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NPR Viewpoint

Insights into The Chemical Logic and Enzymatic Machinery of NRPS Assembly Lines

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Appreciation that some cyclic peptide antibiotics such as gramicidin S and tyrocidine were nonribosomally synthesized has been known for 50 years. The past two decades of research including advances in bacterial genetics, genomics, protein biochemistry and mass spectrometry have codified the principles of assembly line enzymology for hundreds of nonribosomal peptides and in parallel for thousands of polyketides. The advances in understanding the strategies used for chain initiation, elongation and termination from these assembly lines have revitalized natural product biosynthetic communities.

Appreciating Nonribosomal Peptide Synthetase Assembly (NRPS) Lines:

Our research on natural product enzymatic assembly lines has focused on nonribosomal peptides, from the twin points of view of understanding both the chemical logic and enzymatic machinery of how amino acids are assembled, morphed and elongated, particularly into constrained heterocyclic and macrocyclic scaffolds ([1, 2]).In one sense my group came late to the study of natural product biosyntheses, with our first publications on purification and characterization of the priming phosphopantetheinyl transferases ([3,4]) in the mid 1990s. In another sense I had been sensitized to nonribosomal peptide assembly strategies and protein machinery thirty years previously from my time as a graduate student in the research group of Professor Fritz Lipmann in the 1960s at The Rockefeller University. Two of the postdoctoral fellows in the group at that time, Wieland

Gevers from South Africa and Horst Kleinkauf from Germany, were discovering and reporting the early characterizations of gramicidin and tyrocidin synthetases:as ATP-dependent enzymes that installed the starting amino acids as covalent thioesters to tethered phosphopantetheinyl groups ([5],[6]).

For much of the first twenty years, starting in 1972, my research group focused on deciphering unusual chemical transformations in enzyme-catalyzed processes. At the end of the 1980s a new graduate student in my group Jun Liu was interested in the mechanism of conversion of chorismate to isochorismate. He established that transformation by the enzyme EntC (ent as acronym for enterobactin) proceeded via a net 1,5 proton addition/elimination reaction ([7]). We then followed the aromatization of isochorismate to 2,3 dihydroxybenzoate (DHB) by the next two enzymes in the enterobactin biosynthentic pathway, Ent A and Ent B ([8, 9]). In turn, this stimulated our interest in how 2,3-DHB was next cyclotrimerized in the presence of three molecules of the amino acid L-serine into the iron-chelating trilactone enterobactin (**Figure 1A**), whose K_D for ferric iron is estimated to be 10^{-52}M ([10]). The relevant enzyme macrolactonizing catalyst turned out to be a 140 kDa protein EntF ([11]). On purification we could show that EntF reversibly formed seryl-AMP. We suspected it would contain covalently attached phosphopantetheine (shades of the 1960s efforts in the Lipmann lab). In those days before the biological mass spectrometry revolution, we intuited the presence of a P-pantetheine prosthetic group in EntF by acid hydrolysis and microbiological analysis to detect released β-alanine with an *E. coli* strain deleted in the aspartate decarboxylase gene ([11, 12])and so requiring added β-alanine for growth.

At that point we became seriously interested in full reconstitution of the three amide and three ester bonds of enterobactin and committed to dissecting the logic and catalytic mechanisms for assembly of not only this peptide siderophore ([13, 14, 15]) but also the siderophores pyochelin ([16]), vibriobactin ([17, 18]), and yersiniabactin ([19, 20, 21]) which have thiazoline and oxazoline rings as ligands for iron (**Figure 1B**). They were formed on short, fully reconstitutable NRPS assembly lines which allowed pioneering studies of the many steps in assembly line operations. This was

the side door that got us into twenty years of research and a myriad of publications on NRPS assembly lines. During the past two decades our research group enjoyed fruitful collaborations with Prof Mohamed Marahiel and his group in Marburg and also with the combined efforts of Profs Cane and Khosla in their studies on polyketide biosynthesis ([4,22,23,24,25]). To help decipher the principles of assembly line enzymology, the four research groups met every other year over the course of a decade for open discussions of new concepts, approaches, and methodologies.

We also have had an abiding interest in antibiotics, including mechanisms of action, modes of resistance development, and routes of biosynthesis ([25]). In particular, vancomycin caught our attention during the early 1990s when it was often used as a life-saving antibiotic "of last resort" for patients coming down with fulminant bacterial infections in the middle of cycles of cancer chemotherapy. The dramatic rise of vancomycin resistant enterococci (VRE) in hospital-based infections with high mortality rates motivated my group to decipher the molecular basis of resistance ([26,27,28]). We then took our second avenue into nonribosomal peptide biosynthetic logic in the hope of getting around clinical resistance: to understand the rules by which the producing microbe *Amycolatopsis orientalis* strung together a heptapeptide, with five nonproteinogenic amino acid monomers, and then regiospecifically crosslinked it three times to produce the rigidified cup-shaped architecture of the mature vancomycin scaffold. (**Figure 2**) ([29]).

We have undertaken research into amino acid selection, posttranslational priming of carrier protein domains, the processes of chain initiation, elongation, and termination from covalent tethering on the assembly lines, and post translational tailoring enzymes that create the mature natural product scaffolds, including some novel chemical transformations and catalyst types ([2,30]).

Priming of Assembly Lines: The phosphopantetheinyl transferase superfamily: Perhaps the most generally useful enzymology we have contributed to NRPS (nonribosomal peptide synthetase) and PKS (polyketide synthase) assembly lines was the purification and characterization of the

phosphopantetheinyl transferases (PPTases) ([3, 4,13]). There are two related PPTase families which convert the inactive *apo* forms of carrier protein domains to the active *holo* forms in both acyl carrier protein (ACP) contexts of PKS and FAS (fatty acid synthase)assembly lines and also in peptidyl carrier proteins (PCP) of NRPS assembly lines (**Figure 3**). In so doing we also found, in collaboration with the Marahiel group ([4]), that the Sfp protein (required for surfactin production) was a broad specificity PPTase that primed apo forms of many ACPs and PCPs. These discoveries built on initial studies of Elovson and Vagelos ([31]) on partial purification of the *E coli* priming PPTase for the free standing acyl carrier protein of FAS. The work has enabled subsequent use of Sfp and congeners as a broad specificity priming PPTase for a wide range of PKS, NRPS and hybrid NRPS/PKS protein components in heterologous bacterial and fungal hosts (see Beld et al[32], for a recent review). The *apo* to *holo* chemical transformation is related to the action of serine-specific protein kinases, but in this case it is CoASH not ATP that is the electrophilic donor and the product is a protein-Ser phosphodiester rather than the monoester in the case of the protein kinases (Figure 3).

NRPS Assembly Lines

Chain Initiation: Once NRPS (and PKS) assembly lines are primed at every carrier protein subunit/domain, the assembly lines can begin to run. At every module, an amino acid is selected by a 50 kDa adenylation (A) domain and activated as the aminoacyl-AMP mixed anhydride by attack on Pα of Mg-ATP by the bound aminoacyl carboxylate. The thermodynamic activation is then preserved on covalent tethering of the aminoacyl group in thioester linkage to the terminal thiolate of the P-pantetheine arm on each holo-carrier protein domain.

In the initiation module for gramicidin synthetase we dissected rates of L-Phe activation, covalent loading, and epimerization to yield the D-Phe_1-S-GrsA and of the cognate tyrocidine synthetase module, before chain elongation to the downstream Pro_2-S-GrsA occurs ([33],[34-36]). We and others evaluated mechanisms in lipopeptide

biosynthesis for loading of acyl chains onto the free amine of the aminoacyl$_1$-S-module 1 ([37]).

Chain Elongation: During chain elongation at the internal modules of an NRPS assembly line, in addition to peptide bond formation catalyzed in each such module by a 50 kDa condensation (C) domain, two kinds of optional modifications occur. One is "on assembly line" epimerization of L-aminoacyl- and L-peptidyl thioesters to the corresponding D-residues before transfer to the next downstream module. Whereas Phe1 of gramicidin S is epimerized as the aminoacyl-S-GrsA before condensation while Phe 4 is epimerized after condensation but before transfer to the downstream module. These studies led to subclassification of C domains into $^{L}C_{L}$ and $^{D}C_{L}$ to indicate donor and acceptor stereochemistry ([38]). No $^{D}C_{D}$ domains were detected even when paired D-residues were found in the mature peptide products (such as vancomycin), consistent with the observed timing of epimerization of elongating peptidyl thioester chains.

The second common modification during NRPS assembly line elongation steps is N-methylation, catalyzed by methyltransferase (MT) domains, inserted into A domains. This appears to be a straightforward transfer of an electrophilic methyl group equivalent from S-adenosylmethionine to the amine of the thiol-tethered amino acid. Detection of intermediates covalently accumulating on NRPS and PKS assembly lines was improved by development of mass spectrometry methodology by Pieter Dorrestein, while a join postdoctoral fellow in our group and that of Neil Kelleher to observe ejected acyl/peptidyl-pantetheine fragments ([39, 40]).

A third set of chemical alterations during elongation of siderophore chains can occur on Ser, Thr, and Cys residues, with cyclization to oxazoline, methyloxazoline, and thiazoline respectively, ([41, 42, 43, 44]) as shown in figure 1B. The nitrogen atoms in these conformationally constrained heterocycles are basic and serve as ligands to $Fe^{(III)}$.

These five ring heterocycles can undergo redox transformations. In yersiniabactin and pyochelin a thiazoline gets reduced to the thiazolidine, while in the NRP-PK

hybrids epothilone and bleomycin the thiazolines are oxidized up to thiazoles, heteroraromatic rings where the nitrogens show reduced basicity (**Figure 4**) ([45, 46]). The planar bithiazole in bleomycin is reputed to be a DNA-intercalating element.

Chain Termination: Many bacterial NRPS and PKS assembly lines have a 35 kDa thioesterase (TE) domain as the most downstream domain in the last module to function as a release catalyst for the full length chain. By contrast many fungal NRPS instead have a condensation (C) domain that mediates chain release, often by macrocyclization ([47]). The TE domains are members of the serine hydrolase super family and the full length chain moves from *thioeste*r linkage on the last ACP/PCP to *oxoester* linkage as an acyl-O-TE covalent intermediate.

Two major chain release fates of the acyl-O-enzyme intermediate are known. One is straightforward hydrolysis, releasing the peptide free acid, as occurs for yersiniabactin and the vancomycin aglycone (**Figure 5A**). The chemically more interesting fate is intramolecular capture of the acyl-O-TE by an internal nucleophile within the chain, yielding conformationally constrained macrocycles. For gramicidin S and tyrocidine it is the free amino terminus of D-Phe1 that acts as the internal nucleophile, yielding a cyclic macrolactam from head to tail condensation. When the kinetically competent internal nucleophile is a hydroxyl oxygen, the result is instead a cyclic macrolactone. In collaborations with the Marahiel group we could show that apo-PCP-TE didomains could be loaded with peptidyl-S-CoA derivatives by the Sfp PPTase and function as regio-and stereoselective macrocyclization catalysts ([48-50]).

Among the variant release routes is NADPH-mediated reduction found in the yeast lysine biosynthetic pathway. There is no TE domain in the Lys2/Lys5 enzyme pair. Instead there is an NADPH-utilizing reductase doman that acts as chain terminator. The aminoadipoyl thioester is reduced to the thiohemiacetal which spontaneously unravels to release the aldehyde product ([51]) which exists predominantly as the cyclic imine (**Figure 5B**). In lyngbyatoxin A formation the chain termination step involves double reduction, via 2 NADPH molecules, converting the thioester to aldehdyde and ultimately to the alcohol ([52]). One additional route that has been

characterized in fungal NRPS assemblages is Dieckmann cyclization for chain release ([53]) as shown for cycloacetyl-tryptophan (Figure 5B).

Tailoring Enzymes: Dedicated tailoring enzymes are often encoded within biosynthetic gene clusters. They can act to provide nonproteinogenic amino acids as building blocks, tailor elongating chains still on assembly lines, or act as post-assembly line tailoring catalysts. We have noted how several pyrrole-containiing natural products arise from enzymatic oxidation of prolyl-S-PCPs, and then subsequent mono- and di-halogenation by flavin-dependent halogenases (**Figure 6A**) ([54]). We discovered a second set of halogenases, iron-based O_2 consuming catalysts, that halogenate aminoacyl-S-PCPs at unactivated carbon sites ([55, 56]) (**Figure 6B**). Remarkably such cryptic chlorination occurs during conversion of a tethered *allo*-isoleucine to the corresponding cyclopropyl aminoacyl thioester in coronamic acid generation. Six of the seven amino acids in the vancomycin scaffold are nonproteinogenic. In addition to the chlorohydroxy-Tyr residues at residues 2 and 6 are the 4-OH-PheGly at 4 and 5 and a 3,5-$(OH)_2$-PheGly as the seventh residue. We characterized eight enzymes encoded in the chloroeremomycin gene cluster that build these PheGly amino acid frameworks by remarkable distinct pathways (summarized in Hubbard & Walsh, [29]).

Our efforts to evaluate selectivity, timing, and mechanism of post-assembly line tailoring enzymes resulted in mechanistic and structural characterization of the three glycosyltransferases involved in conversion of the vancomycin aglycone to the mature chloroeremomycin antibiotic ([57-61]).

NRP/PK hybrid assembly lines: Among the most interesting natural product scaffolds built by assembly lines are NRP-PK hybrid molecules. Those assembly lines require that growing chains are passed without a hitch between PKS and NRPS modules ([22]). We examined these requirements in the reconstitution of the yersiniabactin and epothilone assembly lines and in studies on installation of unusual α-methyl substituents in bacillaene formation ([62-64], as well as characterization of assembly lines where α-keto acids, rather than amino acids,

were selected by some A domains and then reduced on assembly line to either D- or L-hydroxyacyl thioesters ([65]). The first three modules of the epothilone assembly line were examined in detail and reconstituted to show how a PKS-NRP and then an NRPS-PKS interface could function to elongate a hybrid chain (**Figure 7**). Once the N-acetyl-Cys bond is formed by the C domain of the NRPS module, it is cyclized to the thiazoline and oxidized to the heteroaromatic thiazole before passage on to the second PKS module ([43, 66]).

Challenges and Opportunities:

The dramatic explosion in microbal genome sequences and the development of bioinformatic programs to identify as yet uncharacterized NRPS and PKS and hybrid modules offers opportunities for finding new molecular scaffolds. A widely utilized discovery framework is the antismash program (antibiotics and secondary metabolite analysis shell) ([67], antismash.secondarymetabolites.org). These would serve as starting points for engineered evolution of new activities and new pathways from synthetic refactoring efforts. The recent excitement around teixobactin ([68]) as an NRP lipopeptidolactone antibiotic acting as a novel lipid II antagonist continues to indicate the rewards of accessing the previously uncutivatable microbiomes. Many of the known therapeutically active molecules require several steps of post assembly line back end tailoring, by dedicated sets of enzymes. Manipulation of the tailoring catalysts offer routes to expanded structures and activities. On the front end of NRPS assembly lines exploration of additional nonproteinogenic amino acid building blocks such as *ortho*-aminobenzoate has begun to offer insights into short pathways to fungal peptidyl alkaloid complexity ([69]) while *para*-aminobenzoate moieties have turned up in cystabactamids, new DNA gyrase inhibitors ([70]). There is no reason to believe that all the nonproteinogenic amino acid building blocks have yet been found for NRPS and hybrid NRPS-PKS assembly lines.

Figure Legends:

Figure 1: (A) Three amide and three ester bonds are created in generation of the siderophore enterobactin by a two module nonribosomal peptide synthetase assembly line involving proteins Ent BDEF. (B) The siderophores pyochelin, vibriobactin, and yersiniabactin contain two types of ligands for chelation of $Fe^{(III)}$: phenols or catechols, and thiazoline or oxazoline heterocycles.

Figure 2: The heptapeptide framework of vancomycin includes five nonproteinogenic amino acids, with three rigidifying crosslinks.

Figure 3: The phosphopantetheinyl transferases (PPTases) are posttranslational modification enzymes that transfer the phosphopantetheinyl moiety from coenzyme A to a specific side chain in the folded forms of peptidyl carrier proteins (PCPs) or acyl carrier proteins (ACPs), converting inactive apo to active holo forms. The holo form of the carrier protein contains a serine phospho diester linkage; the chemistry has analogy to formation of serine phospho monoester linkages in protein kinase action.

Figure 4: Cyclodehydration of X-Ser/-Thr/-Cys moieties generates five membered oxazoline/thiazoline heterocycles. Subsequent redox reactions can lead to two electron reduction to thiazolidine/oxazolidine while two electron oxidation yields the heteroaromatic oxazole/thiazole ring systems.

Figure 5: Chain termination strategies for release of covalently tethered thioesters from NRPS modules: (A) Two major fates involve chain transfer to terminal thioesterase (TE) domains and then either intermolecular transfer to water(hydrolysis) or intramolecular transfer to hydroxyl (macrolactone) or amino groups (macrolactam). (B) Chain release can occur reductively, either by two electrons to the free aldehyde or by four electrons to the alcohol. A Dieckmann condensation is involved in cyclizing release of acetoacetyl-Trp-S-PCP.

Figure 6: Oxidative, on assembly line tailoring of aminoacyl-S-PCPs: (A) Proline after activation and installation on a holo-PCP can be oxidized by four electrons to the corresponding pyrrolyl-S-PCP and then chlorinated or chain-extended in hybrid

assembly lines. (B) chlorination of C_4 occurs on Thr-S-PCP, and three chlorines can be transferred to Leu-S-PCP in barbamide formation by nonheme oxygen-dependent halogenases. Monochlorination of an *allo*-Ile-S-PCP produces an intermediate that then undergoes conversion to the amino-carboxy-cyclopropane framework in coronamic acid formation.

Figure 7: The first three modules of the epothilone synthase assembly line contains two PKS modules and one NRPS module. They act sequentially to generate a methylthiazolyl intermediate on EpoB that is chain extended by a methylmalonyl-derived unit on EpoC.

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