

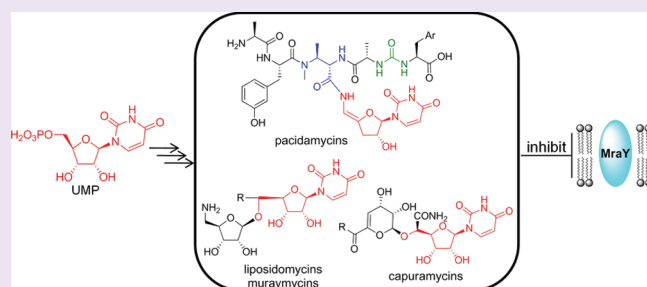
Chemical Logic and Enzymatic Machinery for Biological Assembly of Peptidyl Nucleoside Antibiotics

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ABSTRACT: Peptidyl nucleoside antibiotics are a group of natural products targeting *MraY*, a bacterial translocase involved in the lipid-linked cycle in peptidoglycan biosynthesis. In this Perspective, we explore how Nature builds complex peptidyl nucleoside antibiotic scaffolds from simple nucleoside and amino acid building blocks. We discuss the current stage of research on biosynthetic pathways for peptidyl nucleoside antibiotics, primarily focusing on chemical logic and enzymatic machinery for uridine transformation and coupling to peptides. We further survey the nonribosomal biosynthetic paradigm for a subgroup of uridyl peptide antibiotics represented by pacidamycins, concluded by diversification opportunities for antibiotic optimization.



Microbes make a variety of natural products with antibiotic activity, including molecules with polyketide frameworks (erythromycins and tetracyclines), oligosaccharide frameworks (aminoglycosides), and those with peptide scaffolds. The peptide scaffolds can be of ribosomal origin with extensive post-translational modification to rigidify the architecture, as in lantibiotics¹ and thiazolyl peptides,² or they can be made on nonribosomal peptide synthetase (NRPS) assembly lines, most famously the tripeptide precursors to penicillins and cephalosporins.³ The nonribosomal peptide antibiotics are often elaborated during biosynthesis to lipopeptides such as daptomycin⁴ or to glycopeptides such as vancomycin⁵ to alter physical properties and provide targeting.

Microbes can also join nucleosides to peptide scaffolds to create peptidyl nucleosides that function as antibiotics. Examples include the cytosine derivative blasticidin S and the adenine derivative puromycin, which target bacterial and fungal ribosomes,^{6–8} and uridine-based nikkomycins and polyoxins, which target cellulose biosynthesis in fungi.^{9,10} A larger group of uridyl peptide natural product antibiotics, including pacidamycins and mureidomycins, liposidomycins and caprazamycins, capuramycin and muraymycins (Figure 1), target the enzyme *MraY* involved in the first membrane-dependent step in bacterial peptidoglycan assembly.¹¹

■ MRAY AND BACTERIAL CELL WALL BIOSYNTHESIS

MraY is an essential bacterial enzyme with 10 predicted trans-membrane helices that embed it within the cytoplasmic membrane of bacteria¹² where it can interact with one of its substrates, the C_{55} isoprenoid carrier lipid known as bactoprenol-phosphate (bactoprenol-P). The cosubstrate is the UDP-*N*-acetylmuramoyl

(UDP-MurNAc) pentapeptide (-L-Ala₁-D- γ -Glu₂-Lys/DAP₃-D-Ala₄-D-Ala₅-COOH) that accumulates from action of the enzymes MurA-F in the bacterial cytoplasm. The MurNAc-pentapeptide will need to be subsequently glycosylated with an *N*-acetylglucosamine (GlcNAc) residue and flipped to the outer surface of the cytoplasmic membrane where the disaccharyl pentapeptide units can be used to elongate peptidoglycan chains necessary for cell division (Figure 2). The UDP moiety, the canonical biologic sugar activating group, has two negatively charged phosphate groups and cannot cross membranes. Thus transfer of the MurNAc-pentapeptide to the membrane-soluble C_{55} bactoprenol-P by *MraY* is a necessary prequel to getting peptidoglycan building blocks to the outer surface of the membrane. For this reason, *MraY* is sometimes termed a translocase because it moves the sugar pentapeptide unit from the cytoplasm to the membrane. The *Bacillus subtilis* *MraY* has been solubilized, purified, and characterized kinetically, but there are no structural data to guide the *MraY* inhibitor design.¹³ It is also unclear if the phospho-MurNAc-pentapeptide group is directly translocated to bactoprenol-P or this transfer process involves a covalent enzyme intermediate. If direct, this would be a precedented displacement of UMP as a good leaving group by a nucleophilic phosphate oxygen, in this instance the C_{55} phosphate monoester (Figure 2).

■ URIDYL PEPTIDE ANTIBIOTICS TARGETING MRAY

As often the case for antibiotics Nature has served up some scaffolds that give insights into mode of action and further

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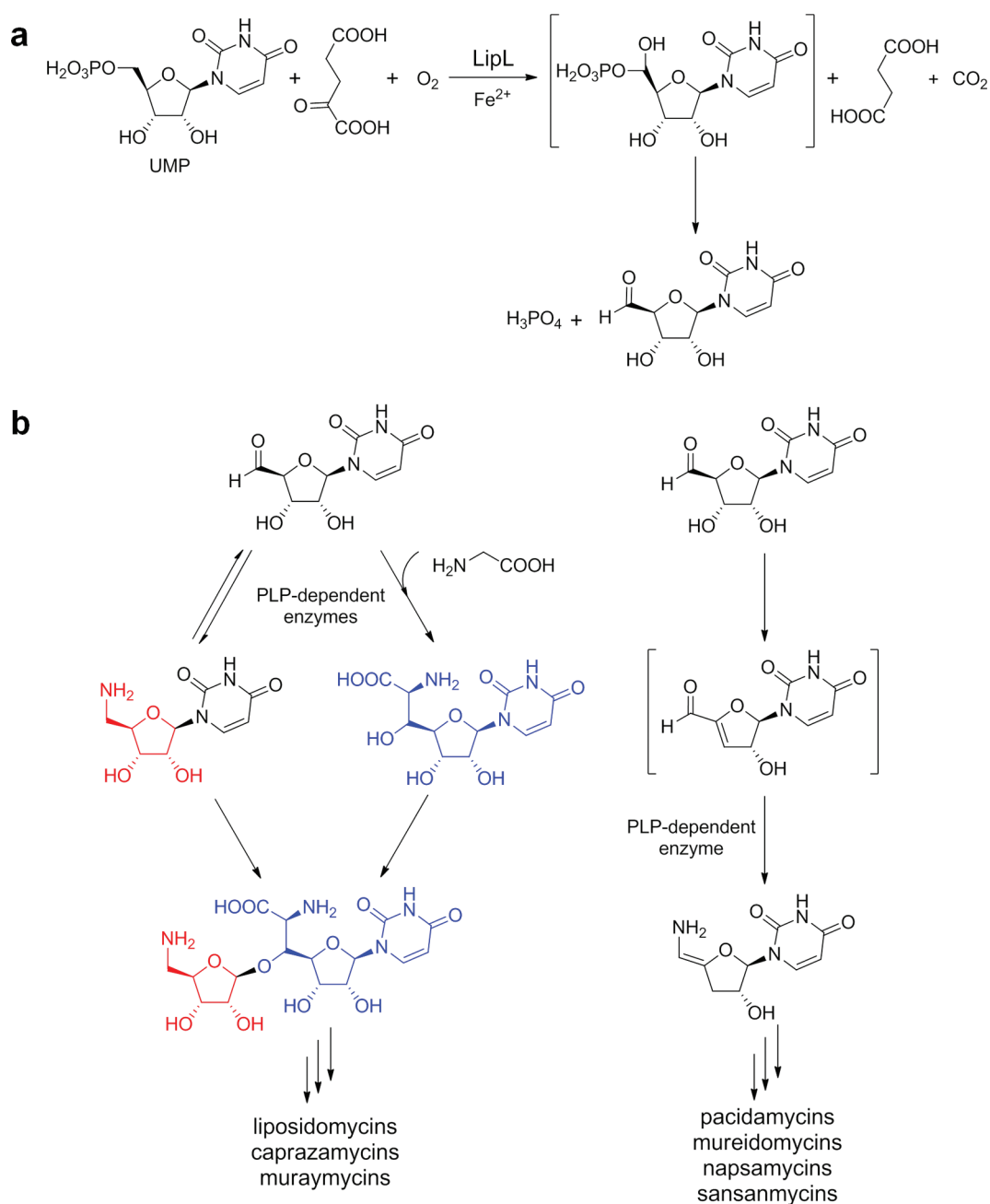


Figure 3. Schematic of nucleoside modification for peptidyl nucleoside antibiotics biosynthesis. (a) Characterization of LipL as a Fe(II)- and α -ketoglutarate-dependent hydroxylase catalyzing uridine-5'-aldehyde formation from UMP. (b) Proposed roles of uridine-5'-aldehyde and uridine-5'-amine as electrophilic and nucleophilic condensation partners.

chains of various lengths can be esterified, giving rise to the name liposidomycins. Capuramycin forms a third subgroup in which C-5' of the uridine moiety has a pendant one-carbon carboxamide moiety that has been introduced during biosynthesis. As one might anticipate from these structures, the producing microbes have thus built molecules that mimic the *MraY* substrate UDP-MurNAc-pentapeptide and act as competitive inhibitors with the uridine moiety as a key recognition element. None of the molecules is a potent broad spectrum antibiotic ready for human clinical use, but they have selectivity against bacterial *MraY* and are generally not toxic,¹⁴ suggesting they could be improved. This is in contrast to tunicamycins and

related metabolites (Figure 1); these uridyl-sugar-lipids lack peptide moieties and are prohibitively toxic by inhibiting a wide variety of membrane-associated glycosyl transferases including those involved in eukaryotic glycoprotein maturation.

■ BIOSYNTHETIC GENE CLUSTERS TO PROVIDE ASSEMBLY HYPOTHESES

In the past two years genome sequencing, gene cloning, and bioinformatics-guided analyses have identified biosynthetic gene clusters for liposidomycins, caprazamycins, capuramycin, pacidamycins, and napsamycins.^{15–21} A combination of individual

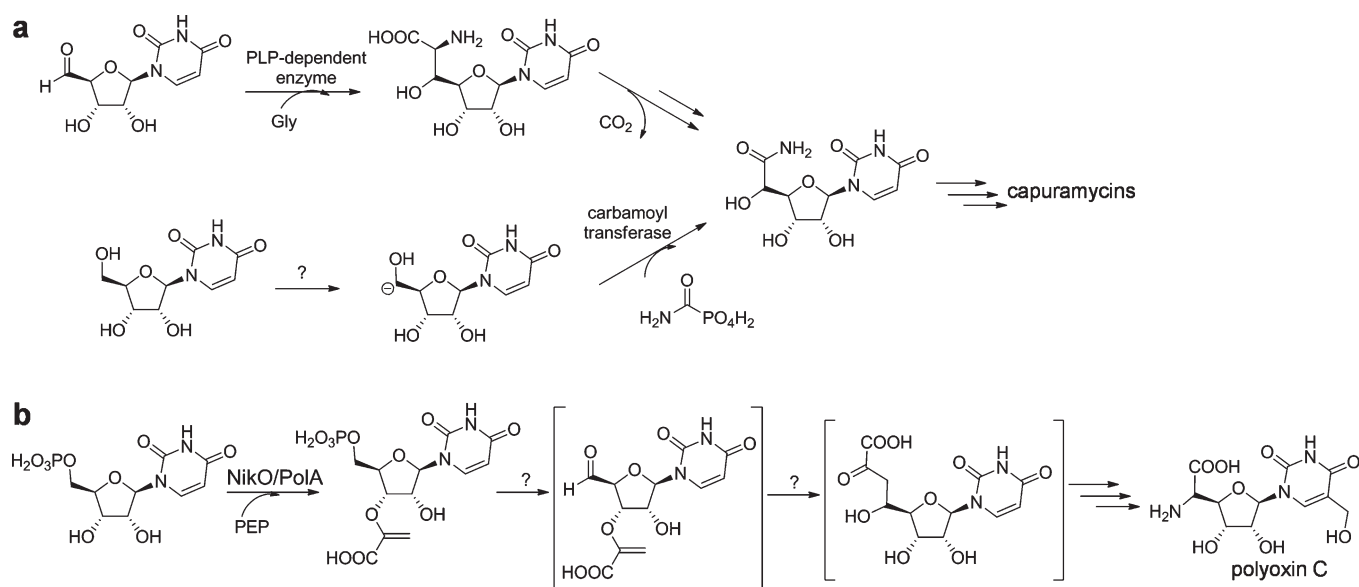


Figure 4. Proposed nucleoside transformations leading to capuramycin (a) and polyoxin C (b).

gene mutagenesis, heterologous expression of gene clusters in *Streptomyces* hosts, and mechanistic study of purified pathway enzymes have given insight into the chemical logic for coupling of uridine moieties to the peptide backbones, as well as appreciation for the nonribosomal peptide assembly lines used to build the noncanonical peptide scaffolds. These pathways have also turned up novel chemistry in biological systems.

■ URIDINE-5'-ALDEHYDE AND URIDINE-5'-AMINE TO PROVIDE ELECTROPHILIC AND NUCLEOPHILIC CONDENSATION PARTNERS

Inspection of C-5' of the ribose moiety in liposidomycins, caprazamycins, muraymycins (and also tunicamycins) has suggested that enzymatic oxidation of the 5'-alcohol to the aldehyde level would create an electrophilic center for C–C bond formation. This uridine metabolite, the 5'-aldehyde, had never been reported in the literature, and several efforts to find a uridine-specific alcohol dehydrogenase/oxidase activity have not succeeded. In 2011, Van Lanen and colleagues reported that an oxygenative rather than a dehydrogenative metabolic strategy is in play.²² The enzyme LipL encoded in the A90289 cluster (a caprazamycin type antibiotic) is a mononuclear iron-containing, α -ketoglutarate-dependent hydroxylase acting on C-5' of UMP (not free uridine) to generate what is presumed to be the phospho form of the aldehyde hydrate, which decomposes to inorganic phosphate and uridine-5'-aldehyde (Figure 3, panel a). Characterization of the LipL reaction is a transformative event in uridine oxidative metabolism and places uridine-5'-aldehyde firmly on the metabolic board as a player. Whether other pathways also use this oxygenative strategy on UMP or act on uridine via a NADH-dependent dehydrogenation has yet to be resolved.

Inspection of C-5' in the mureidomycin and pacidamycin subgroup suggests uridine-5'-amine could be on the pathway, which is bolstered by the observation that the liposidomycins, caprazamycins, and muraymycins all have 5-aminoribose groups in β -1'',5'-linkage to the uridine moieties. These glycosidic linkages could arise by displacement of uracil from a 5'-aminouridine by the 5'-OH of an intermediate via C-1'-N cleavage. Thus

it is highly likely that the initial uridine-5'-aldehyde or its derivative undergoes enzymatic transamination by pyridoxal-phosphate (PLP)-dependent enzymes to yield uridine-5'-amine building blocks in both subfamilies of antibiotic producers (Figure 3, panel b).

The uridine-5'-aldehyde can now function as an *electrophilic* partner not just with amine nucleophiles, as in the postulated transamination, but also with carbon nucleophiles on amino acid frameworks. The classic route to stabilize α -amino acid carbanions in biology is to form aldimines in the active sites of PLP-dependent enzymes and remove H α to give the delocalized carbanion equivalent. As shown in Figure 3, panel b, reaction with such a glycine carbanion equivalent would build the key C–C bond and lead to uridyl-5'-glycine as a prototypic building block in the muraymycin framework. Analogously the value of a uridine-5'-amine partner, such as 3'-deoxy-4',5'-enamino-uridine, is that it can be used with reverse polarity, as a *nucleophile*, to build the uridine-carboxamide bond to the peptide backbone in all of the pacidamycins, mureidomycins, napsamycins, and sansamycins. As we will note below, the electrophilic partner in those amide-forming condensations are tetrapeptidyl or pentapeptidyl thioesters tethered to the phosphopantetheinyl arms of thiolation domain proteins in NRPS assembly lines.

Capuramycin and Polyoxin Mysteries. Still mysterious are the transformations that occur at C-5' of uridines to build the connectivity in such uridyl peptides as capuramycin and polyoxins, e.g., polyoxin C (Figure 4, panel a). In a formal sense the one-carbon carboxamide branching at C-5' in capuramycin could arise by action of a carbamoyltransferase with the primary metabolite carbamoyl phosphate as an electrophilic acyl donor. This requires C-5' of the uridine moiety to be nucleophilic if it is a typical carbamoyl transfer reaction. It is not clear how carbanion character would be generated, nor is it clear when the hexuronic acid glycoside link is made to the C-5' alcohol. Another route to the one-carbon carboxamide might involve uridyl-5'-glycine, the same intermediate proposed in muraymycin assembly. Decarboxylation followed by oxygenation at 5'-glycine would lead to the C-5' carboxamide branching. For polyoxins and related nikkomycins, C-5' has both an amine and a carboxylate substituent.

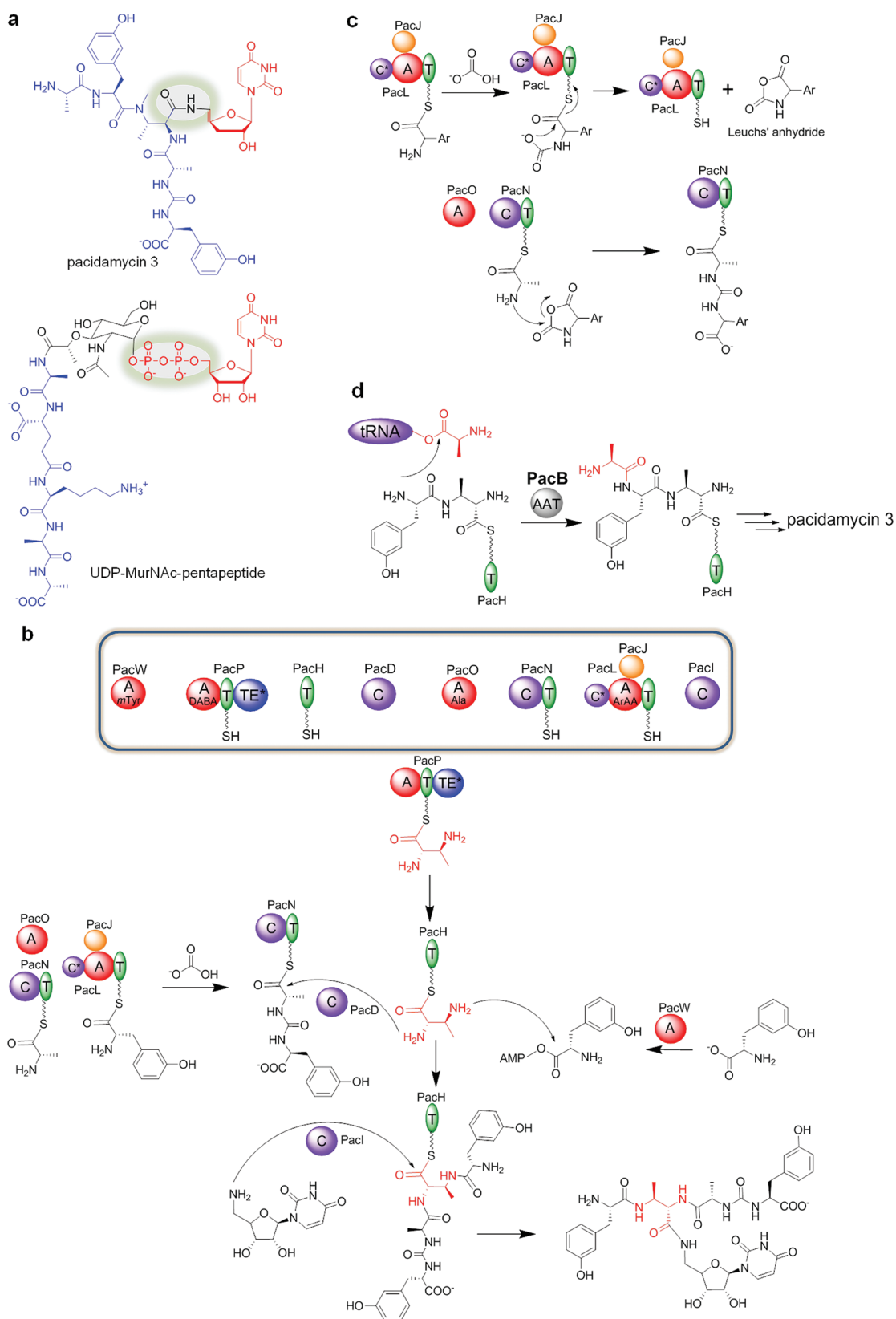


Figure 5. Pacidamycin uridylyl peptide scaffold assembly. (a) Structure comparison of *MraY* inhibitor pacidamycin pentapeptide with *MraY* substrate UDP-MurNAc-pentapeptide. (b) Schematic of pacidamycin highly fragmented NRPS assembly line. (c) Proposed mechanism leading to ureido linkage found in pacidamycins. Ar = aromatic side chain of Phe, *m*-Tyr, or Trp. (d) Characterization of PacB as a tRNA-dependent aminoacyltransferase acting on NRPS assembly line PacH.

Surprisingly the carboxylate derives from C-3 of glycerate,²³ and 3'-O-enolpyruvyl UMP appears to be an early biosynthetic intermediate.^{24,25} It has been proposed, but not shown, that the pyruvyl moiety could migrate and rearrange, presumably with C-5' at the aldehyde oxidation state. The four-carbon γ -hydroxy- α -keto acid side chain would need to be oxidatively decarboxylated, trimmed, and reductively aminated to get to polyoxin C (Figure 4, panel b).

■ PACIDAMYCIN PEPTIDE SCAFFOLD: NONRIBOSOMAL PROVENANCE

In the first subgroup of uridyl peptide antibiotics, the pacidamycins have been most thoroughly examined and most probably represent the paradigm for the other members. Depending on fermentation conditions, both tetrapeptide and pentapeptide versions of pacidamycins can be isolated. The pentapeptide versions have Ala or Gly as residue 1 at the N-terminus. The tetrapeptide versions are all missing that first residue and have a nonproteinogenic *meta*-Tyr₂ (*m*-Tyr₂), an Ala₂, or a tetrahydroisoquinoline moiety seen as the predominant residue in the napsamycin series at the N-terminus.^{26–28} Residue 3 is the nonproteinogenic 2S,3S-diaminobutyrate (DABA); residue 4 is Ala; and residue 5 is any one of aromatic amino acids Phe, Trp, or *m*-Tyr. The *m*-Tyr building block is produced by a novel regioselective iron(II)-dependent phenylalanine-3-hydroxylase that resembles its phenylalanine-4-hydroxylase counterpart, using molecular oxygen and tetrahydrobiopterin as cosubstrates.²⁹ One might have expected that the tetrapeptides arose by aminopeptidase-mediated degradation of the pentapeptides during or after secretion, but that expectation was invalidated during careful study of the assembly line logic as described below.

The multiple forms and the utilization of nonproteinogenic amino acid building blocks raised the expectation that the peptide scaffolds would be put together by mRNA-independent NRPS assembly lines. Furthermore, residues 4 and 5 are connected not by a normal peptide bond, but by a highly unusual ureido linkage (hence the name mureidomycins). Closer inspection of the peptide scaffold running from the N-terminus to the C-terminus indicates the chain direction is actually reversed twice. First, the connectivity between Ala₂/*m*-Tyr₂ and DABA₃ is not to the α -amine of DABA₃ to form a normal peptide bond but instead to the β -amine (a β -peptide bond); then, the next residue Ala₄ is not connected to the DABA₃ carboxyl but instead to the α -amine of DABA₃ through the carboxyl group of Ala₄. Ala₄ is thus in a retro direction and offers its amino to the fifth and last residue. The last peptidyl chain connectivity is through the ureido linkage, which again reverses the polarity, so that *m*-Tyr₅ is back in the correct direction and the peptide chain ends with a free carboxyl moiety.

One last consequence of the use of DABA₃, not as a regular amino acid building block but effectively as a diamine spacer, is that its carboxyl is not tied up in an amide linkage and becomes available for reaction with the uridine-5'-amine. The uridine amide linkage is built as a branch point at residue 3 using carboxyl of DABA₃, rather than using the C-terminal carboxylate of residue 5. A side by side comparison of a pacidamycin pentapeptide with the UDP-MurNAc-pentapeptide substrate for MrAY (Figure 5, panel a) shows a branched *versus* linear connectivity in the molecular scaffolds and a replacement of the diphosphate linker in the substrate with the enamide linkage in the antibiotic.

■ A HIGHLY FRAGMENTED NRPS ASSEMBLY LINE

Examination of the Pac proteins revealed a highly dissociated assembly line (Figure 5, panel b), in contrast to typical NRPS organizational logic where multiple modules are typically encoded within a single protein, the prototype being the three-module ACV synthetase in penicillin biosynthesis. There are four proteins containing adenylation (A) domains, two of them free-standing. There are likewise four proteins containing predicted 8–10 kDa thiolation (T) domains with a conserved serine residue likely to undergo post-translational phosphopantetheinylation, adding a thiol group for covalent tethering of amino acids and the growing peptide chain as a series of elongating thioesters. There are three proteins with condensation (C) domains that typically make amide bonds during NRP chain elongation on the pantetheinyl arms of T domains, even though five amino acid monomers need to be joined. Ultimately a combination of genetic deletions, the heterologous expression and purification of nine Pac proteins from *E. coli* and the following assays in various combinations and permutations have allowed complete *in vitro* reconstruction of the highly fragmented NRPS assembly line.^{20,30,31}

■ UNUSUAL BUILDING BLOCKS AND UREIDO LINKAGE

Notable biosynthetic features include the fact that the tetrapeptidyl chain (residues 2–5) is built on the free-standing T domain PacH (10 kDa), allowing high resolution mass spectrometry (HRMS) characterization of all covalently tethered intermediates. The chain is built from the middle outward in both directions, *via* DABA₃ tethered through its carboxyl as a thioester to PacH. The *m*-Tyr₂ is regioselectively coupled to the β -amine of the DABA₃ residue, while the Ala₄-ureido-*m*-Tyr₅ dipeptidyl piece is coupled to the α -amine of the tethered DABA₃. The ureido linkage is most probably introduced during chain elongation, *via* reaction of CO₂ with the amine of *m*-Tyr₅ to yield the *N*-carboxyaminoacyl-thioester. This may cyclize to a Leuchs' anhydride and diffuse to the active site of the Ala₄-S-PacN (C-T didomain protein), where it can be regioselectively opened to give only the indicated PacN-S-Ala₄-ureido-*m*-Tyr₅-COOH (Figure 5, panel c).^{20,32} Ureido linkages have been seen in some other peptide natural products including syringolins, which are mechanism-based inactivators of the proteasome, and *N*-carboxyaminoacyl-thioesters are also intermediates for those ureido linkages.^{33–35} The resultant *m*-Tyr₂-DABA₃-Ala₄-ureido-*m*-Tyr₅-COOH is stable in thioester linkage to PacH, and the cluster does not encode a standard thioesterase for chain release. Instead the free-standing condensation protein PacI catalyzes the aminolysis reaction with a synthetic 5'-aminouridine to build a tetrapeptidyl nucleoside scaffold (Figure 5, panel b).

■ ALANYL-TRNA AS DONOR TO COMPLETE PENTAPEPTIDE SCAFFOLD

A combination of genetic deletions and structure-based predictions suggested that PacB might be the missing enzyme for converting uridyl tetrapeptide to uridyl pentapeptide frameworks in the pacidamycin and related subfamilies.³⁶ This was validated with purified PacB from *E. coli* heterologous expression: pure PacB uses alanyl-tRNA as the aminoacyl donor to *m*-Tyr₂-DABA₃-S-PacH to create Ala₁-*m*-Tyr₂-DABA₃-S-PacH for subsequent coupling with the Ala₄-ureido-*m*-Tyr₅-COOH moiety

(Figure 5, panel d). Thus, these peptidyl nucleoside assembly lines show an intersection between an RNA-templated protein synthesis component, an aminoacyl-tRNA, and the mRNA-independent NRPS assembly line machinery. Furthermore, it is remarkable that these assembly lines do not start at residue 1 and proceed downstream to the C-terminal residue 5 but instead build in both directions from the trifunctional DABA in the middle.

MECHANISMS FOR DIVERSIFICATION

The producing *Streptomyces* already practice combinatorial biosynthesis at residue 2 (Ala, *m*-Tyr from two different adenylation enzymes)³¹ and residue 5 (Phe, Trp, *m*-Tyr) in these uridyl peptide antibiotic families and will incorporate exogenous building blocks such as halotryptophans during fermentations for mutasynthesis.³⁷ The 10 protein assembly line for pacidamycins suggests some of the A and C domains will be evolvable and/or interchangeable with proteins from other NRPS assembly lines. It will be worth exploring for promiscuity to use the *Streptomyces coeruleorubidus* tRNA^{Ala} loaded with different aminoacyl moieties to see what variation can be achieved at residue 1. Other diamino acids may serve as central building blocks in place of DABA₃. Determining how a β -peptide coupling is achieved at Ala₂-DABA₃ may allow export of this machinery to other NRPS antibiotic assembly lines. Finally, the tetra/pentapeptidyl offloading PacI condensation enzyme will use uridines in place of aminouridines,³¹ suggesting further exploration of nucleoside specificity for antibiotic optimization. In summary, characterization of the 10 protein assembly line for pacidamycin antibiotics has revealed new principles of chemical logic to couple nucleosides and peptides, and enzymatic machinery to build morphed peptide frameworks.

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KEYWORDS

Peptidyl nucleoside antibiotics, A group of natural products with both nucleoside and peptide in molecular scaffolds that have antibiotic activities; MraY, An essential bacterial cytoplasmic membrane enzyme catalyzing the formation of lipid intermediate I in peptidoglycan biosynthesis by transferring phosphor-MurNAC-pentapeptide from UDP-MurNAC-pentapeptide to bactoprenol-phosphate; Nonribosomal peptide synthetase (NRPS), A modular enzyme catalyzing biosynthesis of peptides without the aid of mRNA and ribosome; Nonproteinogenic amino acid, An amino acid not typically involved in protein synthesis, and often present in nonribosomal peptides

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