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STRUCTURAL AND BIOCHEMICAL ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS BETWEEN THE ACYL-CARRIER PROTEIN AND PRODUCT TEMPLATE DOMAIN

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Abstract

In fungal non-reducing polyketide synthases (NR-PKS), the acyl-carrier protein (ACP) carries the growing polyketide intermediate through iterative rounds of elongation, cyclization and product release. This process occurs through a controlled, yet enigmatic coordination of the ACP with its partner enzymes. The transient nature of ACP interactions with these catalytic domains imposes a major obstacle for investigation of the influence of protein–protein interactions on polyketide product outcome. To further our understanding about how the ACP interacts with the product template (PT) domain that catalyzes polyketide cyclization, we developed the first mechanism-based crosslinkers for NR-PKSs. Through *in vitro* assays, *in silico* docking and bioinformatics, ACP residues involved in ACP–PT recognition were identified. We used this information to improve ACP compatibility with non-cognate PT domains, which resulted in the first gain-of-function ACP with improved interactions with its partner enzymes. This advance will aid in future combinatorial biosynthesis of new polyketides.

Keywords

Non-reducing polyketide synthase; crosslinking; acyl-carrier protein; product template domain

Fungal polyketide natural products are chemically complex small molecules, many of which have diverse biological activities. Examples include the hepatocellular carcinogen aflatoxin

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Author contribution

The primary author along with N. Gamarra and D. Martinez were responsible for the expression, purification of all protein constructs provided by A. L. Vagstad and A. G. Newman. K. Finzel, J. L. Meier synthesized the crosslinkers. J. F. Barajas, T. R. Valentic and N. Gamarra generated homology models, sequence alignments and various mutants of all the PT and ACP constructs. J. F. Barajas and N. Gamarra conducted *in silico* docking, crosslinking and *in vitro* reconstitution analysis.

B1 and the chemotherapeutic bikaverin [1]. These compounds are synthesized by highly regulated, large iterative multifunctional enzymes known as the type I non-reducing polyketide synthases (NR-PKSs) [2]. The NR-PKS is composed of six enzyme domains that are covalently linked together (Fig. 1A) [3]. These domains are responsible for polyketide elongation, cyclization, and product release (Fig. 1B) [4]. The NR-PKS lacks reducing domains and relies on other pathway enzymes for further tailoring modifications of the polyketide intermediate. Throughout the PKS biosynthetic process, the growing polyketide intermediate is tethered to the acyl-carrier protein (ACP) via a 4′-phosphopantetheine (PPant) arm and shuttled to each partner domain [5]. How the ACP selectively interacts with its NR-PKS partner domains is not well understood. Specifically, very little is known about the protein–protein interactions between the ACP and the product template (PT) domain. Because PT cyclizes the mature poly-β-keto intermediate that is tethered to the ACP, the ACP–PT interactions are hypothesized to be crucial for product outcome.

Covalent crosslinking of protein partners is a powerful tool for investigating protein–protein interactions [6]. In this work, NR-PKS ACP and PT crosslinking efficiency was used to probe domain interactions and compatibility. We prepared a series of probes designed to crosslink PT=ACP (the equal sign designates “crosslinked”) based on the cyclization mechanism of PT. We next demonstrated that crosslinking efficiency correlates with the strength of these ACP–PT interactions. Utilizing the ACP from the fungal bikaverin NR-PKS and the PT from the melanin NR-PKS, we conducted *in silico* docking in parallel with sequence-structure alignments to identify key surface binding residues on the ACP and PT domains. Several ACP and PT mutants were generated to alter ACP–PT compatibility and tested using both *in vitro* assays and mechanism-based crosslinking. These results identified structural characteristics important for protein–protein interactions and provide strategies for improved combinatorial biosynthesis for NR-PKSs.

The PT shares structural and functional similarities to the dehydratase (DH) domains of PKS and FAS (fatty acid synthase) [7]. Both PT and DH are composed of a double hot-dog fold that constitutes a core anti-parallel beta sheet with a central helix insertion (Fig. S2) [7–8]. The PT and DH share a well-conserved catalytic His–Asp dyad (Fig. 2B,C) [9], and the catalytic mechanism is also similar, where the His serves as the active site base and Asp serves to polarize the His residue for nucleophilic attack. Further, positions of the active site His and Asp for DH and PT can be overlaid almost perfectly (PDB: 3HRR, 3KG8). Therefore, we hypothesize that the mechanism-based crosslinkers for DH may also be applicable to PT (Fig. 2D).

Previous studies on *Escherichia coli* FAS yielded the discovery of the first mechanism-based inhibitor of the DH domain by 3-decynoyl-*N*-acetylcystamine (SNAC) [10]. This suicide substrate undergoes α-deprotonation in the DH active site to form an electrophilic allene, which then modifies the active site His of the DH to inactivate the enzyme. One probe that mimics this inhibitor, but with the thioester replaced by a sulfone to increase stability in water, inactivates the DH domain by the same mechanism (Fig. 2E) [11]. Expanding on this knowledge, we previously synthesized a series of analogous mechanism-based crosslinkers that incorporates a variety of known inhibitor scaffolds of DH (Fig. 2D). Based on the consideration that the substrates of DH or PT are tethered to the phosphopantetheine (PPant)

group of the ACP, probes **5-9** were designed to contain two components (Fig. 2D): 1) the pantetheine moiety that can be chemoenzymatically loaded on the active site Ser of the ACP, and 2) the “warhead” segment that selectively binds the catalytic site of the target enzyme (Fig. 2A, D).

Using a coupled chemoenzymatic protocol previously used on the *E. coli* FAS ACP, AcpP [9, 12], crosslinkers **5-9** were successfully loaded onto the heterologously expressed and purified fungal ACP monodomains, bikaverin Pks4 ACP and melanin Pks1 ACP. We were able to crosslink both Pks4 and Pks1 ACP to either the Pks4 or Pks1 PT domains. Because these two ACPs had been shown to be interchangeable for crosslinking to their corresponding partners [6], we chose to continue experiments using Pks4 ACP and Pks1 PT, as both provided the highest protein expression yields. We found that among **5-9**, crosslinker **9** with the sulfonyl 3-alkyne moiety displayed the highest crosslinking efficiency (Fig. S3A) and, therefore, was used to conduct subsequent crosslinking analyses. Although probe **9** was extensively studied with FAS DH domains [9, 12], its application for ACP=PT crosslinking has never been attempted. In order to determine if probe **9** indeed crosslinks to the active site His of PT, we conducted site-directed mutagenesis of the Pks1 PT active site. Mutations of the catalytic His or Asp residues with H61A, D248A and H252A abolished crosslinking. Partial crosslinking was observed with H61R and D248E rescue mutants (Fig. S3C). It is important to note that the numbering scheme presented here is for the monodomain constructs and does not represent the absolute numbering for the intact NR-PKS. These results strongly support the view that **9** is a mechanism-based crosslinker for the active site His of Pks 1 PT, and also affirm the hypothesized structural-mechanistic similarities between the DH and PT domains.

To identify surface residues that are important for ACP–PT interactions, we carried out *in silico* docking analysis using homology models of the Pks1 PT and Pks4 ACP (Fig. 3). Docking simulations suggest several electrostatic interactions between the positively charged PT surface and the negatively charged surface of the Pks4 ACP (Fig. 3A–B). K107, K301, and K304 are situated on the surface adjacent to the entrance of the buried active site of Pks1 PT. These residues may interact with negatively charged D60, E76 on the C-terminus of helix II of the Pks4 ACP (Fig. 3A–B). The conserved “DSL” motif in ACPs reveals a salt bridge between D60 of the ACP and K305 of the Pks1 PT. Similar to both the desaturase-ACP and FabA=ACP complex structures (PDB: 4KEH, 2XZ1) (Fig. S1) [13] [14], the region between the C-terminus of helix II and helix III of the Pks4 ACP, specifically D78 and E86 interacts with the Pks1 PT surface (Fig. 3A). These electronegative ACP surface residues were also consistently identified from a study with the KS–ACP interactions of NR-PKSs, where electropositive surface residues of KS and electronegative residues of ACP interact [6].

In order to elucidate the importance of the electronegative residues on the ACP, we generated mutants to reverse the electrostatic charge at the ACP and PT surface. Based on the PT–ACP docking, we generated E73R, E74R, D76R, D78R and E86R of Pks4 ACP, and K301E, K304E and K107E of Pks1 PT. We compared the crosslinking efficiency of all Pks4 ACP and Pks1 PT mutants with the WT constructs at varying time points over 72 hours (Fig. 4). Both K301E and K304E of Pks1 PT demonstrated a loss of crosslinking efficiency (Fig.

4). Rescue of crosslinking efficiency was observed when either K301E or K304E of Pks1 PT was crosslinked to D78R, E73R or D76R of Pks4 ACP in pairwise combinations (Fig. 4). Most notably the wild-type Pks1 PT displayed higher crosslinking efficiency with the D78R Pks4 ACP mutant. These results highlight the significance of electrostatic surface interactions in the ACP and PT complex. In addition, these findings emphasize the significance of an electropositive residue in the Pks4 ACP for Pks1 PT compatibility, which is observed in the electropositive Lys residue of the endogenous second Pks1 ACP "REKSx" sequence (Fig. 3C).

In previous studies, we had shown extensively that the crosslinking efficiencies of wild type and mutant enzyme domains from PKS and FAS are closely related with protein-protein interactions as well as enzyme activities [6, 14]. To further correlate enzyme activity to crosslinking efficiency, and to investigate the role of electrostatic surface interactions during ACP–PT complex formation, we utilized an established *in vitro* reconstruction assay to test the ACP and PT mutants [15]. Recombinant SAT-KS-MAT (SKM) tridomain from the melanin Pks1 NR-PKS pathway was reconstituted with malonyl-CoA, plus a combination of WT or mutant Pks4 ACP and Pks1 PT constructs. The Pks1 SKM, PT and Pks4 ACP with malonyl-CoA TE produce the hexaketide isocoumarin **11**, which can be detected by HPLC at a retention time of 14.7 min (Fig. 5). In the absence of the Pks1 PT, a pyrone shunt product **10** is biosynthesized instead, with a retention time of 7.8 min. Utilizing this assay, we swapped the Pks4 ACP mutants for the WT Pks4 ACP and analyzed the formation of isocoumarin **11** (Fig. 5). As a control, the active site mutant, S61A of Pks4 ACP, reveals no product formation. However, both D78R and E86R of Pks4 ACP showed a two-fold increase in the formation of hexaketide isocoumarin **11** (Fig. 5). In contrast, the ACP-E73R, E74R, and D76R mutants had similar activity to the wild-type control. These results, in parallel with the crosslinking analysis, provide the first evidence that the surface charges on ACP not only govern client domain interactions, but may alter the efficiency of the catalytic cycle. The increase in product formation seen in the D78R and E86R Pks4 ACP mutants confirms the hypothesis that engineering of non-cognate ACP constructs can be used to increase the compatibility of ACP to interact with different FAS and PKS domains.

Bioengineering polyketide biosynthesis via domain swapping requires knowledge of protein–protein interactions between the ACP and its partner domains in the synthase. As more megasynthase genomes become annotated, there is a need for tools to identify residues involved in their intricate protein–protein interactions. The chemical probes described here may be used as a general toolkit for evaluating PT–ACP interactions in both native and engineered systems. Surface residue mutagenesis, in combination with *in silico* docking, crosslinking and *in vitro* reconstitution experiments, identified key residues important in ACP–PT interactions. This study introduces the first application of a mechanism-based crosslinker for the PT domain for the analysis of PT–ACP interactions (Fig. 3–4).

The current mix-and-match effort of PKS domains often results in a complete loss of activity, and past studies had shown that ACP incompatibility is key to such failure in combinatorial biosynthesis [1b, 15]. This work shows how we can engineer ACP to increase the compatibility of ACP and increase enzyme activity (Figure 5B), which is correlated with the crosslinking efficiency (Figure 4) and also consistent with the protein docking model

(Fig. 3A). The knowledge gleaned from this study will serve to potentially guide future efforts in generating an optimized ACP that can serve as an adaptor for different types of mega-synthase domains as well as the development of new polyketide analogues with unique biological activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. a Campbell CD, Vederas JC. Biopolymers. 2010; 93:755–763. [PubMed: 20577995] b Cox RJ. Org Biomol Chem. 2007; 5:2010–2026. [PubMed: 17581644] c Dufosse L, Fouillaud M, Caro Y, Mapari SA, Sutthiwong N. Curr Opin Biotechnol. 2014; 26:56–61. [PubMed: 24679259] d Abrar M, Anjum FM, Butt MS, Pasha I, Randhawa MA, Saeed F, Waqas K. Crit Rev Food Sci Nutr. 2013; 53:862–874. [PubMed: 23768148]
2. Shen B. Curr Opin Chem Biol. 2003; 7:285–295. [PubMed: 12714063]
3. Udwary DW, Merski M, Townsend CA. Journal of molecular biology. 2002; 323:585–598. [PubMed: 12381311]
4. Crawford JM, Thomas PM, Scheerer JR, Vagstad AL, Kelleher NL, Townsend CA. Science. 2008; 320:243–246. [PubMed: 18403714]
5. Beld J, Sonnenschein EC, Vickery CR, Noel JP, Burkart MD. Nat Prod Rep. 2014; 31:61–108. [PubMed: 24292120]
6. Bruegger J, Haushalter B, Vagstad A, Shakya G, Mih N, Townsend CA, Burkart MD, Tsai SC. Chem Biol. 2013; 20:1135–1146. [PubMed: 23993461]
7. Crawford JM, Korman TP, Labonte JW, Vagstad AL, Hill EA, Kamari-Bidkorpeh O, Tsai SC, Townsend CA. Nature. 2009; 461:1139–1143. [PubMed: 19847268]
8. Akey DL, Razelun JR, Tehranisa J, Sherman DH, Gerwick WH, Smith JL. Structure. 2010; 18:94–105. [PubMed: 20152156]
9. a Meier JL, Haushalter RW, Burkart MD. Bioorg Med Chem Lett. 2010; 20:4936–4939. [PubMed: 20620055] b Ishikawa F, Haushalter RW, Burkart MD. J Am Chem Soc. 2012; 134:769–772. [PubMed: 22188524]
10. Kass LR, Brock DJ, Bloch K. J Biol Chem. 1967; 242:4418–4431. [PubMed: 4863739]
11. a Endo K, Helmkamp GM Jr, Bloch K. J Biol Chem. 1970; 245:4293–4296. [PubMed: 5498414] b Helmkamp GM Jr, Brock DJ, Bloch K. J Biol Chem. 1968; 243:3229–3231. [PubMed: 4872868]
12. a Worthington AS, Porter DF, Burkart MD. Org Biomol Chem. 2010; 8:1769–1772. [PubMed: 20449476] b Ishikawa F, Haushalter RW, Lee DJ, Finzel K, Burkart MD. J Am Chem Soc. 2013; 135:8846–8849. [PubMed: 23718183]
13. Guy JE, Whittle E, Moche M, Lengqvist J, Lindqvist Y, Shanklin J. Proc Natl Acad Sci U S A. 2011; 108:16594–16599. [PubMed: 21930947]
14. Nguyen C, Haushalter RW, Lee DJ, Markwick PR, Bruegger J, Caldara-Festin G, Finzel K, Jackson DR, Ishikawa F, O'Dowd B, McCammon JA, Opella SJ, Tsai SC, Burkart MD. Nature. 2014; 505:427–431. [PubMed: 24362570]
15. Newman AG, Vagstad AL, Storm PA, Townsend CA. J Am Chem Soc. 2014; 136:7348–7362. [PubMed: 24815013]

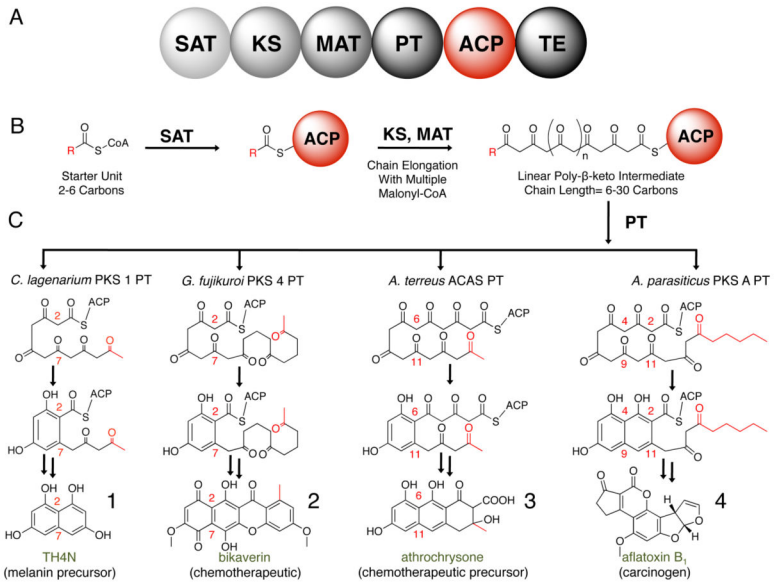

Figure 1.

(A) The typical NR-PKSs are composed of six domains that work iteratively to synthesize polyketide natural products. (B) The SAT domain is responsible for selecting the starting unit (2–6 carbon starter units in these 4 PT-mediated biochemical pathways). Multiple rounds of elongation mediated by the KS and AT generate a linear polyketide intermediate bound to the ACP that is cyclized by the PT domain. (C) NR-PKS natural products are known for their multi-cyclic templates and include TH4N, bikaverin, and mycotoxins athrochrysone and aflatoxin B_1.

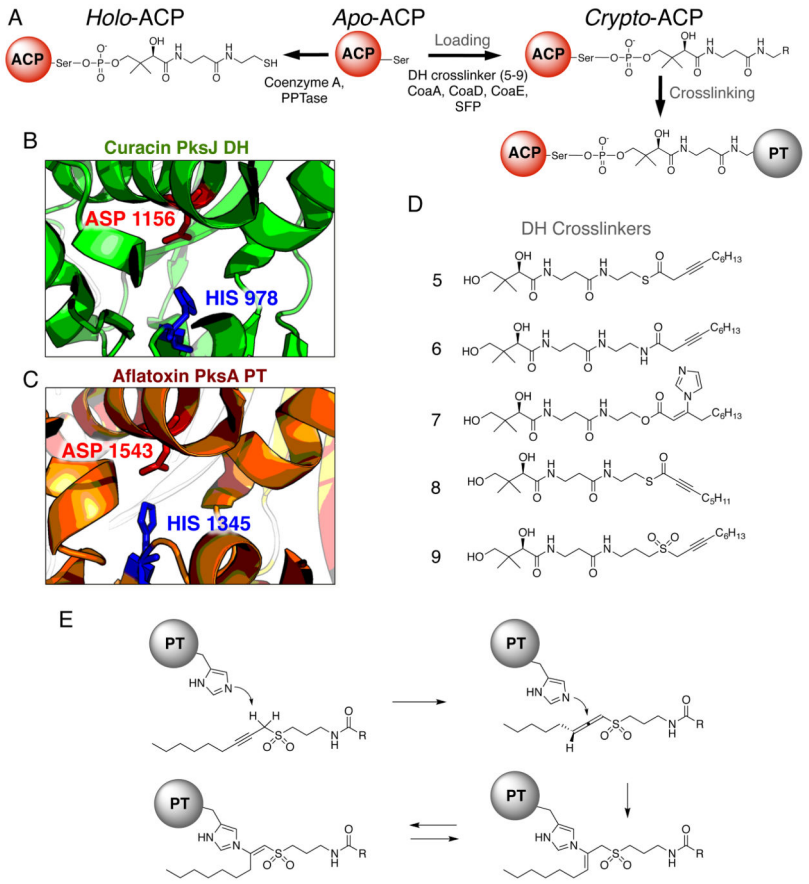

Figure 2.

(A) The ACP is expressed in the inactive *apo* form. A post-translational modification and attachment of the PPant arm generates *holo*-ACP. Crosslinking requires the loading of *apo*-ACP with a crosslinking probe to generate *crypto*-ACP, which is then crosslinked with the PT domain. (B–C) The DH and PT share a similar catalytic dyad. (D) Crosslinkers **5**-**9** were screened against the PT domain. (E) The proposed mechanism for the DH/PT crosslinking reaction with crosslinker **9**.

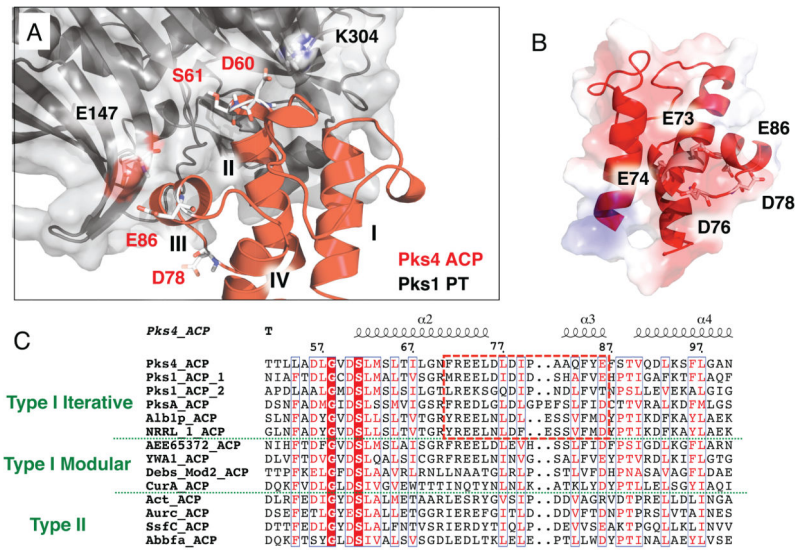

Figure 3.

(A) *In silico* docking of the Pks4 ACP with Pks1 PT reveals several electrostatic interactions, primarily on helices II and III of the ACP. (B) Pks4 ACP contains a highly negative charged surface that interacts with the Pks1 PT. (C) Multiple sequence alignments reveal key sequence motifs at the C-terminus of helix II and helix III that are important for ACP-partner interactions.

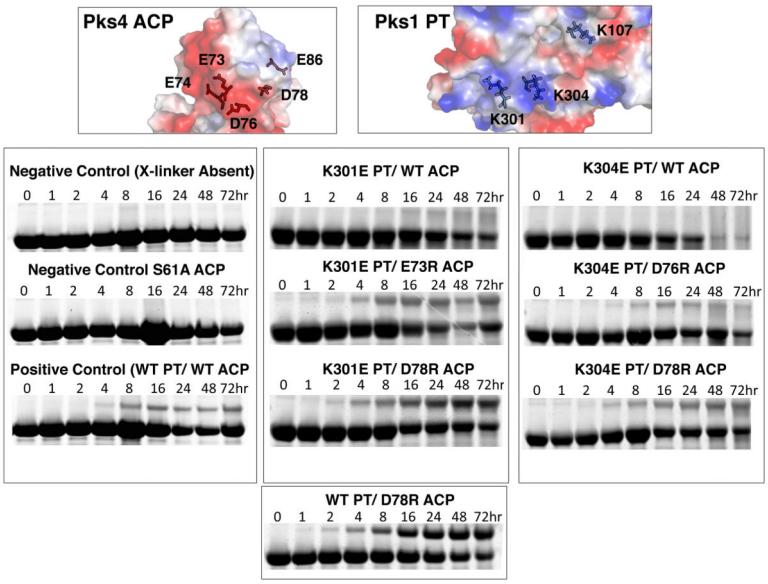

Figure 4.

The generated surface mutants of Pks4 ACP and Pks1 PT are shown on top. The time course crosslinking assay results for these mutants are shown by 15% SDS PAGE band shifts.

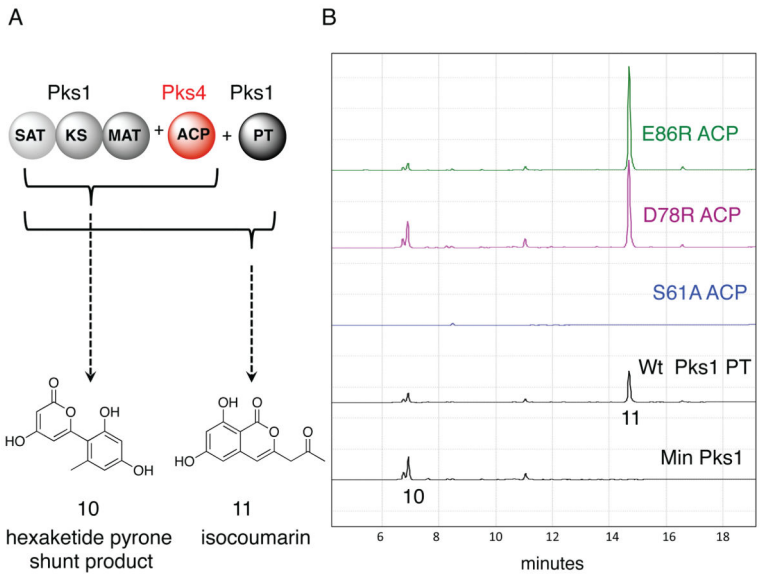

Figure 5.

(A) *In vitro* reconstitution domain swapping experiment. The absence of PT generates the shunt product **10**. The inclusion of WT Pks1 PT results in the isocoumarin product **11**. (B) HPLC analysis of mutant Pks4 ACPs displays higher product formation of **11**.