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Efficient Chemoenzymatic Synthesis of ADP-D-*glycero*-β-D-*manno*-Heptose and a Mechanistic Study of ADP-L-*glycero*-D-*manno*-Heptose 6-Epimerase

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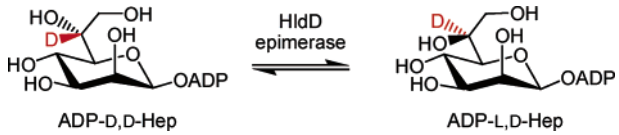
ABSTRACT



ADP-D,D-Hep ADP-L,D-Hep

A chemoenzymatic synthesis of ADP-D-*glycero*-β-D-*manno*-heptose (ADP-D,D-Hep) is described in which D,D-Hep 7-phosphate is converted to ADP-D,D-Hep by two biosynthetic enzymes. This strategy allows access to the 6″-deuterated analogue, which upon incubation with the epimerase showed complete retention of the isotopic label at the 6″-position. This provides evidence for a direct oxidation mechanism in which the hydride initially transferred to the NADP$^+$ cofactor is subsequently returned to the same carbon in a nonstereospecific manner.

Lipopolysaccharide (LPS) is a large glycolipid that comprises the outer surface of the outer membrane in gram-negative bacteria.[1,2] It consists of three main parts: a Lipid A component, which serves as a hydrophobic anchor to the outer membrane; a core oligosaccharide, which serves as a barrier to hydrophobic antibiotics, and; an O-antigenic component, consisting of an immunogenic repeating oligosaccharide distinct to each species. The constituents of the core oligosaccharide are largely conserved among gram-negative bacteria and contain the higher order sugars 3-deoxy-D-*manno*-octulosonic acid (KDO) and L-*glycero*-D-*manno*-heptose (L,D-Hep).

The biosynthesis of the core component L,D-Hep begins with the conversion of sedoheptulose 7-phosphate into D,D-Hep 7-phosphate as catalyzed by the isomerase GmhA (Scheme 1).[3,4] The D,D-Hep 7-phosphate is then converted into D,D-Hep 1,7-bisphosphate by the kinase activity of the bifunctional enzyme HldE. It has recently been determined that the unusual β-anomer is utilized in this biosynthetic scheme. A phosphatase, GmhB, then generates D,D-Hep 1-phosphate, and the second activity of HldE catalyzes a pyrophosphorylase reaction with ATP to give ADP-D,D-Hep. The required stereochemistry for LPS biosynthesis is then introduced by the action of ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (HldD) that interconverts ADP-D,D-Hep and ADP-L,D-Hep by a reversible inversion of stereochemistry at C-6″. Finally, a heptosyltransferase incorporates the L,D-Hep into the LPS structure.

The mechanism employed by ADP-L-*glycero*-D-*manno*-heptose 6-epimerase is of considerable interest in that, unlike the majority of racemases and epimerases, it performs an inversion of stereochemistry at an "unactivated" center.[5,6] Previous biochemical and structural studies have confirmed

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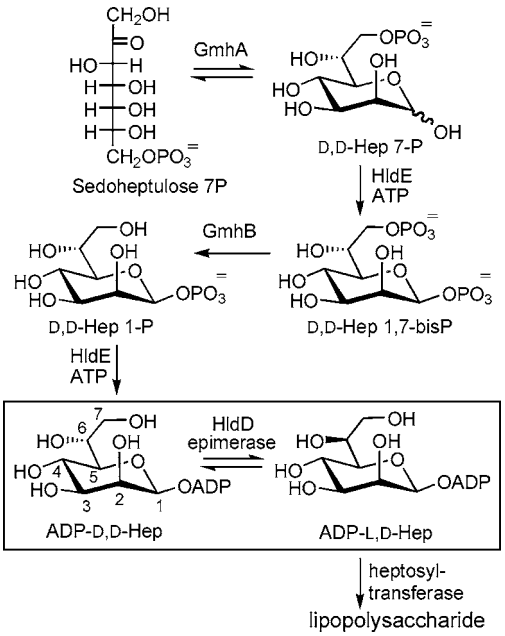
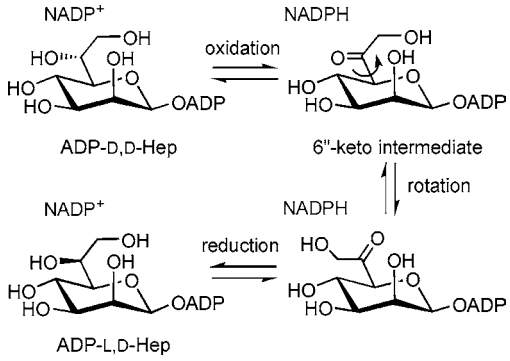
Scheme 1. Biosynthesis of ADP-L-*glycero*-β-D-*manno*-Heptose



that it employs a tightly bound $NADP^+$ cofactor during catalysis.[7,8] Recent studies have supported a direct oxidation−reduction mechanism in which transient oxidation takes place at C-6″ to produce a 6″-keto intermediate (Scheme 2).[9,10]

Scheme 2. Mechanism of the Reaction Catalyzed by ADP-L-*glycero*-D-*manno*-Heptose 6-Epimerase



Following reorientation of the intermediate within the active site, the hydride is then transferred to the opposite face of the carbonyl to yield the epimeric product during catalysis. Evidence in support of this mechanism includes the observation that no solvent-derived isotope (from either ^2H_2O or $H_2{}^{18}O$) is incorporated into product. The demonstration that 7″-deoxy and 4″-deoxy analogues serve as substrates for the epimerase rules out mechanisms involving transient oxidation at either of these positions. Finally, the demonstration that the epimerase is capable of catalyzing the dismutation of a truncated intermediate analogue containing an aldehyde at C-6″ into a 1:1 mixture of 6″-alcohol and 6″-acid strongly supports the notion that oxidation/reduction takes place at the 6″ position during the epimerase mechanism. A necessary consequence of the proposed mechanism is that the C-6″ hydrogen of ADP-D,D-Hep will be transferred to the C-6″ position of ADP-L,D-Hep. In this paper, ADP-[6″-^{2}H]-D,D-Hep is used to test this proposal.

Progress in the study of ADP-L,D-Hep 6-epimerase has been hampered by the difficulty in obtaining the epimeric substrates. ADP-D,D-Hep can be isolated from mutant strains of *Escherichia coli* that lack the epimerase gene; however, only submilligram amounts can be obtained in this fashion.[11] Although the synthesis of L,D-Hep and D,D-Hep has been achieved by several groups,[12] the synthesis of both epimers of ADP-β-Hep has only recently been reported and involves lengthy synthetic sequences with several challenging steps (15 steps for ADP-D,D-Hep).[13] Notably, it is necessary to install the correct stereochemistry at both C-6″ and C-1″ (the latter gives the β-manno configuration). Furthermore, the products are sensitive to both acidic and basic conditions, with the latter instability attributed to formation of a cyclic phosphodiester. In our effort to develop a more efficient synthesis of ADP-D,D-Hep, we targeted the biosynthetic intermediate D,D-Hep 7-phosphate that could be converted into ADP-D,D-Hep by the action of the enzymes HldE and GmhB (Scheme 1). This avoids the need to introduce the β-phosphate group and to accomplish the diphosphate coupling reaction. Our synthesis was also planned such that ADP-[6″-^{2}H]-D,D-Hep could be readily obtained with the use of NaB^2H_4. Our chemoenzymatic synthesis of ADP-D-*glycero*-β-D-*manno*-heptose begins with the primary alcohol **1** (Scheme 3), which is available in four steps from D-mannose.[12b] The alcohol was converted to the acid **2** by dichromate oxidation. Acid **2** was reacted with oxalyl chloride to produce the acid chloride, which was added to a solution of diazomethane in ether to afford the α-diazoketone **3**.[14] Nucleophilic displacement of nitrogen with dibenzylphosphoric acid gave the α-ketophosphate;[15] however, this compound was relatively unstable and decomposed on silica gel. Instead of isolating the compound, a reductive workup using either $NaBH_4$ or $NaBD_4$ was employed to give the nondeuterated (**4a** and **5a**) and deuterated (**4b** and **5b**)

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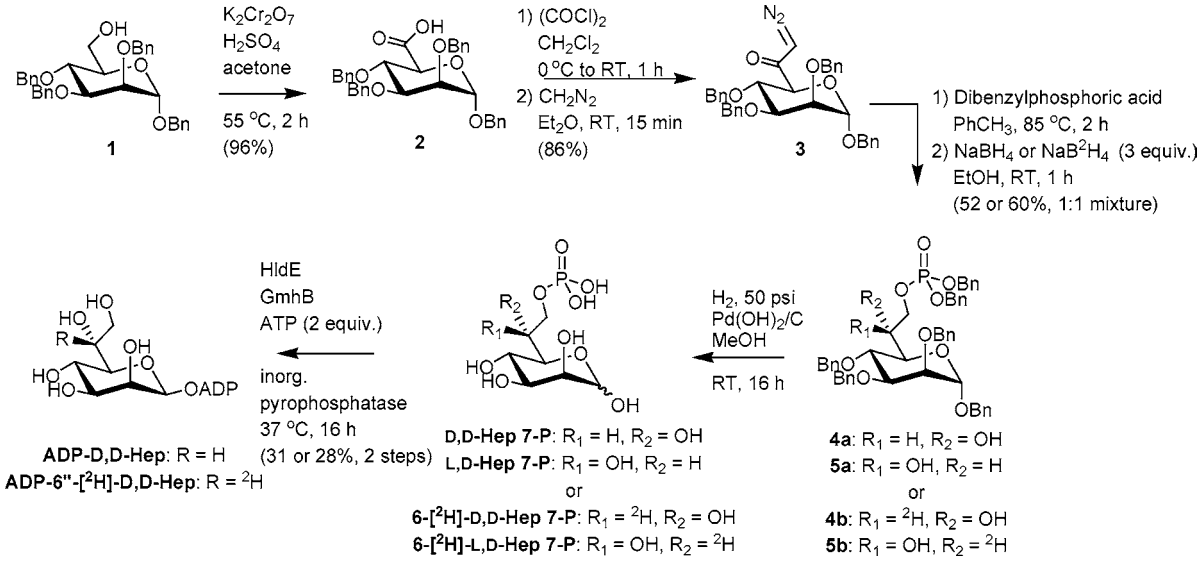
Scheme 3. Synthesis of ADP-D,D-Hep and ADP-[6″-^{2}H]-D,D-Hep



dibenzyl phosphates, respectively, each as an equimolar mixture of diastereomers. An attempt to control the diastereoselectivity of the reduction employed $Zn(BH_4)_2$ as the reducing agent.[16] This gave predominantly one diastereomer; however, further studies confirmed that it was the L,D-isomer that would not be carried forward by the biosynthetic enzymes. A global deprotection of the mixture of **4** and **5** using Pearlman's catalyst under 50 psi of H_2 produced a mixture of D,D-Hep 7-phosphate and L,D-Hep 7-phosphate, which were carried forth without further purification. To accomplish the final enzymatic step of the synthesis, the gene products HldE and GmhB were overexpressed and purified. The enzymes were generated with N-terminal hexahistidine tags to facilitate purification by immobilized metal ion affinity chromatography. The mixture of D,D-Hep 7-phosphate and L,D-Hep 7-phosphate was incubated with HldE, GmhB, and 2 equiv of ATP. Inorganic pyrophosphatase was also added in order to hydrolyze the pyrophosphate produced and drive the reaction to completion. The reaction was monitored by ^{31}P NMR spectroscopy, and it was clear that only one of the isomers was converted into a sugar nucleotide upon overnight incubation. The resulting sugar nucleotide was readily separated from the other reaction components by anion-exchange chromatography and found to be identical with ADP-D,D-Hep that had been generated via chemical synthesis.[10] Thus, the enzymes would readily accept the natural D,D-Hep 7-phosphate as a substrate and would not accept the unnatural L,D-isomer, effectively separating the mixture that had been generated by the borohydride reduction.

With ADP-[6″-^{2}H]-D,D-Hep in hand, it was possible to unequivocally show that the hydride that is transferred to the $NADP^+$ during the initial oxidation is subsequently transferred back to the same carbon of the product. The labeled compound was incubated with ADP-L,D-Hep 6-epimerase, and the reaction was monitored by ^{1}H NMR spectroscopy (Figure 1). A convenient signal that can be used to monitor the epimerization reaction is that from H5″, which appears at 3.32 ppm in ADP-D,D-Hep and 3.23 ppm in ADP-L,D-Hep. In ADP-[6″-^{2}H]-D,D-Hep, the H5″ signal appears as a doublet with a ^3J value of 9.5 Hz due to its coupling

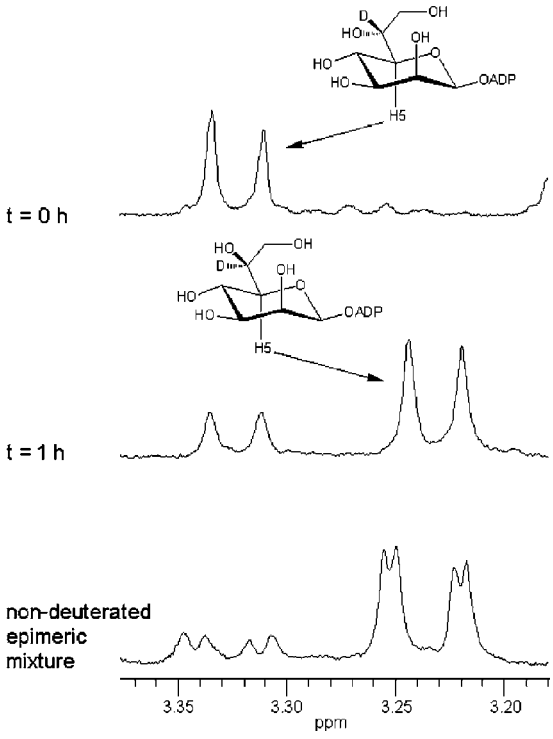


Figure 1. ^{1}H NMR spectra illustrating the enzyme-catalyzed epimerization of ADP-[6″-^{2}H]-D-*glycero*-D-*manno*-heptose.

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with H4″. Upon incubation with the epimerase, this signal was diminished, and a new doublet appeared corresponding to the H5″ signal of the other epimer. The two doublets reached equilibrium after 1 h, and their signals remained unchanged after incubation for an additional 16 h. The fact that the ADP-[6″-^{2}H]-L,D-Hep produced also displays an H5″ signal that is a doublet confirms that the label was retained during catalysis. Its location can be assigned to C-6″ on the basis of the observed large $^{3}J_{4,5}$ value of 9.5 Hz and the knowledge that the $^{3}J_{5,6}$ value is small (see the spectrum of the nondeuterated sample). As expected, the mass spectrum of the epimeric mixture was identical to that of the starting material.

The synthesis outlined in this letter provides a convenient source of deuterated and nondeuterated ADP-D,D-Hep and will greatly aid further mechanistic studies, including the measurement of kinetic isotope effects and isotope crossover experiments. In the latter studies, ADP-[6″-^{18}O]-D,D-Hep could readily be prepared using a minor modification of the chemoenzymatic synthesis and epimerized in the presence of ADP-[6″-^{2}H]-D,D-Hep to test for the formation of compounds containing both isotopic labels. This would test whether the 6″-keto intermediate is transferred between active sites during catalysis.

The observation that the C-6″ hydrogen retains its position during catalysis is completely consistent with the proposed nonstereospecific hydride transfer mechanism (Scheme 2). It rules out mechanisms involving an initial oxidation at one carbon (C-6″ or C-7″), followed by an isomerization of the carbonyl to interconvert ketone and aldehyde intermediates, and a final reduction at a different carbon (C-7″ or C-6″ respectively). Such mechanisms could be invoked in order to avoid proposing a nonstereospecific hydride transfer that requires major reorientation of the substrate and cofactor; however, they are somewhat more complex in terms of the number of chemical steps. Instead, ADP-L,D-Hep 6-epimerase appears to employ the direct two-step oxidation/reduction mechanism that is reminiscent of the approaches taken by the enzymes UDP-galactose 4-epimerase and CDP-tyvelose 2-epimerase.[17,18] It is intriguing to see that this strategy was adopted by three epimerases that are all evolutionarily related yet operate at positions of a sugar nucleotide that are spacially quite distinct (C-2″, C-4″, and C-6″). In each case, the chemical strategy is the same; however, the required reorientation of the oxidized intermediate is dramatically different.

Acknowledgment. This work was supported by the Canadian Institutes of Health Research (CIHR)

Supporting Information Available: Experimental procedures and NMR spectra for compounds **2**, **3**, and ADP-[6″-^{2}H]-D,D-Hep. This material is available free of charge via the Internet at http://pubs.acs.org.

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