLC) traces of PikAIII/PikAIV assays with (red trace) and without NADPH (blue). Peaks for 10-dml, narbonolide (nbl) and thiophenol-activated pentaketide (starting material) are indicated.

**b**, Levels of thiophenol-activated pentaketide consumed in the PikAIII/PikAIV reactions with and without NADPH. All reactions were completed in triplicate and the data are presented as average ± standard deviation.

| Condition   | % substrate (thiophenol-pentaketide) consumed |
|-------------|---------------------------------------------|
| 0.5mM NADPH | 54 ± 1                                      |
| No NADPH    | 30 ± 3                                      |

*Extended Data Figure 5 PikAIII/PikAIV functional assays. a, Example high-performance liquid chromatography (HPLC) traces of PikAIII/PikAIV assays with (red trace) and without NADPH (blue). Peaks for 10-dml, narbonolide (nbl) and thiophenol-activated pentaketide (starting material) are indicated. b, Levels of thiophenol-activated pentaketide consumed in the PikAIII/PikAIV reactions with and without NADPH. All reactions were completed in triplicate and the data are presented as average ± standard deviation.*