JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Biogenesis of the Unique 4′,5′-Dehydronucleoside of the Uridyl Peptide Antibiotic Pacidamycin

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Supporting Information

ABSTRACT: The pacidamycins belong to a class of antimicrobial nucleoside antibiotics that act by inhibiting the clinically unexploited target translocase I, a key enzyme in peptidoglycan assembly. As with other nucleoside antibiotics, the pacidamycin 4′,5′-dehydronucleoside portion is an essential pharmacophore. Here we show that the biosynthesis of the pacidamycin nucleoside in *Streptomyces coeruleorubidus* proceeds through three steps from uridine. The transformations involve oxidation of the 5′-alcohol by Pac11, transamination of the resulting aldehyde by Pac5, and dehydration by the Cupin-domain protein Pac13.

Nucleoside antibiotics exhibit versatile biological properties such as antibacterial, antifungal, antiviral, and antitumor activity.[1,2] The wide scope of such activities stems from the structural similarity of these compounds to nucleosides and nucleotides, which play key roles as energy donors, metabolite carriers, and enzyme cofactors. Puromycin is probably the best known example because of its widespread use in the study of the ribosome and in protein evolution through mRNA display.[3,4] Here we report the first insights into the biogenesis of the pacidamycin aminonucleoside **1** and demonstrate that the unusual dehydroaminonucleoside can be formed in vitro from uridine by three enzymes. The enzymes involved in the generation of this rare aminonucleoside demonstrate substrate flexibility and have the potential to be utilized for biotransformations that will enable access to series of new bioactive compounds.

Figure 1 shows the chemical structures of aminonucleoside moieties found in peptidyl nucleoside antibiotics. The peptide portions of these compounds share no similarities. Despite all the differences, however, the biosyntheses of all the aminonucleosides characterized to date begin with their corresponding nucleotides. Puromycin and the related antibiotic A201A contain a 3′-amino-3′-deoxyadenosine moiety **4** that has been shown to be derived from adenosine-5′-triphosphate (ATP).[5] The biosyntheses of the 5′-deoxynucleoside **3** (found in nikkomycins) and the bisaminonucleoside **2** (found in liposidomycins) begins with uridine-5′-monophosphate (UMP).[6−8]

The biosynthesis of the aminonucleoside moiety **1** of the pacidamycin group of uridyl peptide antibiotics has not been investigated until now. The uridyl peptide antibiotics, such as pacidamycin, napsamycin, and mureidomycin, are translocase I inhibitors. This mode of action is shared with the liposidomycins. The aminonucleoside motif **1** of the pacidamycin-like antibiotics

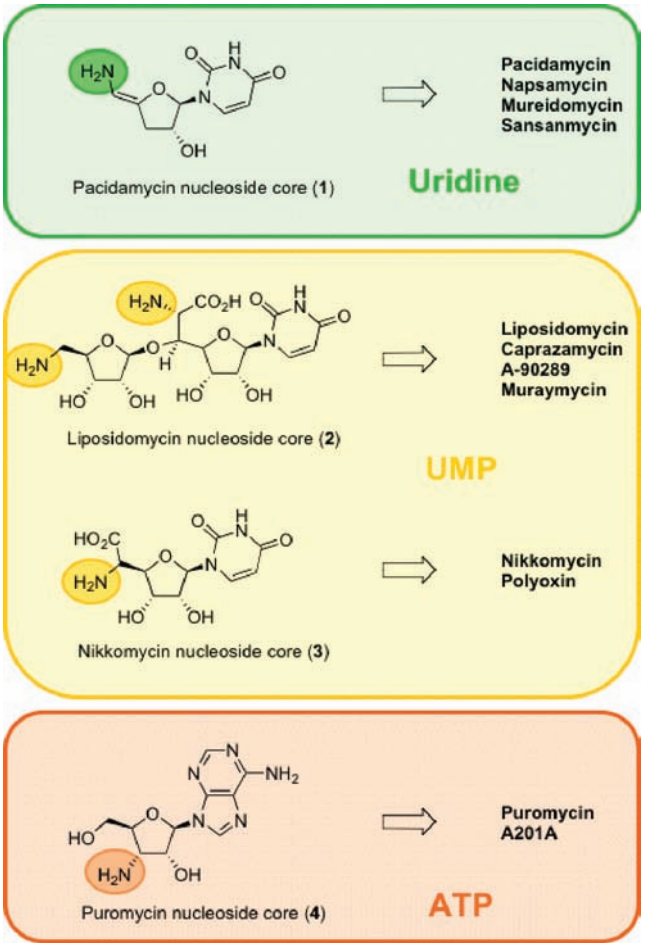

Figure 1. Aminonucleoside portions of peptidyl nucleoside antibiotics.

differs from other aminonucleosides in that it lacks the 3′-hydroxyl group and has an exocyclic double bond. The unique features of **1** imply a novel biosynthetic pathway.

On the basis of sequence analysis of the pacidamycin biosynthetic cluster, we had suggested the involvement of the flavin-dependent oxidoreductase Pac11 and the aminotransferase Pac5 in the biosynthesis of the aminonucleoside.[9] These two enzymes together could potentially provide 5′-amino-5′-deoxyuridine **7** from uridine or UMP. We postulated that at least one additional enzyme was needed to account for the double bond in the

Received: July 3, 2011
Published: August 23, 2011

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dx.doi.org/10.1021/ja206163j | *J. Am. Chem. Soc.* 2011, 133, 15288–15291

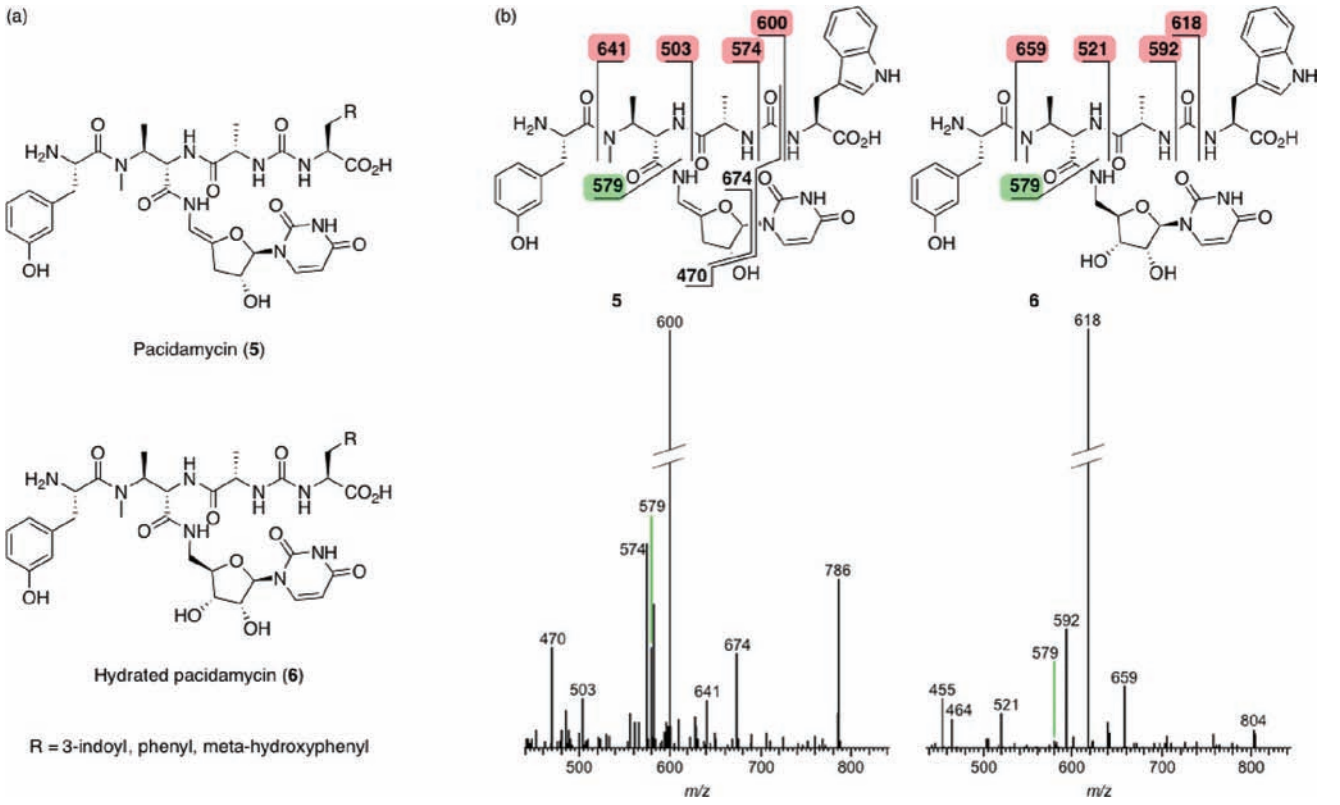


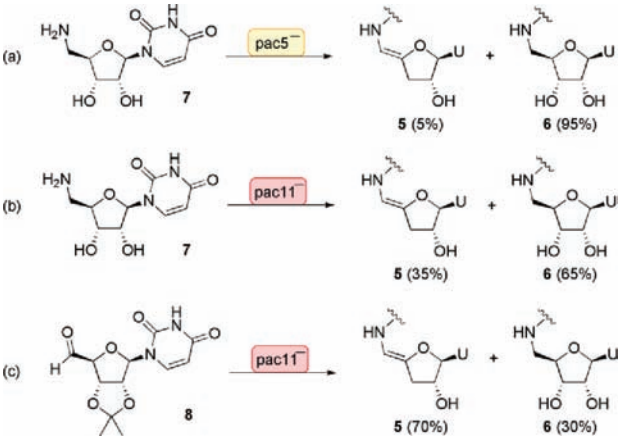
Figure 2. (a) Structures of pacidamycins found in wild-type *S. coeruleorubidus* (**5**) and the *pac13* mutant (**6**). (b) MS/MS analyses of **5** and **6**.

pacidamycin aminonucleoside **1**. Of the 22 genes in the pacidamycin core cluster, only *pac6*, *pac7*, and *pac13* had not been assigned a function. Pac6 shows sequence homology to non-heme iron dioxygenases belonging to the TauD family of enzymes[10] and was therefore an alternative candidate for formation of the uridyl aldehyde. However, none of the strictly conserved iron-binding residues are found in Pac6 [see the Supporting Information (SI)], indicating that the enzyme may not be active as a dioxygenase. Pac7 has no significant homologies to characterized proteins, and Pac13 belongs to the Cupin family of proteins. The Cupin family is characterized by a metal-binding motif, and members of this protein family have a diverse set of functions.[11]

In order to determine which of these genes were involved in the biosynthesis of the aminonucleoside, knockout mutants for each of the five genes were constructed. Analysis of the culture extracts by liquid chromatography−tandem mass spectrometry (LC−MS/MS) showed that none of the mutant strains were able to generate the typical pacidamycin suite of compounds. Upon closer inspection of the metabolite profile of the *pac13* mutant, hydrated pacidamycins **6** were detected (Figure 2). These pacidamycins were not detected in any of the other mutant strains or in the wild-type organism. The presence of hydrated pacidamycins strongly suggests that Pac13 acts as the dehydratase. Furthermore, this finding indicates that the aminonucleoside **7** is a chemically competent substrate for attachment to the pacidamycin peptide portion in vivo, as had been previously demonstrated in an in vitro study with the pacidamycin nonribosomal peptide synthetases.[12]

The origins of the aminonucleoside portion in primary pyrimidine metabolism were established by synthesizing and

Scheme 1. Feeding of Precursors to Pacidamycin Mutants



feeding deuterated ribose, uracil, and uridine to the wild-type pacidamycin producer. All three compounds resulted in the incorporation of the deuterium label into the nucleoside portion of **5**, as established through MS/MS (Figures S7−S9 in the SI). Next, we sought to re-establish pacidamycin biosynthesis in the mutant strains through chemical complementation. To this end, putative biosynthetic intermediates for the aminonucleoside portion were synthesized (see the SI) and fed to cultures of the *S. coeruleorubidus* mutants.

Production of wild-type pacidamycins **5** was restored in the *pac5* and *pac11* mutants through addition of aminonucleoside **7** (Scheme 1a,b). The hydrated pacidamycins **6** were detected

dx.doi.org/10.1021/ja206163j |*J. Am. Chem. Soc.* 2011, 133, 15288–15291

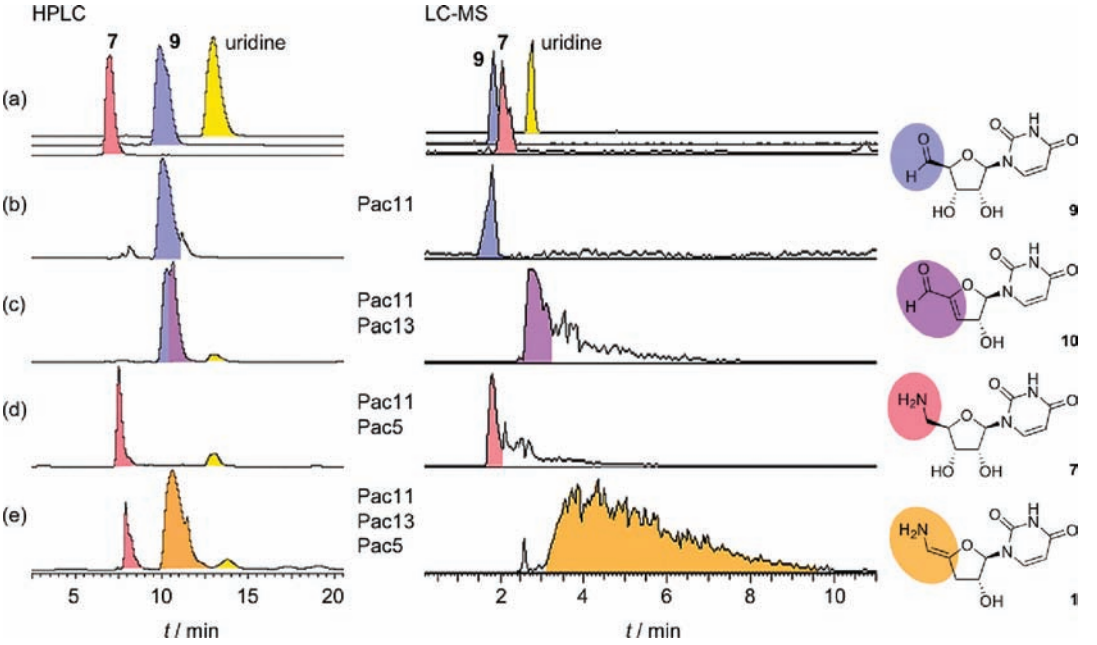


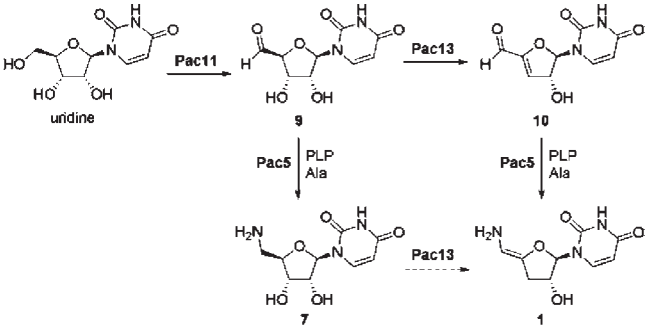
Figure 3. HPLC and LC−MS analyses of (a) standards and (b−e) in vitro assays. The structure of the main product for each enzyme combination is shown. In the LC−MS chromatograms, the extracted ion trace for the main product is shown.

alongside the enamide-containing pacidamycins **5** in both of these extracts. In contrast, no change in metabolite profile was observed in culture extracts of the *pac6* or *pac7* mutants (Figure S10). Further chemical complementation experiments were planned with the protected 5′-uridylaldehyde **8**. We decided to use the protected aldehyde because of stability issues with unprotected 5′-uridylaldehyde **9**, which slowly dehydrates under mildly acidic conditions. We reasoned that deprotection of **8** would occur during culturing, either spontaneously or enzymatically. To test the chemical competence of **8** as a pathway intermediate, we administered its deuterated form [5-^{2}H]-**8** to wild-type *S. coeruleorubidus*. This resulted in the detection of deuterated pacidamycins . Rather surprisingly, however, the deuterated form of the hydrated pacidamycin **6** was also detected (Figure S18). When aldehyde **8** was administered to cultures of the *pac11* mutant, pacidamycin production was restored (Figure S10 and Scheme 1c). Again, hydrated pacidamycins **6** were detected alongside **5** in the *pac11* mutant. Supplementing cultures of the *pac6* and *pac7* mutant strains with **8** did not result in the production of any pacidamycins (Figure S10).

Taken together, the data suggest that *pac5*, *pac11*, and *pac13* are involved in the biosynthesis of pacidamycin aminonucleoside **1**. The involvement of *pac6* or *pac7* in the generation of the aminonucleoside is not supported by our findings. Our experiments clearly indicate that the *pac13*-mediated dehydration is not required for pacidamycin biosynthesis to go to completion. The results obtained from the chemical complementation experiments are consistent with initial action of Pac11 to generate uridyl aldehyde **9** followed by Pac5-catalyzed transamination to give the 5′-aminouridine derivative.

In order to test whether Pac5, Pac11, and Pac13 were sufficient to generate aminonucleoside **1** in vitro, we heterologously expressed the three enzymes in *Escherichia coli* BL21(DE3) as His_6-fusion proteins. The enzymes were purified by affinity chromatography. The putative uridine dehydrogenase Pac11 is homologous to flavin-dependent oxidoreductases of the vanillyl alcohol oxidase family. Many enzymes of this family covalently bind their flavin cofactor.[13] Spectroscopic and mass spectrometric analysis of purified Pac11 confirmed the presence of a covalently attached flavin cofactor (Figures S4 and S5). For the putative aminotransferase Pac5, spectral analysis indicated that pyridoxal-5′-phosphate (PLP) did not copurify with the enzyme (Figure S3). Addition of Pac5 to PLP, however, did result in the shifts characteristic for Schiff base formation between the Pac5 active-site lysine (Lys270) and PLP.[14]

Scheme 2. Biosynthesis of Pacidamycin Nucleoside 1



Enzyme reactions were allowed to incubate at 28 °C for 18 h prior to LC−MS or HPLC analysis of the products. The identities of **7**, **9**, and **10** were established through comparison of their MS and MS/MS data to those obtained from synthetic standards (Figures S22−S24) and HPLC coinjections with these synthetic standards (Figure S21). Pac11 was capable of oxidizing uridine to uridyl aldehyde **9** (Figure 3). Traces of dehydrated aldehyde **10** were also detected (Figure S22). Although rare, molecular oxygen can act as a terminal electron sink for flavin, thus bypassing the need for nicotinamide-mediated cofactor recycling. No conversion was observed when UMP was used as the substrate instead of uridine (Figure S21). This is the first firm

dx.doi.org/10.1021/ja206163j |*J. Am. Chem. Soc.* 2011, 133, 15288–15291

experimental evidence that the biosynthesis of pacidamycin nucleoside **1** starts from a different precursor than for liposidomycin-type nucleoside **2** or nikkomycin-related nucleoside **3**. The reaction of Pac13 with uridine did not yield any product (Figure S21), which strongly suggests that Pac11 is the first enzyme to act in aminonucleoside biosynthesis. The reaction of Pac11 in combination with Pac13 generated **10** as the major product from uridine, and uridyl aldehyde **9** was detected as a minor product (Figure 3 and Figure S22). Enzyme assays using Pac5 were performed in the presence of PLP and alanine (see section 4.3 in the SI). As expected, aminonucleoside **7** was obtained when uridine was incubated with Pac11 and Pac5.

When all three enzymes were incubated together, a new peak appeared as the major product. Though under the conditions of the HPLC its retention time was similar to those of **9** and **10**, in LC−MS it differed (Figure 3 and Figure S24). Its molecular ion and MS/MS fragmentation pattern were consistent with pacidamycin nucleoside **1** (see the SI). This product was detected only when Pac5, Pac11, and Pac13 were present. The new compound exhibited extreme tailing under the acidic conditions employed for LC−MS, possibly because of the presence of interconverting isomers. Acetylated nucleoside **1** model compounds were shown to be surprisingly stable toward double-bond isomerization, but the free amine **1** was never tested in those studies.[15] Interestingly, in the three-enzyme mixture, aminonucleoside **7** was also present alongside residual amounts of aldehydes **9** and **10** (Figure S24). Pac13 showed the ability to convert **7** to **1** (Figure S21). Pac13 was also incubated with hydrated pacidamycins purified from a *pac13* mutant, but the enzyme was unable to mediate the dehydration of these species (Figure S25).

In summary, we have identified the genes that are required for formation of pacidamycin nucleoside **1**. The biosynthesis of the nucleosidic portion follows a pathway that is different from previously characterized nucleoside antibiotic pathways. The biogenesis of the nucleoside has been demonstrated to start from uridine, which is converted to its aldehyde **9** by the flavin-dependent dehydrogenase Pac11. This is in contrast to the first step in the biosynthesis of liposidomycin nucleoside **2**, where UMP is oxidized to aldehyde **9** through the action of LipL, a non-heme iron α-ketoglutarate-dependent enzyme.[8] To the best of our knowledge, this is the first example wherein the biosynthesis of a nucleoside antibiotic starts from the nucleoside and not the nucleotide. Furthermore, we have demonstrated that the dehydration is mediated by the Cupin family enzyme Pac13 and that the transamination is catalyzed by Pac5. Both enzymes are relatively flexible in their substrate requirements, allowing the biosynthesis of **1** to follow a randomized order, as shown in Scheme 2.

The generation of the unnatural pacidamycin **6** that occurs upon feeding of aldehyde **8** and amine **7**, hints at the inherent substrate flexibility within the pathway.[16,17] While the observed metabolic plasticity leads to problems in pinpointing the timing of the dehydration step, it will facilitate the generation of pacidamycin analogues with altered nucleoside portions. We are carrying out further structural and biochemical assessment of the enzymes on this pathway and exploring the use of these enzymes in the generation of nucleoside analogues for antiviral therapies.

■ ASSOCIATED CONTENT

Supporting Information. Full experimental details, LC−MS chromatograms, MS/MS spectra, and protein sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We thank Prof. Julia Butt (UEA) and Dr. Miles Cheeseman (UEA) for useful discussions, Prof. M. J. Bibb (John Innes Centre) for provision of *E. coli* BW25113/pIJ790 and plasmid pIJ773, and Dr. L. M. Hill (JIC) for assistance with LC−MS/MS. Funding by the Egyptian Ministry of Higher Education and the Leverhulme Trust (F/00204/AF) is acknowledged.

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