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Biosynthetic Origin and Mechanism of Formation of the Aminoribosyl Moiety of Peptidyl Nucleoside Antibiotics

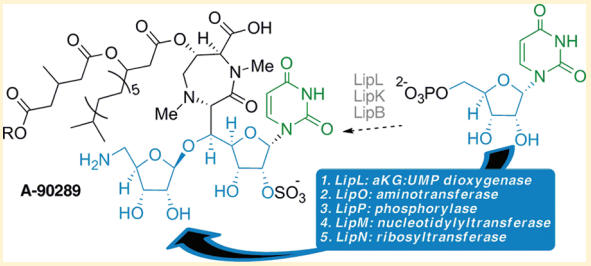
Xiuling Chi,[†] Pallab Pahari,[†] Koichi Nonaka,[‡] and Steven G. Van Lanen*[,†]

[†]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone, Lexington, Kentucky 40536, United States

[‡]Biopharmaceutical Research Group I, Biopharmaceutical Technology Research Laboratories, Pharmaceutical Technology Division, Daiichi Sankyo Company, Ltd., 389-4 Aza-ohtsurugi, Shimokawa, Izumi-machi, Iwaki-shi, Fukushima 971-8183, Japan

Supporting Information

ABSTRACT: Several peptidyl nucleoside antibiotics that inhibit bacterial translocase I involved in peptidoglycan cell wall biosynthesis contain an aminoribosyl moiety, an unusual sugar appendage in natural products. We present here the delineation of the biosynthetic pathway for this moiety upon in vitro characterization of four enzymes (LipM-P) that are functionally assigned as (i) LipO, an L-methionine:uridine-5′-aldehyde aminotransferase; (ii) LipP, a 5′-amino-5′-deoxyuridine phosphorylase; (iii) LipM, a UTP:5-amino-5-deoxy-α-D-ribose-1-phosphate uridylyltransferase; and (iv) LipN, a 5-amino-5-deoxyribosyltransferase. The cumulative results reveal a unique ribosylation pathway that is highlighted by, among other features, uridine-5′-monophosphate as the source of the sugar, a phosphorylase strategy to generate a sugar-1-phosphate, and a primary amine-requiring nucleotidylyltransferase that generates the NDP-sugar donor.



INTRODUCTION

Glycosylation is a common modification found in several microbial natural products of therapeutic value, and the sugar appendage typically has a profound effect on the biological activity.[1,2] The modus operandi by which sugars are generally incorporated into molecular scaffolds such as natural products is (i) formation of a sugar-1-phosphate derived from a glycolytic intermediate or galactose, a catalytic process that requires either a phosphosugar mutase or an anomeric sugar kinase; (ii) conversion of the sugar-1-phosphate to usually an NDP-sugar (also called an activated sugar), a reaction catalyzed by a sugar-1-phosphate nucleotidylyltransferase; and (iii) transfer of the sugar to an acceptor substrate by a glycosyltransferase to typically generate a new O-, N-, or aryl-C-glycosidic bond. A remarkable feature of glycosylated natural products is the high degree of variability and functionality that can be incorporated into the sugar moiety by reductases, epimerases, methyltransferases, and aminotransferases, among others—enzymatic modifications that generally occur at the level of the activated sugar prior to the glycosyltransferase-catalyzed reaction.[1,2]

A distinct strategy has recently been identified for generating glycosidic bonds with ribose units during natural product biosynthesis. The ribosyl moiety of the aminoglycoside antibiotic butirosin was shown to be derived from 5-phospho-α-D-ribose-1-diphosphate,[3] from which BtrL transfers ribose-5-phosphate to the acceptor disaccharide neamine to generate an O-glycosidic bond and a second enzyme BtrP catalyzes dephosphorylation to form the final trisaccharide scaffold (Supporting Information Figure S1a). This tandem, enzyme-catalyzed process is also utilized during O-ribosylation of decaprenyl-phosphate to initiate the biosynthesis of mycobacterial arabinogalactan.[4] Along with the wealth of *N*- and *C*-ribosides that originate via phosphoribosyltransfer from 5-phospho-α-D-ribose-1-diphosphate (Figure S1a),[5] it would appear that ribosylation is an exception to the typical glycosylation paradigm.

We have been studying the biosynthesis of several families of nucleoside antibiotics that inhibit the enzyme bacterial translocase I (MraY) involved in peptidoglycan cell wall biosynthesis,[6] and all of these antibiotics contain unusual sugar appendages. The lipopeptidyl nucleoside family of translocase I inhibitors, which includes A-90289s from *Streptomyces* sp. SANK 60405,[7] caprazamycins from *Streptomyces* sp. MK739-62F,[8] FR-900493 from *Bacillus cereus* No. 2045,[9] and muraymycins from *Streptomyces* sp. NRRL 30471[10] contain an aminoribosyl moiety—a 5-amino-5-deoxyribose—attached via an O-glycosidic bond to a heptofuranose nucleoside component, 5′-*C*-glycyluridine (Figure 1). Structure−activity relationship studies using simplified synthetic analogues of these compounds have revealed this aminoribosyl moiety,[11] and specifically the primary amine functionality,[12] is critical for optimal antibiotic activity.

The first insight into how the disaccharide core is assembled was unveiled upon cloning of the biosynthetic gene clusters for four lipopeptidyl nucleoside antibiotics,[7,13−15] which revealed six

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shared genes encoding a putative serine hydroxymethyltransferase (*lipK* for the A-90289 gene cluster), a nonheme, Fe(II)-dependent dioxygenase (*lipL*), a putative nucleotidylyltransferase (*lipM*), glycosyltransferase (*lipN*), aminotransferase (*lipO*), and uridine phosphorylase (*lipP*). We have demonstrated that LipL is a nonheme, Fe(II)-dependent α-ketoglutarate:UMP dioxygenase that catalyzes the conversion of UMP to uridine-5′-aldehyde (**1**) during A-90289 biosynthesis (Figure 2).[16] In turn, we proposed that **1** serves as the substrate for LipK, which catalyzes an aldol-type reaction using glycine as a co-substrate to generate 5′-*C*-glycyluridine (Figure 2). On the basis of the conjecture that the most efficient overall biosynthetic pathway will be employed, we subsequently hypothesized **1** is also an intermediate in the pathway leading to the aminoribosyl moiety, which would necessitate aminotransfer, phosphorolysis, ribose activation, and ribosyltransfer by LipO, LipP, LipM, and LipN, respectively (Figure 2).

The apparent involvement of a nucleotidylyltransferase and glycosyltransferase suggested the pathway leading to the aminoribosyl moiety does not parallel the known ribosylation pathways that are 5-phospho-α-D-ribose-1-diphosphate-dependent but instead utilizes an NDP-sugar as the activated, sugar donor. We now present the delineation of the biosynthetic pathway for incorporating this moiety by functionally assigning four enzymes, LipM−P; the results reveal a unique O-ribosylation pathway that indeed parallels the typical glycosylation paradigm yet with significant distinctions that are disclosed herein.

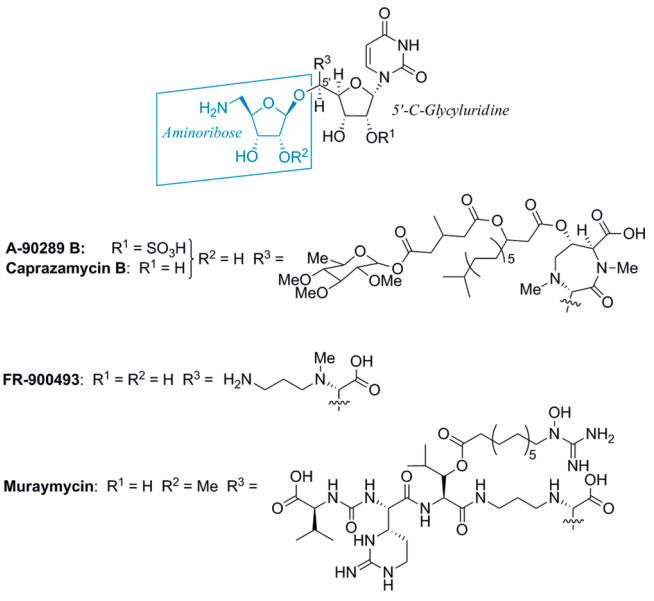

Figure 1. Structure of representative lipopeptidyl nucleoside antibiotics containing an aminoribosyl moiety.

RESULTS

Functional Assignment of LipO as an L-Methionine:1 Aminotransferase. LipO has modest sequence similarity to several proteins predicted to belong to the pyridoxal-phosphate (PLP)-dependent aspartate aminotransferase (Type I) superfamily.[17,18] Of note is the sequence similarity of LipO to PacE (37% identity/51% similarity) involved in the biosynthesis of the pacidamycin family of antibiotics. The pacidamycins consist of an enamide-containing nucleoside with a 5′-amine functionality as the sole ribose-derived unit, which we and others have previously speculated proceeds through **1** as an intermediate[16,19−21] (Figure S2), which would necessitate transamination at the nucleoside level to yield the nucleoside building block. Thus, we envisioned an analogous biosynthetic pathway such that LipO catalyzes aminotransfer utilizing the corresponding nucleoside **1**.

To interrogate the mechanism of amine incorporation, *lipO* was expressed in *Escherichia coli*; however, the recombinant protein was insoluble using a variety of growth and induction conditions. Therefore, we turned to *Streptomyces lividans* TK64 as a host, which resulted in the successful preparation of soluble, recombinant LipO (Figure S3a) that was shown by UV/vis spectroscopy to co-purify with the cofactor PLP (Figure S3b). The mutant LipO(K282A), expected to be unable to form an internal aldimine with Lys and hence be inactive, was also produced in *S. lividans* TK64 to yield a purified protein devoid of bound PLP (Figure S3b).

The activity of LipO was next monitored by HPLC using diode array for detection, and to simplify the analysis, the putative product 5′-amino-5′-deoxyuridine (**2**) was synthesized. When LipO was incubated with **1** and L-glutamate or L-aspartate, two common amine donors for the Type 1 aminotransferase superfamily, a small peak representing <1% conversion appeared in both cases, which was confirmed as **2** by LC−MS and co-injections with authentic **2** (Figure S3c); in contrast, no new peak was observed with LipO(K282A) (Figure S3d). Further analysis revealed several potential amine donors were substrates—findings

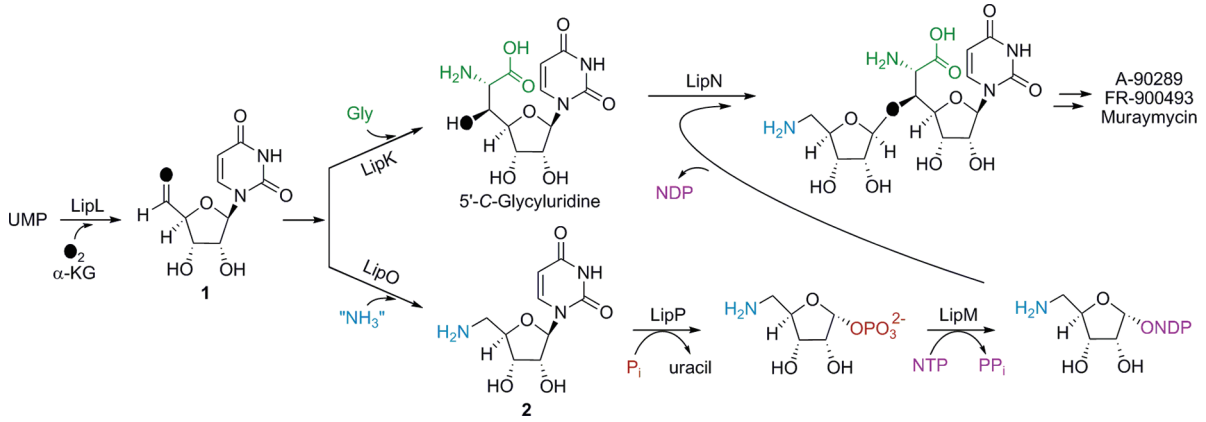

Figure 2. Proposed biosynthesis of the aminoribosyl moiety of A-90289 antibiotics.

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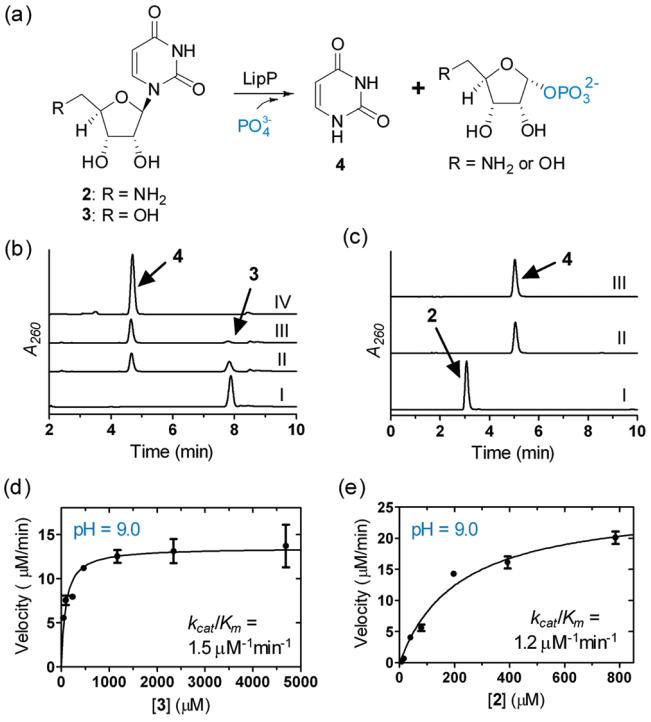

Figure 3. Characterization of LipP. (a) Phosphorylase reaction catalyzed by LipP. (b) HPLC analysis using **3** after (I) 30 min without LipP, (II) 5 min reaction, (III) 30 min reaction, and (IV) authentic **4**. (c) HPLC analysis using **2** after (I) 30 min without LipP, (II) 30 min reaction, and (III) authentic **4**. (d) Single-substrate kinetic analysis with variable **3**. (f) Single-substrate kinetic analysis with variable **2**. A_{260}, absorbance at 260 nm.

similar to many other characterized aminotransferases;[17,18] however, the highest specific activity was obtained with L-methionine followed by *N*-acetylcysteine, L-arginine, and *S*-adenosyl-L-methionine (Table S1). Finally, the reverse reaction of LipO using **2** and 4-methylthio-2-oxobutanoate or other amine acceptors as substrates was not observed, suggesting that the equilibrium of aminotransfer favors formation of **2**. The results are consistent with the functional assignment of LipO as a methionine:**1** aminotransferase and, importantly, that **2** is the likely intermediate in the biosynthesis of the aminoribosyl moiety of A-90289 (Figure 2).

Functional Assignment of LipP as a Low Specificity Phosphorylase. Bioinformatics analysis revealed LipP has sequence similarity to proteins annotated as uridine phosphorylases (Udp), an enzyme family that catalyzes the phosphorolysis of uridine (**3**) or—less efficiently—thymidine nucleosides to generate α-D-ribose-1-phosphate and uracil (**4**) or thymine, respectively, to initiate nucleotide salvage pathways (Figure 3a).[22] Although inactive with the 5′-monophosphorylated nucleotide, both human and mouse Udp have been shown to have modest activity with unnatural 5′-deoxynucleosides.[23,24] However, the potential activity of Udp with other 5′-modifications has not been reported, so it remained unclear as to the chemical identity of the LipP substrate.

The *lipP* gene was cloned and expressed in *E. coli* to yield soluble protein (Figure S4a). For comparisons, the *udp* gene from *E. coli* was also cloned and expressed to yield recombinant *Ec*Udp (Figure S5a). Initial activity tests using HPLC revealed **3** is rapidly converted to **4** and α-D-ribose-1-phosphate by both LipP (Figure 3b) and *Ec*Udp (Figure S5b) as expected. Identical to prior reports with *Ec*Udp, the LipP-catalyzed reaction was dependent upon phosphate and a nonphosphorylated nucleoside (Figure S4b), and the reaction proceeded with thymidine (Figure S4c) but not cytidine (Figure S4d) or purine nucleosides as substrates (data not shown). In addition to the formation of the pyrimidine base, LC−MS analysis of the reaction revealed an $(M - H)^-$ ion at m/z = 228.8 that was absent in the control (Figure S6), a mass that is consistent with the molecular formula $C_5H_9NO_8P$ of the expected product α-D-ribose-1-phosphate. Further activity tests revealed the hypothetical pathway intermediates **1** and **2** were also converted to **4** and the respective sugar-1-phosphate by LipP (Figure S7 and Figure 3c, respectively) and *Ec*Udp (Figure S5e,f), thus warranting further kinetic investigation for both enzymes.

The pH profile for LipP and *Ec*Udp was initially examined using **2** or **3** as a substrate and detection of **4** by UV/vis spectroscopy (Figure S8a),[25] revealing an apparent optimal activity for LipP at pH = 9.0, which is moderately higher than reported for *Ec*Udp (pH ∼ 7.5) (Figure S8b).[26] As a result, single substrate kinetic analysis was performed at both pH 9.0 and 7.5 with all hypothetical pathway intermediates (Figure 3d,e and Figures S5 and S8c−f). The extracted kinetic constants (Table 1) revealed that LipP has comparable efficiency with **2** and **3** at pH 9.0; thus, both appear to be viable substrates in vivo. Saturation kinetics could not be reached with **1** (Figure S8f), and the relatively low first-order rate constant of $k = (3.0 \pm 0.5) \times 10^{-2}$ min^{-1} suggests that **1** is less probable as the in vivo substrate. Although the K_m and k_{cat} for each respective substrate were lower for LipP relative to *Ec*Udp, the efficiencies and kinetic trends for both enzymes were quite comparable. Furthermore, bisubstrate kinetic analysis of LipP resulted in intersecting double reciprocal plots consistent with a sequential kinetic mechanism, also akin to the mechanism reported for *Ec*Udp (Figure S8g).[27,28]

Functional Assignment of LipM as a Primary Amine-Requiring Nucleotidylyltransferase. Bioinformatics analysis of LipM revealed sequence similarity to proteins annotated as putative nucleotidylyltransferases. Similarly to LipO, the gene product of *lipM* was only soluble when expressed in *S. lividans* TK64 (Figure S9a). The activity of LipM was tested with α-D-ribose-1-phosphate or 5-amino-5-deoxy-α-D-ribose-1-phosphate (**5**) generated in situ by LipP or *Ec*Udp, and analysis of these reactions revealed a new peak was formed only in the presence of **5** and UTP or—to a lesser extent—TTP, CTP, or GTP (Figure 4a,b and Figure S9b−d). In contrast no new peaks were detected with commercially available α-D-glucose-1-phosphate, α-D-galactose-1-phosphate, or α-D-glucosamine-1-phosphate and co-substrate UTP. Unexpectedly, the new peak derived from activity tests with **5** and the different NTPs had the same retention time and UV/vis spectrum as the respective nucleotide monophosphate (NMP), and co-injections with authentic material and LC−MS revealed this to be the case.

We hypothesized that NMP was generated by the degradation of the product via intramolecular attack of the 2-hydroxy group of the aminoribosyl unit on the proximal phosphate, a phenomenon that was previously observed upon characterization of apiofuranosyl-1,2-cyclic phosphate as the product of the plant bifunctional UDP-apiose/UDP-xylose synthase that catalyzes decarboxylation of UDP-glucuronic acid.[29] To detect any potential amine-containing product, the reaction components were first modified with *o*-phthalaldehyde (OPA) prior to injection, and two new peaks were identified by HPLC (Figure 4c). The first peak was identified as residual OPA-modified **5**, while LC−MS analysis of the remaining peak yielded an $(M - H)^-$ ion at m/z = 385.6, consistent

Table 1. Extracted Kinetic Constants for LipP and *Ec*Udp

enzyme	pH	substrate[a]	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	relative efficiency
LipP	9.0	Phosphate	73 ± 6.0	(1.3 ± 0.1) × 10^2		
		Uridine (**3**)	87 ± 21	(1.3 ± 0.1) × 10^2	1.5	100
		Thymidine	(2.8 ± 0.2) × 10^3	5.3 ± 0.2	1.9 × 10^{-3}	0.13
		ADU (**2**)	(2.1 ± 0.3) × 10^2	(2.6 ± 0.2) × 10^2	1.2	80
		2′-deoxyU	(3.1 ± 0.4) × 10^3	(2.1 ± 0.8) × 10^2	6.8 × 10^{-2}	4.5
	7.5	Uridine	NA[b]	NA[b]		
		ADU (**2**)	(2.6 ± 0.5) × 10^2	34 ± 1.6	1.3 × 10^{-1}	8.7
*Ec*Udp	9.0	Uridine (**3**)	(1.3 ± 0.2) × 10^3	(2.6 ± 0.1) × 10^3	2	100
		ADU (**2**)	(1.0 ± 0.2) × 10^3	(9.7 ± 0.7) × 10^2	1.0	49
	7.5	Uridine (**3**)	(1.5 ± 0.1) × 10^3	(1.6 ± 0.1) × 10^3	1.1	55
		ADU (**2**)	(2.0 ± 0.5) × 10^3	(3.2 ± 0.4) × 10^2	1.6 × 10^{-1}	8

[a] ADU, 5′-amino-5′-deoxyuridine; 2′-deoxyU, 2′-deoxyuridine. [b] Not applicable, non-Michaelis−Menten kinetics was observed.

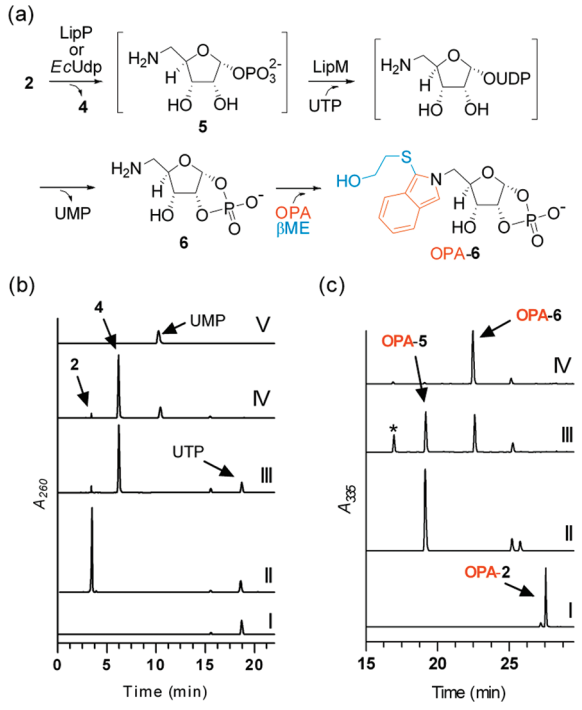

Figure 4. Characterization of LipM. (a) Reaction catalyzed by LipM including in situ generation of the substrate and amine derivatization with *o*-phthalaldehyde (OPA). (b) HPLC analysis starting with **2** after (I) 30 min without **2**, (II) 30 min without LipP or *Ec*Udp, (III) 30 min reaction, (IV) 3 h reaction, and (V) authentic UMP. (c) HPLC analysis of reaction mixtures with OPA derivatization starting with **2** after (I) 30 min without LipP or *Ec*Udp, (II) 30 min without LipM (II), 30 min reaction (III), and 3 h reaction (IV). A_{260}, absorbance at 260 nm; A_{335}, absorbance at 335 nm.

with the molecular formula $C_{15}H_{18}NO_7PS$ of an OPA-modified 5-amino-5-deoxy-α-D-ribose-1,2-cyclicphosphate, **6** (expected m/z = 386.1) (Figure 4a and Figure S10a,b). Large-scale purification of OPA-**6** and subsequent LC−MS and 1D and 2D NMR spectroscopic characterization (Figures S10c,d−S14)—notably the $^1H-^{31}P$ HMBC data that was consistent with the very recently discovered metabolite α-D-ribose-1,2-cyclicphosphate[30] (Figure S15)—revealed the expected degradation product (an isoindol-1-one) for OPA-**6**.[31] Although the genuine identity of the product of the LipM-catalyzed reaction remained elusive at this stage, the results did reveal that LipM only utilizes a sugar-1-phosphate containing a primary amine functionality.

We rationalized that a stable LipM product would be attainable by using a 2-deoxyribose-containing surrogate substrate. *Ec*Udp as well as other UDPs are known to catalyze phosphorolysis using thymidine or 2-deoxy-**3**,[26,27] and similarly LipP catalyzed the reaction with either substrate (Figure S4c,e), although the latter was determined to be nearly 35-fold more efficient (Table 1 and Figure S16). Identical to the results utilizing 5′-hydroxy nucleosides (i.e., **3**), in situ generation of 2-deoxy-α-D-ribose-1-phosphate did not yield a product when tested with LipM. Subsequently, the potential substrate 5′-amino-2′,5′-dideoxyuridine (**7**) was synthesized from 2′-deoxy-**3**, and HPLC analysis with this surrogate substrate revealed it was processed by both LipP and LipM, generating two new peaks with **3**-like chromophores (UV_{max} ∼ 260 nm) (Figure 5b). While the minor peak was identified as UDP, the major peak did not co-elute with any known **4**-containing metabolite. LC−MS analysis of the purified new peak revealed an $(M - H)^-$ ion at m/z = 517.6, consistent with the molecular formula $C_{14}H_{23}N_3O_{14}P_2$ of UDP-5-amino-2,5-dideoxyribose (**8**) (expected m/z = 518.1) (Figure S17). NMR analysis, including ^1H-, ^{13}C-, and ^{31}P NMR, $^1H-^1H$ COSY, and $^1H-^{13}C$ HMBC (Figures S18−S20), confirmed the identity of the product as **8**, thus consistent with the function of LipM as a UTP:5-amino-5-deoxy-α-D-ribose-1-phosphate uridylyltransferase.

Functional Assignment of LipN as a 5-Amino-5-deoxyribosyltransferase. We finally turned our attention to LipN, which has low sequence identity to a small number of proteins annotated as putative glycosyltransferases. Once again, soluble protein was only obtained upon heterologous expression in *S. lividans* TK64 (Figure S21a). We were disheartened to find out that **8** was not stable upon storage, in this case degrading to UDP and an undetermined product likely by hydrolysis of the anomeric bond (Figure S21b). Thus, we again relied on the in situ generation of substrate beginning with **2** or **7**. While no glycosyltransferase activity was observed with uridine-5′-diphosphoglucose, PRPP, or ribose-1-phosphates generated with LipP, HPLC analysis of LipN reactions using surrogate acceptor **3** revealed a new, small peak using either **2** (Figure 6) or **7** (Figure S21c) as the ultimate sugar donor. Our pilot experiments suggested higher yields were obtained with the genuine sugar donor UDP-5-amino-5-deoxy-α-D-ribose, and thus, no further experiments were undertaken with **8**. Following large-scale purification of the LipN-product generated from **2** and **3**, mass and complete NMR spectroscopic

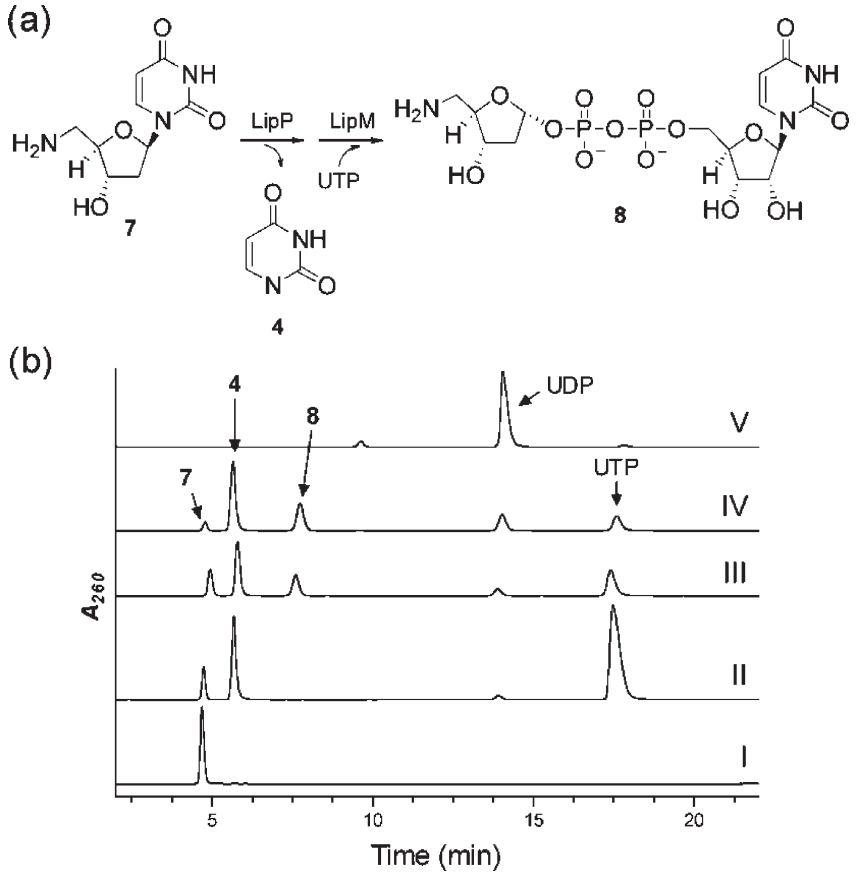

Figure 5. Characterization of LipM. (a) Enzymatic preparation of the substrate using LipP and the dideoxyuridine analogue **7** and the reaction catalyzed by LipM to generate **8**. (b) HPLC analysis of the reaction starting with **7** after (I) 3 h without UTP and LipP, (II) 3 h without LipM, (III) 1 h reaction, (IV) 3 h reaction, and (V) authentic UDP. A_{260}, absorbance at 260 nm.

analysis (Figures S22−25)—notably the $^{1}H-^{13}C$ HMBC demonstrating the H-1″ and C-5′ correlation (Figure S26)—revealed the identity of the new product as 5′-*O*-(5″-amino-5″-deoxy-β-D-ribose)-uridine, **9** (Figure 6). Thus, the function of LipN is assigned as a 5-amino-5-deoxy-α-D-ribosyltransferase that catalyzes the terminal step in the biosynthesis of the aminoribosyl moiety.

DISCUSSION

Bacterial natural products are notorious for their diverse array of sugar modifications that are typically critical for their biological activity. As a result there has been a significant effort toward understanding the molecular details behind incorporation of these sugars, in part with the expectation that the results will ultimately enable rationale manipulation of sugar biosynthetic pathways as a strategy to produce novel glycosylated compounds.[1,2] Undoubtedly, the identification and characterization of new pathways will afford more tools for applying such a structural diversification approach, and thus, we initiated studies toward delineating the biosynthetic mechanism of an unique pentofuranose—an aminoribosyl moiety found in several lipopeptidyl nucleosides of potential therapeutic significance as antibiotics.

Our strategy to unravel the pathway was to reconstitute the enzyme activities in vitro, which ultimately required the use of the heterologous host *S. lividans* TK64 to obtain three of the four enzymes in soluble form. Our prior results with LipL demonstrated this enzyme had strict specificity for the substrate UMP to initiate the pathway,[16] an attribute that was not reciprocated by the four enzymes assigned in this study. LipO utilized a variety of amine donors with L-methionine as the slightly preferred amine source, the biological relevance of which is currently under investigation. Likewise, the phosphorylase LipP was equally efficient with hypothetical pathway intermediates **2** and **3**. Sequence analysis of the whole genomes of the Actinomycetales has revealed minimally one 5′-nucleosidase is encoded within the chromosomal DNA suggesting these organisms have the capability to convert the nucleic acid building block UMP to **3**. Thus, based solely on the results with LipP, the identity of the in vivo substrate could not be established. However, the realization that the amine needs to be incorporated prior to formation of activated sugar would necessitate an additional, unidentified enzyme to oxidize α-D-ribose-1-phosphate prior to LipO catalysis if **3** was indeed the pathway precursor. Thus, we currently prefer a pathway originating from UMP without the involvement of **3** as shown in Figure 2.

While we were initially disheartened by the lack of substrate specificity of LipP, this low specificity turned out to be critical for discovering the function of LipM by enabling the preparation of a surrogate substrate that was converted to a less unstable product for structural elucidation. Similarly, we took advantage of the promiscuity of LipN with respect to the sugar acceptor to define its function as a ribosyltransferase. Interestingly, although the genuine sugar donor UDP-5-amino-5-deoxy-α-D-ribose was initially only indirectly identified as the LipM-product based on the identification of UMP and OPA-**6** and—despite exhaustive

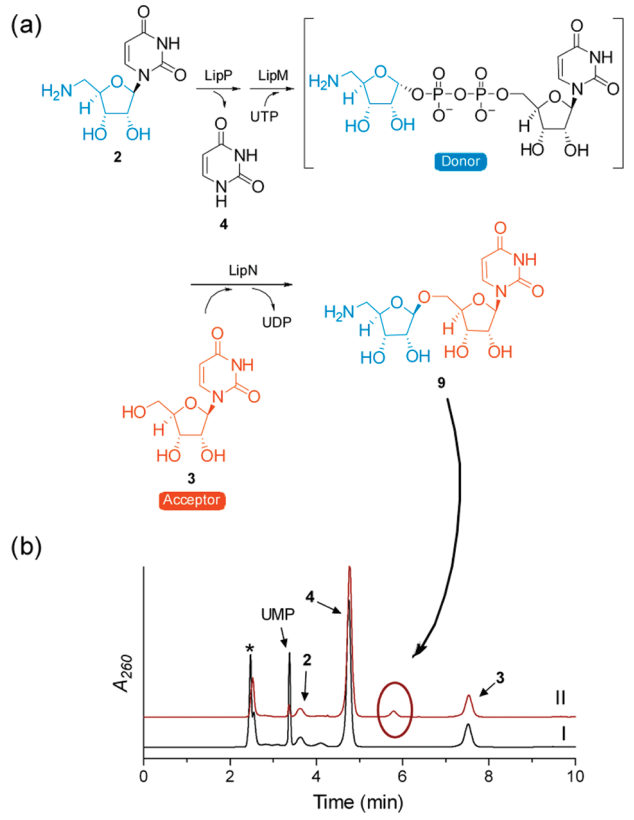

Figure 6. Characterization of LipN. (a) Enzymatic preparation of the sugar donor substrate using LipP and LipM, and the reaction catalyzed by LipN using the surrogate acceptor substrate **3** to generate **9**. (b) HPLC analysis of the reaction starting with **2** after (I) 3 h without LipN and (II) 3 h reaction. Asterisk (*) indicates expected retention time for residual UTP and co-product UDP. A_{260}, absorbance at 260 nm.

attempts—could not be directly observed by MS, our successful analysis of LipN with in situ generated UDP-5-amino-5-deoxy-α-D-ribose suggests that this activated sugar is formed yet is refractive to direct characterization using the conditions employed here. Although it remains a possibility that **6** is generated enzymatically by a contaminating protein, several activated sugars are known to be unstable; for instance, it has recently been reported that the expected product of UDP-apiose/UDP-xylose synthase can be transiently detected by high-field NMR prior to degrading to the isolable apiofuranosyl-1,2-cyclic phosphate[29] and the activated carbocycle NDP-valienol can only be detected by MS within the crude reaction mixture.[32]

Although the glycosylation process predominantly involves the incorporation of hexoses, it is not limited to these sugars: several pentose units, for example, are derived from this glycosylation mechanism that typically proceeds with decarboxylation of an NDP-glucuronic acid (Figure S1b).[29,33−37] Alternatively, sugar salvage pathways from plants,[38,39] Leishmania,[40] and the thermophilic bacterium *Thermus caldophilus* GK24[41] have been characterized that utilize broad-specificity nucleotidylyltransferases capable of activating the pentopyranosyl phosphates of D-xylose and L-arabinose albeit with lower efficiencies than the α-D-hexopyranosyl phosphates of glucose and galactose. Furthermore, a nucleotidylyltransferase from *Salmonella enterica typhimurium* LT2 (E_p) has been extensively studied and shown to utilize α-D-xylose-1-phosphate along with dozens of α-D-hexopyranosyl phosphates.[42−44] Although a more in depth investigation is underway, our results identified LipM as an unusual α-D-pentofuranosyl-1-phosphate-activating enzyme with an apparently more refined specificity and hence well-defined role in A-90289 biosynthesis. Furthermore, LipM was shown to have an absolute requirement for the primary amine functionality, which is likewise unusual relative to other bacterial nucleotidylyltransferases that have been demonstrated to activate unnatural aminohexoses, although with equal or less efficiency relative to the hydroxylated counterpart.[43−47]

CONCLUSION

In summary, we have completed the functional assignment of five enzymes—including four here—involved in the modification and incorporation of the aminoribosyl moiety of A-90289 antibiotics. The pathway is initiated by the LipL-catalyzed conversion of a novel ribosyl donor UMP to form the aldehyde **1**.[16] Following introduction of the amine by LipO, LipP catalyzes phosphorolysis to initiate "salvage" of the modified sugar and LipM subsequently catalyzes the formation of an activated pentofuranose that serves as the final sugar donor. Finally, the ribosyltransferase LipN completes the pathway by incorporation of the aminoribosyl moiety, the identity of which is apparently dictated by the specificity of the pathway-initiating enzyme LipL and the penultimate enzyme LipM. The end result is a sugar biosynthetic pathway highlighted in Figure 2 that not only establishes an alternative mechanism for ribosylation, but also features intriguing variations of the established paradigm for glycosylation.

EXPERIMENTAL METHODS

Cloning, Gene Expression, and Protein Production. Genes for heterologous expression in *E. coli* were cloned into pET30Xa-LIC (Supplementary Methods). Genomic DNA from *E. coli* DH5α and cosmid pN1[7] was used as templates for PCR, and the fidelity of the resulting product was confirmed by DNA sequencing. Standard conditions were used for expression of *lipP* and *EcUdp* using BL21(DE3) with the respective plasmid.[48] The remaining genes, *lipM*, *lipN*, *lipO*, and *lipO*(K282A) were subcloned into pUWL201pw and expressed in *S. lividans* TK64.[49] Protein purification following cell harvesting was performed using immobilized metal affinity chromatography according to standard procedures (see Supporting Information for details).

In Vitro Characterization of LipO. Reactions consisted of 50 mM potassium phosphate, pH 7.5, 2 mM **1**, 2 mM amine donor, 200 μM PLP, and 1 μM LipO at 30 °C, and the reaction was terminated by the addition of cold TCA to 5% (w/v) or by ultrafiltration using a Microcon YM-3. Alternatively, PLP was eliminated from the reaction mixture to give comparable results. Following centrifugation to remove protein, the reaction components were analyzed by HPLC using a C-18 reverse-phase column. A series of linear gradients was developed from 0.1% TFA in 5% acetonitrile (A) to 0.1% TFA in 90% acetonitrile (B) in the following manner (beginning time and ending time with linear increase to % B): 0−4 min, 100% B; 4−24 min, 50% B; 24−26 min, 100% B; 26−32 min, 100% B; and 32−35 min, 0% B. The flow rate was kept constant at 1.0 mL/min, and elution was monitored at 260 nm. LC−MS was performed using a linear gradient from 0.1% formic acid in water to 0.1% formic acid in acetonitrile over 20 min. The flow rate was kept constant at 0.4 mL/min, and elution was monitored at 254 nm.

In Vitro Characterization of LipP. Reactions consisted of 25 mM potassium phosphate, pH 7.5, 2 mM **3** or analogue, and 100 nM LipP at

30 °C, and terminated by the addition of cold TCA to 5% (w/v) or by ultrafiltration using a Microcon YM-3. HPLC analysis of the reaction was similar to that described for LipO.

The effect of pH on LipP activity was carried out in 50 mM indicated buffer, 2.5 mM potassium phosphate, 2.5 mM **3**, and 1 μM LipP for 5 min at 30 °C. The reactions were terminated with 0.1 M sodium hydroxide, and **4** formation was determined by UV/vis spectroscopy with $\Delta\varepsilon_{290\,nm}$ = 5700 M^{-1} at pH 13.[26] To determine the kinetic constants with respect to co-substrate phosphate, reactions were carried out in 50 mM Tris-HCl pH 9.0 consisting of 1.5 mM **3**, variable phosphate (2.5−2500 μM), and 100 nM LipP at 30 °C under initial velocity conditions (<10% product formation). To determine the kinetic constants with respect to the co-substrate nucleoside, reactions were carried out in 50 mM Tris-HCl pH 9.0 consisting of saturating phosphate (1.5 mM) and variable nucleoside (10−4700 μM **3**, 10−10 000 μM 2′-deoxy-**3**, 8−800 μM **2**, 20−10 000 μM thymidine, or 30−3000 μM **1**), and 100 nM LipP at 30 °C under initial velocity conditions. Product formation when utilizing thymidine was determined by UV/vis spectroscopy with $\Delta\varepsilon_{300\,nm}$ = 3700 M^{-1} at pH 13.[26] Single substrate kinetics at pH 7.5 was carried out using identical conditions except with increased LipP (730 nM). Kinetic analysis with *Ec*Udp was carried out with final enzyme concentrations of 100 nM at pH 9.0 and 200 nM at pH 7.5.

In Vitro Characterization of LipM. Reactions consisted of 50 mM potassium phosphate, pH 7.5, 5 mM $MgCl_2$, 2 mM **3** or analogue, 5 mM nucleotide triphosphate, 5 μM LipP, and 1 μM LipM at 30 °C, and the reaction terminated by the addition of cold TCA to 5% (w/v) or by ultrafiltration using a Microcon YM-3. The activity of LipM was tested with sugar-1-phosphates generated in situ from synthetic **3**, 2′-deoxy-**3**, **2**, or **7** and the co-substrate nucleotide UTP, dUTP, TTP, rCTP, dCTP, dGTP, rGTP, dATP, or rATP. The activity of LipM was also tested with commercial glucose-1-phosphate or glucosamine-1-phosphate with co-substrate UTP. Following centrifugation to remove protein, the reaction components were analyzed by HPLC using a C-18 reverse-phase analytical column. A series of linear gradients was developed from 40 mM phosphoric acid-triethylamine pH 6.5 (C) to 20% methanol (D) in the following manner (beginning time and ending time with linear increase to % D): 0−8 min, 100% D; 8−18 min, 60% D; 18−25 min, 95% D; 25−32 min, 95% D; and 32−35 min, 0% D. The flow rate was kept constant at 1.0 mL/min, and elution was monitored at 260 nm. LC−MS was performed as above.

In Vitro Characterization of LipN. Reactions consisted of 50 mM potassium phosphate, pH 7.5, 2 mM **2**, 2.8 μM LipP at 30 °C for 2 h. LipP was removed by ultrafiltration, and 86 μL of the filtrate was added to a solution of 5 mM $MgCl_2$, 2 mM UTP, 1 mM **4**, 12 μM LipM, and 7 μM LipN (final volume of 100 mL) and incubated at 30 °C for the indicated time points. HPLC analysis was performed using a TFA mobile phase as described above.

■ ASSOCIATED CONTENT

Supporting Information. Instrumentation, chemicals, synthetic procedures and spectroscopic analysis, OPA-modification procedure, large-scale preparation of enzyme products for spectroscopic analysis, NMR analyses, Table S1, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
svanlanen@uky.edu

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