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In Vitro Characterization of LmbK and LmbO: Identification of GDP-D-*erythro*-α-D-*gluco*-octose as a Key Intermediate in Lincomycin A Biosynthesis

Chia-I Lin, Eita Sasaki, Aoshu Zhong, and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry, University of Texas at Austin, Austin, Texas 78712, United States

Supporting Information

ABSTRACT: Lincomycin A is a clinically useful antibiotic isolated from *Streptomyces lincolnensis*. It contains an unusual methylmercapto-substituted octose, methylthiolincosamide (MTL). While it has been demonstrated that the C_8 backbone of MTL moiety is derived from D-fructose 6-phosphate and D-ribose 5-phosphate via a transaldol reaction catalyzed by LmbR, the subsequent enzymatic transformations leading to the MTL moiety remain elusive. Here, we report the identification of GDP-D-*erythro*-α-D-*gluco*-octose (GDP-D-α-D-octose) as a key intermediate in the MTL biosynthetic pathway. Our data show that the octose 1,8-bisphosphate intermediate is first converted to octose 1-phosphate by a phosphatase, LmbK. The subsequent conversion of the octose 1-phosphate to GDP-D-α-D-octose is catalyzed by the octose 1-phosphate guanylyltransferase, LmbO. These results provide significant insight into the lincomycin biosynthetic pathway, because the activated octose likely serves as the acceptor for the installation of the C1 sulfur appendage of MTL.

Carbohydrates are indispensable for all living organisms and play important roles in determining the biological activities of diverse glycoconjugates, including glycosylated secondary metabolites.[1] Altering the glycosylation pattern of targeted natural products by manipulating the corresponding sugar biosynthetic machinery holds promise to enhance or vary the biological properties of the parent molecules. To achieve this goal, it is essential to know how unusual sugars are biosynthesized. Recent research efforts in this area have advanced our understanding of the formation of deoxyhexoses in nature[2] and made possible the glycodiversification of natural products, taking advantage of substrate promiscuity found for many glycosyltransferases.[3] However, information about the construction, activation, and modification of high-carbon sugars (>7 C's) is scarce.[4] For example, the biosynthesis of naturally occurring octoses remains unexplored, except for 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P), which is derived from arabinose 5-phophate and phosphoenolpyruvate (PEP).[5]

Lincomycin A (**1**), isolated from *Streptomyces lincolnensis*,[6] is a clinically useful antibiotic against Gram-positive bacteria.[7] It interacts with the peptidyltransferase domain of the 50S ribosomal subunit due to its structural resemblance to the 3′ end of L-Pro-Met-*t*RNA and deacetylated *t*RNA. This, in turn, inhibits the bacterial protein synthesis.[8] The structure of lincomycin A consists of an amino acid, *N*-methyl-4-propyl-L-proline (**2**), and an unusual methylthiolincosamide (MTL, **3**) moiety.[9] Similar thiooctoses are also found in antimicrobial agents, Bu-2545 (**4**) and celesticetin (**5**) (see Figure 1).[10]

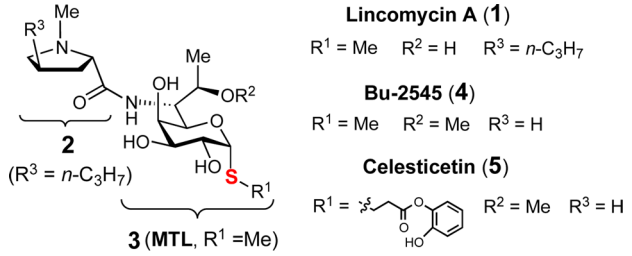
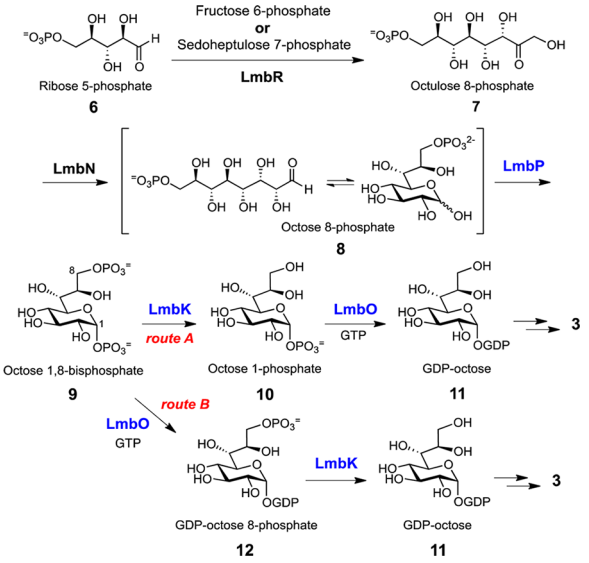


Figure 1. Examples of natural products containing thiooctose moiety.

Scheme 1. Proposed Biosynthetic Pathway for MTL (3)



Recently, we reported that the C_8 backbone of MTL[11] is generated via a transaldol reaction catalyzed by LmbR using D-fructose 6-phosphate or D-sedoheptulose 7-phosphate as the C_3 donor and D-ribose 5-phosphate as the C_5 acceptor (Scheme 1).[12] Subsequent isomerization catalyzed by LmbN converts the

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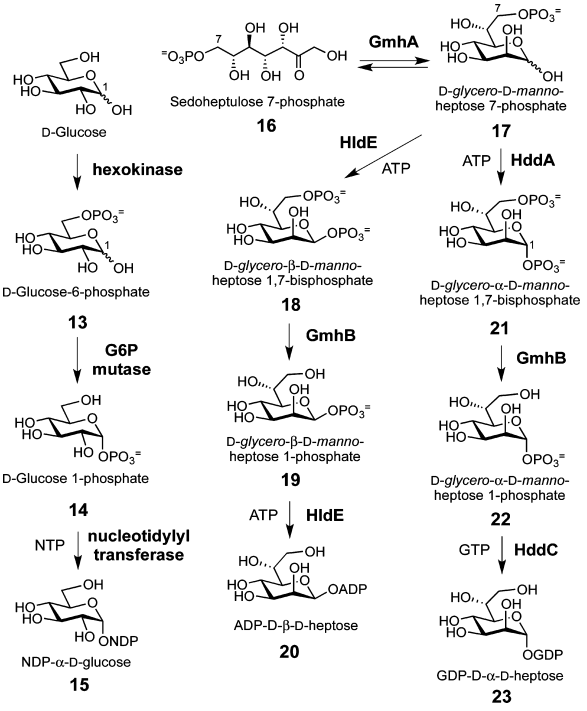
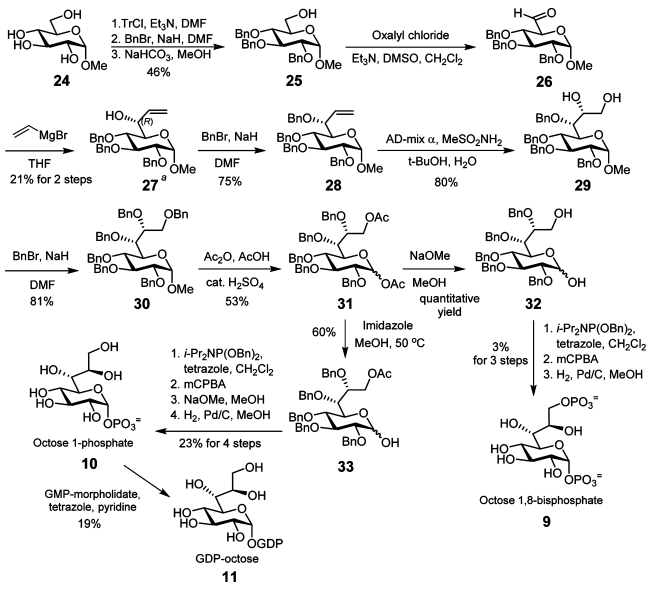
Scheme 2. Biosynthetic Pathways to NDP-Hexose and NDP-Heptoses



Scheme 3. Synthesis of Octose 1,8-Bisphosphate (9), Octose 1-Phosphate (10), and GDP-D-α-D-octose (11)[a]



[a]Both 6*S*- and 6*R*-isomers of **27** were generated, and the desired 6*R*-isomer was isolated using silica gel column chromatography.[12]

resulting octulose 8-phosphate (**7**) to octose 8-phosphate (**8**).[12] The fact that several genes homologous to those found in various NDP-deoxyhexose pathways[13] exist in the lincomycin gene cluster (designated as *lmb*) prompted us to hypothesize that an NDP-activated octopyranose is a possible intermediate in the MTL biosynthetic pathway.[12] However, this hypothesis lacks literature precedence because NDP-octose has never been reported to be an biosynthetic intermediate. Thus, investigations of the existence of this putative NDP-octose intermediate and its transformation from **8** have been the focus of our research effort. In this report, we describe the identification of GDP-D-*erythro*-α-D-*gluco*-octose (GDP-D-α-D-octose, **11**) as the key intermediate in the MTL biosynthetic pathway.

In a typical NDP-sugar biosynthetic pathway, the sugar precursor is first converted to the corresponding sugar 1-phosphate prior to nucleotidyl transfer to yield the NDP-sugar product (e.g., **14**→**15** to make NDP-α-D-glucose, Scheme 2). For the biosynthesis of NDP-deoxyhexoses, generation of the hexose-1-phosphate precursor is achieved either by direct C-1 phosphorylation or via C-6 phosphorylation followed by a mutase-catalyzed 6→1 phosphoryl migration (**13**→**14**, Scheme 2).[2] A different scenario was observed in the biosynthesis of NDP-heptoses (Scheme 2). Studies of the formation of ADP-D-*glycero*-β-D-*manno*-heptose (ADP-D-β-D-heptose, **20**) and GDP-D-*glycero*-α-D-*manno*-heptose (GDP-D-α-D-heptose, **23**) revealed a kinase/phosphatase cascade to produce heptose 1-phosphate in three steps: (1) isomerization of D-sedoheptulose 7-phosphate to D-*glycero*-D-*manno*-heptose 7-phosphate (**16**→**17**); (2) anomeric phosphorylation of heptose 7-phosphate to heptose 1,7-bisphosphate by a kinase (**17**→**18** and **17**→**21**); (3) conversion of heptose 1,7-bisphosphate to heptose 1-phosphate via a phosphatase-catalyzed hydrolysis (**18**→**19** and **21**→**22**).[14]

Sequence analysis of the *lmb* gene cluster from *S. lincolnensis* ATCC 25466 found no mutase homologue required for installation of the C1 phosphoryl group via a C_N→C1 migration route. However, comparison of the *lmb* gene cluster with the NDP-heptose biosynthetic gene clusters from *Aneutinibacillus thermoaerophilus* DSM 10155/G+[15a] and *Escherichia coli* K-12[15b] showed several genes, including *lmbN*, *lmbP*, *lmbK*, and *lmbO*, whose translated amino acid sequences have similarities to those involved in NDP-heptose biosynthesis (Table S2). From this information, we speculated that MTL biosynthesis might follow a kinase/phosphatase cascade analogous to that observed in the NDP-heptose biosynthetic pathway to make NDP-octose.

As depicted in Scheme 1, LmbN catalyzes the C1→C2 isomerization to produce the corresponding octose 8-phosphate (**7**→**8**) in a manner similar to the GmhA-catalyzed isomerization (**16**→**17**).[12] The putative kinase, LmbP, might phosphorylate the C1 hydroxyl group of octose 8-phosphate (**8**) to afford octose 1,8-bisphosphate (**9**). The C8 phosphate group of this bisphosphate intermediate might be hydrolyzed by LmbK (annotated as phosphatase) to give octose 1-phosphate (**10**), which is then converted to the nucleotide-activated octose (**11**) by LmbO (annotated as nucleotidylyltransferase) (route A). It is also conceivable that the reaction proceeds first with nucleotidylyltransfer by LmbO (**9**→**12**), followed by LmbK-catalyzed C8 dephosphorylation (**12**→**11**) (route B). Because LmbP and LmbO exhibit sequence similarities only to their counterparts in the GDP-D-α-D-heptose biosynthetic pathways (Table S2), the C1-activated octose intermediate in lincomycin biosynthesis is likely a GDP-D-α-D-octose. However, it is well known that prediction of substrate specificity for enzymes based solely on sequence alignment can be erroneous; thus the chemical nature of sugar phosphodinucleotide intermediate in this pathway must be experimentally verified.[2c,d]

To determine whether the proposed pathways are valid, the *lmbP*, *lmbK*, and *lmbO* genes were amplified from the genomic DNA of *S. lincolnensis* ATCC 25466 using polymerase chain reaction and were individually cloned into pET28 vectors. The recombinant LmbP, LmbK, and LmbO with *N*-terminal His_6 tags were overexpressed in *E. coli*. LmbK could be purified to near homogeneity as a soluble protein (Figure S1), but both LmbP

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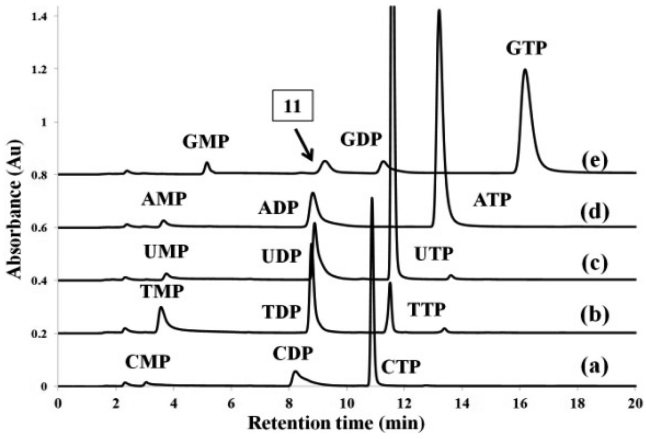

Figure 2. Activity assays for LmbK and refolded LmbO. All reactions contain the synthetic bisphosphate **9**, LmbK, and refolded LmbO. (a) CTP, (b) TTP, (c) UTP, (d) ATP, and (e) GTP.

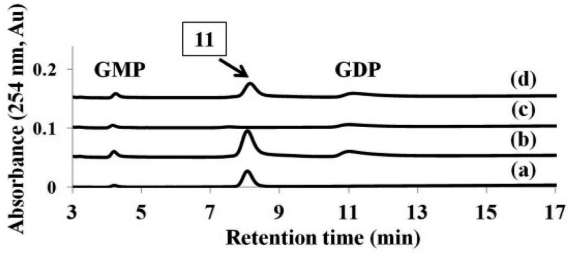

Figure 3. Activity assays for the refolded LmbO: (a) synthetic GDP-octose (**11**) standard; (b) LmbK and refolded LmbO with bisphosphate **9** and GTP; (c) refolded LmbO with bisphosphate **9** and GTP; and (d) refolded LmbO with the synthetic octose 1-phosphate (**10**) and GTP.

and LmbO were expressed only as inclusion bodies. The denatured LmbO could be refolded (protocol detailed in the Supporting Information), but attempts to obtain the soluble form of LmbP using a similar approach were not successful. To circumvent this obstacle, we synthesized the putative substrate for LmbK (compound **9**). The synthetic scheme is shown in Scheme 3.

The synthetic bisphosphate **9** was first incubated with LmbK, and the reaction mixtures were analyzed by HPLC using a Corona charged aerosol detector (CAD) and a Dionex CarboPac PA-1 anion exchange column. A new peak was detected (Figure S2). This new product was collected and characterized by NMR spectroscopy. The $^{1}H-^{31}P$ heteronuclear multiple quantum correlation (HMQC) spectrum of the substrate **9** displayed two ^{31}P signals, one at δ 2.53 coupled to the two C-8 proton signals at δ 3.86 and 3.83, and the other at δ 0.49 coupled to the anomeric proton at δ 5.35 (Figure S3). In contrast, the NMR spectrum of the isolated product displayed a single ^{31}P signal at δ 3.17 coupled to the anomeric proton at δ 5.34. Loss of the C8 phosphoryl group from **9** during the LmbK-catalyzed reaction is thus evident. To gain support for the structural assignment of the isolated product, an octose 1-phosphate (**10**) standard was also synthesized (Scheme 3). The LmbK product was found to coelute with the synthetic **10** on HPLC, and the spectral characteristics of the enzymatic product are in good agreement with the synthetic standard. These results fully established the identity of the LmbK product as **10** starting from **9**.

After formation of **10**, the next reaction step is likely nucleotide activation catalyzed by the putative nucleotidylyltransferase,

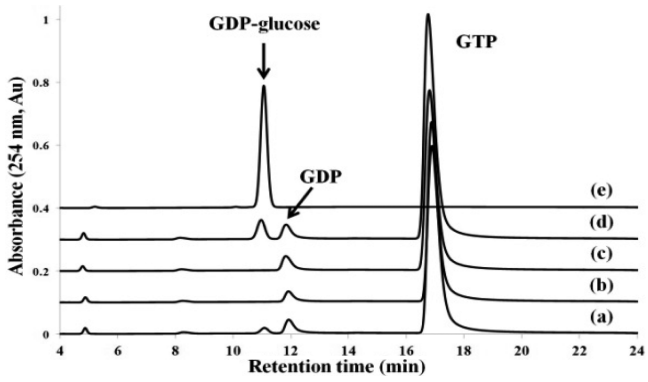

Figure 4. Activity assays for LmbK and refolded LmbO: (a) refolded LmbO with α-glucose 1-phosphate and GTP; (b) refolded LmbO with β-glucose 1-phosphate and GTP; (c) LmbK and refolded LmbO with α-glucose 1,6-bisphosphate and GTP; (d) co-injection of the sample from LmbO reaction with α-glucose 1-phosphate (trace a) and the GDP-D-α-glucose standard; and (e) GDP-D-α-glucose standard.

LmbO (route A). To test this hypothesis and verify which nucleotide is utilized in the lincomycin biosynthetic pathway, the synthetic bisphosphate **9** was incubated with LmbK and the refolded LmbO in the presence of ATP, CTP, GTP, TTP, or UTP (see Supporting Information section S6 for details). The resulting mixtures were analyzed by HPLC (Figure 2). Although no new peak was detected in the reaction mixture using ATP, CTP, TTP, or UTP, a sample derived from the incubation mixture with GTP showed a new peak with a retention time of 9.45 min (Figure 2, trace e). The same results were obtained when the synthetic **10** was incubated with the refolded LmbO (Figure S4). This new product peak was collected and subjected to ESI-MS. The recorded molecular mass (calcd for $C_{18}H_{28}N_5O_{18}P_2^-$ [M−H$^+$], 664.0910; obsd, 664.0922) is consistent with the proposed product, GDP-D-α-D-octose (**11**), which was also chemically prepared from **10**, as shown in Scheme 3. As expected, the isolated product from the LmbK/LmbO reaction displayed a HPLC retention time identical to that of the synthetic standard (Figure 3, traces a and b).

The above results clearly indicate that the refolded LmbO is catalytically active and suggest a pathway in which C8 dephosphorylation by LmbK preceeds prior to nucleotidylyl-transfer catalyzed by LmbO (Scheme 1, route A). However, a reversal of the sequence of these two reactions is also conceivable (route B). To check this possibility, **9** was incubated with the refolded LmbO in the presence of GTP. As shown in Figure 3, trace c, no new peak in the range of GDP-activated sugars is observed. In contrast, formation of **11** is clearly visible (Figure 3, trace d) when the synthetic **10** was incubated with the refolded LmbO and GTP. Evidently, C8 dephosphorylation is a prerequisite for the subsequent nucleotidyl activation. These results unequivocally establish that **11** is indeed an intermediate in MTL biosynthesis and its formation follows route A, not route B.

In heptose biosynthesis, the bisphosphate products (e.g., **18** and **21**) generated in the kinase reactions have defined anomeric configurations that correlate with the anomeric specificities of the subsequent nucleotidylyltransferase (e.g., HldE and HddC)-catalyzed transformations (e.g., **19**→**20** and **22**→**23**, Scheme 2). An analogous anomeric specificity also appears to be conserved in LmbK- and LmbO-catalyzed reactions because their products are all α-anomers. To investigate whether LmbO tolerates flexibility in its anomeric specificity, the refolded LmbO was

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incubated separately with GTP and each of the two anomers of glucose 1-phosphate, used as substrate analogues. Since formation of GDP-α-glucose was found only in the sample of α-glucose 1-phosphate (Figure 4, traces a and b), LmbO shows stringent α-anomeric stereospecificity for both octose and hexose substrates. Interestingly, although LmbO is capable of processing six-carbon sugar 1-phosphate, LmbK could not hydrolyze α-D-glucose 1,6-bisphosphate (Figure 4, trace c). A similar observation was reported for heptose 1,7-bisphosphate phosphatase GmhB, which could not recognize α-D-glucose 1,6-bisphosphate as a substrate.[15] Apparently, the location of the phosphoryl group being removed on the substrate is essential for these phosphatases.

Together these results provide significant insight into the lincomycin biosynthetic pathway, part of which is reminiscent of the NDP-heptose pathway. Transformation of octose 8-phosphate (**8**) to GDP-D-α-D-octose (**11**) is shown to involve kinase and phosphatase reactions as intermediary steps. Our data also reveal that the dephosphorylation step, catalyzed by LmbK, is critical for the nucleotide activation reaction. However, direct demonstration of the predicted kinase activity of LmbP was unsuccessful due to the difficulty in refolding insoluble LmbP. Nevertheless, the involvement of a kinase-catalyzed step and the α-stereospecificity of LmbP reaction are supported by the effective reconstitution of the biosynthesis of GDP-D-α-D-octose using the synthetic bisphosphate **9** as substrate.

Formation of a nucleotide-activated octose intermediate in the lincomycin biosynthetic pathway likely serves two purposes: (1) the phosphonucleotidyl group might be an important recognition/binding element for the later enzymes in the pathway, and (2) it can function as a good leaving group in a nucleophilic substitution reaction, which may eventually allow the installation of the C1 thiol group. Furthermore, the identification of the GDP-octose intermediate has settled a long-standing dispute over whether the proteins encoded in the *lmb* gene cluster with sequence similarity to some NDP-deoxyhexose modifying enzymes play any roles in lincomycin biosynthesis.[12] It should also be noted that most GDP-activated sugars are used in the biosynthesis of bacterial cell-wall polysaccharides and eukaryotic glycans.[2,16] Only GDP-mannose has been demonstrated or suggested to be a biosynthetic precursor of the sugar subunit in some secondary metabolites, including the polyene macrolide nystatin, amphotericin and candicidin,[17] the aminoglycoside hygromycin,[18] and the antitumor drug bleomycin.[19] These results identify several key intermediates in the lincomycin pathway and expand our knowledge of the roles of nucleotide-activated sugars in natural product biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, ESI-MS, and HPLC traces. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

h.w.liu@mail.utexas.edu

Notes

The authors declare no competing financial interest.

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