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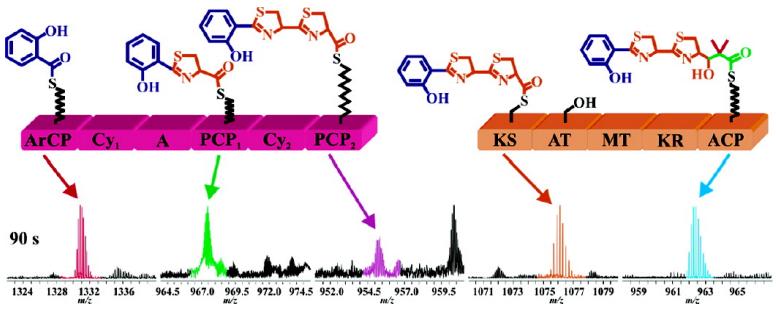

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Monitoring Multiple Active Sites on Thiotemplate Enzymes in Parallel: A Molecular Movie of Yersiniabactin Bioassembly

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Several natural products with complex structures, such as antibiotics, immunosuppressants, and bacterial virulence factors, are synthesized on multifunctional enzyme scaffolds using covalent catalysis in an assembly line manner. The modular nature of these enzymes, known as nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs),[1,2] has spurred interest in their reengineering to create "unnatural" natural products with improved clinical characteristics.[3,4] Such efforts, however, have been hindered by both the complexity of the enzymes and a lack of direct assays that provide information regarding multiple biosynthetic steps in tandem. Recently, Quadrupole Fourier Transform Mass Spectrometry (Q-FTMS) has been employed to directly interrogate peptides harboring covalently modified residues on multiple NRP/PK synthetases.[5−7] The MS-based approach provides valuable information with minimal bias concerning intermediate localization, structural heterogeneity, and occupancy on carrier sites without the use of radioactive substrates. Since this approach can be employed to monitor the covalent states of multiple active site peptides over time, a complete picture of natural product assembly can be obtained. Consequently, the MS-based approach was extended here to provide the most comprehensive molecular pictures to date of a thiotemplate assembly line in action.

Yersiniabactin synthetase, a NRP/PK hybrid, is responsible for synthesizing an iron-chelating virulence factor (siderophore) for bubonic plague,[8,9] yersiniabactin.[10] The siderophore is synthesized using four enzymes: YbtE (a salicyl AMP ligase), HMWP2 (a 230 kDa 3 module NRPS), HMWP1 (a 350 kDa 2 module NRP/PK hybrid), and YbtU (a thiazolinyl reductase), as shown in Figure 1.[10−12] A five domain truncated version of HMWP1, encompassing the polyketide module, was mixed in a 1:1 molar ratio with HMWP2, using catalytic quantities of Sfp and YbtE. Subsequent proteolysis in cyanogen bromide followed by reversed phase fractionation and mass analysis resulted in the detection of species 6526.05, 21 044.3, and 14 971.7 Da in mass. These species all correlate to holo-form peptides harboring the covalently modified serines of the ArCP, PCP1, and PCP2 domains, and elute in chromatographic fractions 24, 33, and 33, respectively (Supporting Figure 1). Further analysis revealed two peptides (5084.40 and 6367.96 Da), which correlated to the peptides harboring the apo-form active site cysteine of the KS domain and the holo-form active site serine of the ACP domain, eluting in fractions 26 and 29, respectively (Supporting Figure 1). The identities of all the aforementioned peptides were verified using tandem mass spectrometry (Supporting Figures 2−6).

A 60 min reconstitution with all the required substrates and cofactors was interrogated to identify the complex intermediates. While small quantities of holo- and monomer-loaded forms were detectable, examination of the fractions permitted the identification of the species 6646.10, 21 247.3, and 15 259.8 Da corresponding to the formation of three complex intermediates: salicyl-*S*-ArCP,

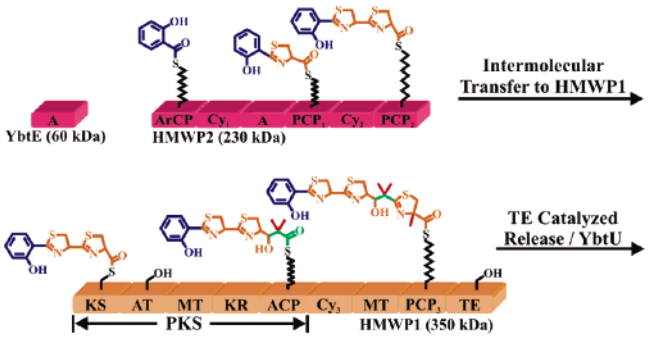

Figure 1. Assembly of yersiniabactin from YbtE, HMWP2, HMWP1, and YbtU. HMWP2 is responsible for forming the HPTT intermediate tethered to the PCP2 domain. Subsequent transfer and processing through HMWP1 creates authentic yersiniabactin after reduction by YbtU. The five domain truncated version of HMWP1 is denoted above as "PKS."

hydroxyphenylthiazolyl-*S*-PCP1 (HPT-*S*-PCP1), and hydroxyphenylthiazolylthiazolinyl-*S*-PCP2 (HPTT-*S*-PCP2), respectively (Figure 2, top). Further measurement of RPLC fractions revealed two species 5372.47 and 6728.17 Da in mass corresponding to the HPTT-*S*-KS and the HPTT-β-hydroxy-2,2-dimethyl-*S*-ACP intermediates, respectively. All of these intermediates were found to be 2 Da underweight, corresponding to the favorable oxidation of the distal thiazoline ring to its thiazole oxidation state, conjugating with the phenyl ring. However, the 2 Da loss at the ACP domain could also result from a lack of β-keto reduction. Therefore, to assign the identity of the intermediate, the reaction was redone using 3,3-d_2-L-cysteine. A species of mass 6731.09 Da, which incorporated three deuteriums, indicated the presence of the thiazole and β-hydroxy oxidation states unambiguously (Supporting Figure 6).

To visualize the assembly process, these complex intermediates were measured in a continuous kinetic assay with time points spanning 2−90 s (Figure 2). After CNBr digestion and Q-FTMS, formation of the complex intermediates on HMWP2 was found to be very rapid, accumulating occupancy within the first 2−4 s. While the fractions could not be successfully pooled to semiquantitatively determine the occupancy of each active site,[5,6] the overall speed of intermediate formation could be inferred by monitoring the absolute abundance of the target peptide and its changes with respect to coeluting peptides that are not covalently modified. While product formation occurs rapidly, the buildup of the HPT-*S*-PCP1 and HPTT-*S*-PCP2 to high occupancy requires ~20 s to reach saturation. The ACP active site peptide demonstrated complex intermediate formation as well, in as little as 4 s, with a subsequent rapid buildup to 8 s. In contrast, however, the intermediate on the KS domain did not begin accumulating product until 6 s had elapsed, indicating that the rate of intermediate formation at the ACP domain is initially faster than intermediate condensation at the KS domain. As the condensed intermediate at the ACP domain accumulated, the KS domain subsequently became backlogged with abundant HPTT-*S*-

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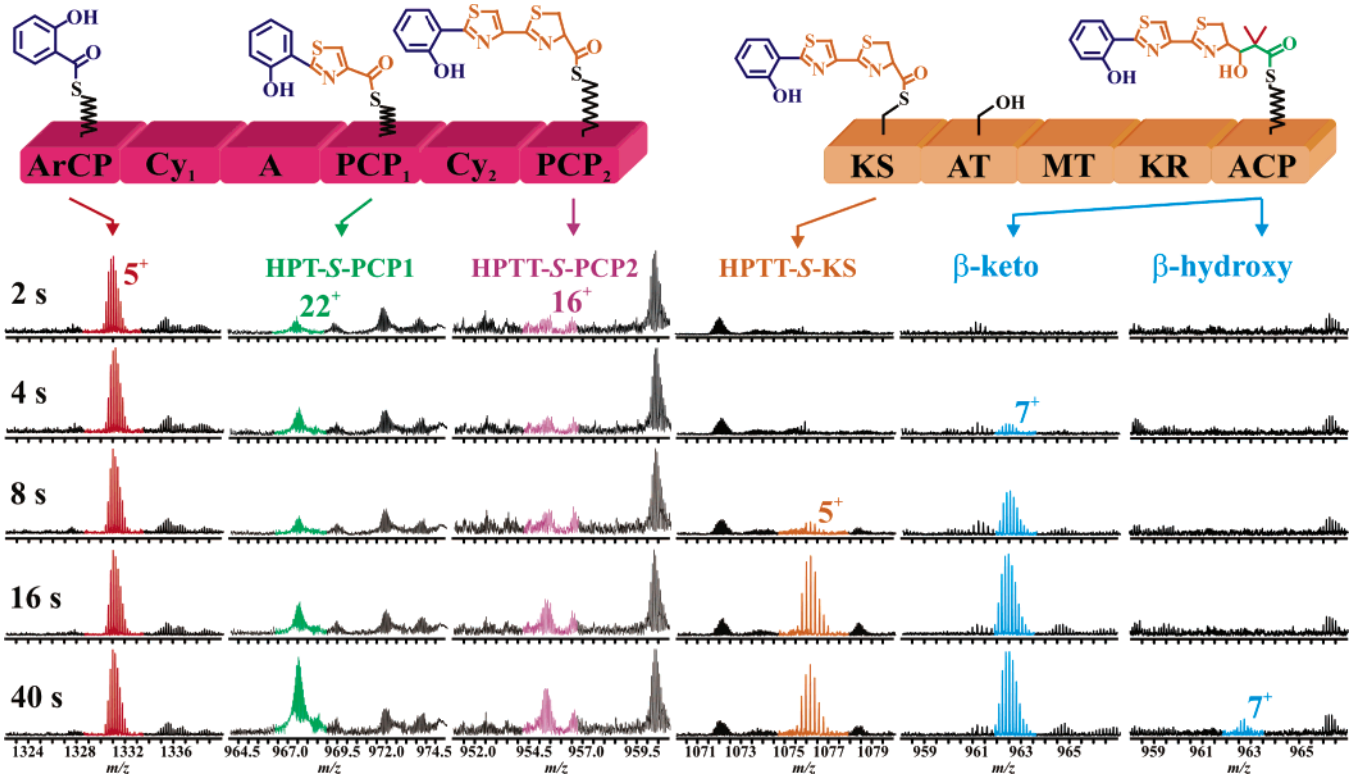

Figure 2. The bioassembly of yersiniabactin synthetase through the fourth module revealed by Q-FTMS. Each highlighted distribution correlates to the on-pathway complex intermediate, whose expected structure is noted at the top. The ACP domain produced both the HPTT-β-keto-2,2-dimethyl-*S*-enzyme and the HPTT-β-hydroxy-2,2-dimethyl-*S*-enzyme intermediates.

intermediate, which was ready to condense to the ACP carrier site upon hydrolysis of the acyl-*S*-ACP intermediate. The ACP intermediate appeared in its expected bismethylated state; however, it was 2 Da further underweight (6726.09 Da). Since this may indicate oxidation of the second thiazoline ring, the reaction was repeated in 3,3-d_2-L-cysteine. The subsequent incorporation of three deuteriums, however, indicated that the loss was due to a lack of β-keto reduction (Supporting Figure 6). By monitoring the neighboring chromatographic fraction, the ACP peptide harboring the β-hydroxy intermediate (6728.07 Da) was observed, but began to accumulate after 40 s (Figure 2, bottom right). Since the two tailoring reactions have been shown to occur independently (Supporting Figure 7) and the β-hydroxy intermediate is predominant at long time points (*vide supra*), it is apparent that under the conditions of this assay the rate of β-keto reduction is limiting. This phenomenon, however, may be due to the use of a truncated HMWP1 construct or withholding YbtU from the reaction.

The use of high performance mass spectrometry continues to evolve as a powerful complement to more widespread assays for the analysis of NRP/PK synthetases. For yersiniabactin synthesis, direct interrogation of peptides harboring covalently modified residues demonstrated rapid formation of the complex intermediates, with the rate of HPTT-β-hydroxy-2,2-dimethyl-*S*-ACP production consistent with the observed rate of yersiniabactin biosynthesis of 1.4 min^{-1}.[10] The majority of intermediate flux is in fact on the pathway, and few dead end products were observed. The work contained herein represents the first example of monitoring intermediate formation on a large number of active sites in tandem, not only providing further insights into yersiniabactin assembly but also setting a precedent for similar analyses on entire NRPS and PKS assembly lines.

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Supporting Information Available: Experimental procedures, active site mapping information, tandem mass spectra and loading experiments at the KS/ACP active sites are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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