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Chemoenzymatic synthesis of ADP-D-glycero-β-D-manno-heptose and study of the substrate specificity of HldE

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ABSTRACT

An efficient one-pot three enzymes strategy for chemoenzymatic synthesis of ADP-D-glycero-β-D-manno-heptose (ADP-D, D-heptose) was reported using chemically synthesized D, D-heptose-7-phosphate and the ADP-D, D-heptose biosynthetic enzymes HldE and GmhB. Moreover, the result of investigating substrate specificity of the kinase action of HldE revealed that HldE had highly restricted substrate specificity towards structurally modified heptose-7-phosphate analogs.

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1. Introduction

Lipopolysaccharides (LPS) are important outer membrane components of gram-negative bacteria. Being exposed to the surface, LPS are responsible for many pathophysiological responses to bacterial infections and are potent endotoxins responsible for high rates of mortality due to septic shock.[1] LPS consist of three main parts: an endotoxic lipid A comprising an acylated β-(1→6) linked glucosamine disaccharide with bisphosphorylation, a core oligosaccharide, and an *O*-antigenic component specific to each bacterial species.[1] The core oligosaccharide region can be further divided into an inner core oligosaccharide region consisting of 3-deoxy-D-manno-octulosonic acid (Kdo) residues as well as D-glycero-β-D-manno-heptose (D, D-heptose) or L-glycero-β-D-manno-heptose (L, D-heptose) residues with the L, D-heptose residues more abundant; and an outer core region that can contain heptoses, hexoses, or hexosamines (Fig. 1).[1,2] The inhibition of the biosynthesis of these carbohydrates, or their assembly, could be exploited for novel antimicrobial drug design.[3,4]

Heptoses are important seven carbon sugars that occur in numerous structural variations in several domains of the bacterial cell envelope. These heptoses manifest themselves in cell envelope anchored oligo- and polysaccharides in a wide variety of conformations and stereo chemistries, both in the pyranose ring and side chain. The heptoses with a glycero-D-manno configuration are common constituents of the inner core oligosaccharides of many gram-negative bacteria such as *Escherichia coli, Salmonella, Pseudomonas*, *Neisseria* and other pathogenic strains of bacteria.[5] In many strains, heptoses are expressed in the inner core region of LPS as branched trisaccharides or disaccharides. Heptoses in LPS are significant in bestowing a hydrophilic character in the cell envelope to keep out hydrophobic residues. Heptoses also contribute to cell envelope stability through the interacting of their phosphate groups with divalent cations.[6,7] The core oligosaccharide region of LPS is largely conserved amongst gram-negative bacteria, with most strains consisting of 3-deoxy-D-manno-octulosonic acid (Kdo) and D, D-heptose or L, D-heptose (Fig. 1).[1,2]

The biosynthesis of the nucleotide activated heptose precursors for assembly of LPS has been extensively studied.[1] These nucleotide activated heptoses mainly include ADP-D-glycero-β-D-manno-heptose (ADP-D, D-heptose), ADP-L-glycero-β-D-manno-heptose (ADP-L, D-heptose), and a less common GDP-D-glycero-α-D-manno-heptopyranose (GDP-D, D-heptose).[8] GDP-D, D-heptose has been described in bakers' yeast and identified as the substrate for the bacterial glycosyltransferase involved in the assembly of the S-layer glycoprotein glycan in *Aneurinibacillus thermoaerophilus*.[8,9] ADP-D, D-heptose and ADP-L, D-heptose have been isolated and identified from *Shigella sonnei* and *Salmonella minnesota* mutants.[10,11] Heptosyltransferases from *E. coli* can accept ADP-D, D-heptose and ADP-L, D-heptose as substrates for core oligosaccharide assembly.[1,12] The biosynthetic pathway of ADP-L/D, D-heptose initiates with the formation of sugar sedoheptulose-7-phosphate by the

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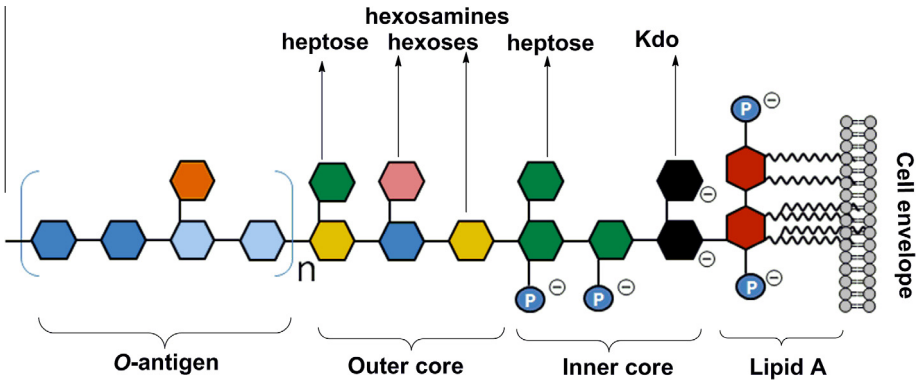


Figure 1. The structure of LPS.

transketolase (TktA, EC 2.2.1.1) which catalyzes the reaction of xylulose-5-phosphate with ribose-5-phosphate (Fig. 2).[13,14] Sedoheptulose-7-phosphate is then converted into D-glycero-D-manno-heptose-7-phosphate by keto-aldose isomerase called GmhA (EC 5.3.1.28), followed by anomeric phosphorylation by the kinase activity of HldE (EC 2.7.1.167) exclusively forming the β-anomer, namely, D-glycero-β-D-manno-heptose-1,7-bisphosphate. HldE comprises two independently functional domains: an N-terminal region with homology to the ribokinase superfamily and a C-terminal region with homology to the cytidylytransferase superfamily.[6] The ADP-D, D-heptose is generated by the sequential dephosphorylation at C-7 of D-glycero-β-D-manno-heptose-1,7-bisphosphate by the phosphatase (GmhB, EC 3.1.3.82) and adenylylation of the resulting D-glycero-β-manno-heptose-1-phosphate by the second activity of HldE (EC 2.7.7.70). Epimerization at C-6 by the epimerase (HldD, EC 5.1.3.20) produces ADP-L, D-heptose.[13,15,16] Heptosyltranferases use this product as the substrate and incorporate it into LPS assembly. ADP-D, D-heptose has also been shown to be a substrate for these heptosyltransferases, but with much lower efficiency.[12]

Chemical synthesis of ADP-L/D, D-heptose suffers from lengthy reaction steps, low yields, tedious separations and purification steps.[12,17] For example, the synthesis of penta-acetyl glycero-β-D-manno-heptose-1-phosphate is accompanied by the formation of the α-anomer (penta-acetyl glycero-α-D-manno-heptose-1-phosphate), which must be separated from the desired β-anomer products.[13] This process of separation is time-consuming and must be done utilizing laborious separation techniques. Moreover, removal of acetyl groups from protected ADP-heptose leads to formation of the by-product (1,2-cyclic phosphate heptose) with release of AMP.[12] Herein, we reported an efficient chemoenzymatic approach to synthesis of ADP-D, D-heptose based on its biosynthetic pathway. Furthermore, using substrate analogs, we revealed highly restricted substrate specificity of the kinase action of HldE.

2. Results and discussion

2.1. Chemoenzymatic synthesis of ADP-D-glycero-β-D-manno-heptose

D, D-Heptose-7-phosphate **2** was chemically synthesized as illustrated in Scheme 1. First, D-mannose **9** as the starting material was subjected to benzylation at the anomeric carbon using benzyl alcohol and acetyl chloride to give benzyl α-D-mannopyranoside **10** in 81% yield.[18] Subsequently, the primary hydroxyl of compound **10** was selectively silylated using *t*-butyldiphenylsilyl (TBDPS) chloride to afford the 6-*O*-TBDPS ether **11** in 82% yield. Benzylation of compound **11** using benzyl bromide and NaH in DMF gave the benzyl ether **12** in 93% yield. Desilylation with tetrabutylammonium fluoride (TBAF) in THF afforded free 6-hydroxyl compound **13** in 93% yield. The primary hydroxyl of compound **13** was subjected to Dess–Martin oxidation to provide the desired aldehyde, which can be directly used for Wittig reaction without further purification to afford alkene **14** in 61% yield over two steps.[19,20] Dihydroxylation of alkene **14** with OsO_4 and *N*-methylmorpholine *N*-oxide (NMNO) gave a 5:1 mixture of diastereomers that can be separated by silica gel column to give predominant D, D-heptose **15** with 63% separated yield. The stereochemical

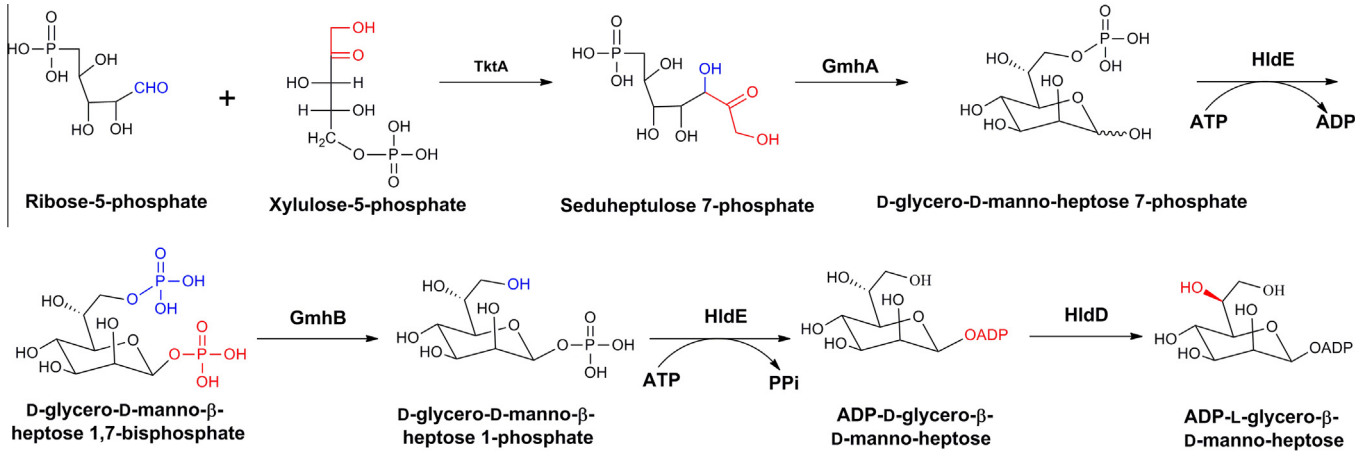
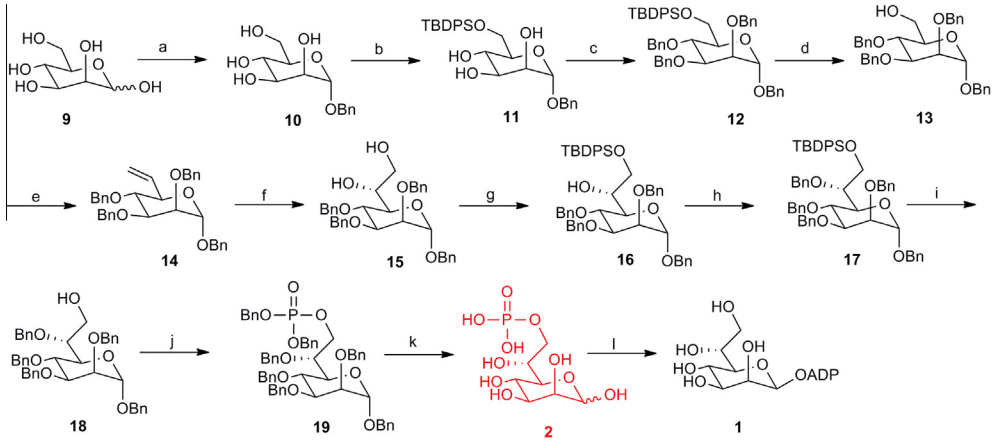


Figure 2. The biosynthetic pathway of ADP-L/D, D-heptose.



Scheme 1. Chemoenzymatic synthesis of ADP-D, D-manno-heptose. Reagents and conditions: (a) BnOH, AcCl, 50 °C, 81%; (b) TBDPSCl, imidazole, DMF, 82%; (c) NaH, BnBr, DMF, 93%; (d) TBAF, THF, 92%; (e) Dess–Martin reagent, DCM; Ph_3PCH_3Br, *n*-BuLi, THF, 61% (two steps); (f) OsO_4, NMNO, acetone/H_2O 9:1, 63%; (g) TBDPSCl, imidazole, DMF, 74%; (h) NaH, BnBr, TBAI, THF, 93%; (i) TBAF, THF, 92%; (j) dibenzyl *N*, *N*-diisopropyl phosphoramidite, *1H*-tetrazole, *m*-CPBA, 82%; (k) $Pd(OH)_2/C$, H_2, MeOH, 92%; (l) HldE, GmhB, ATP, inorganic phosphatase, 42%.

outcome of this dihydroxylation was in accordance with Kishi's empirical rule.[21] Selective silylation of the primary hydroxyl of compound **15** gave the primary silyl ether **16** using TBDPSCl and imidazole in 74% yield. Benzylation of compound **16** using benzyl bromide and NaH in THF with tetrabutylammonium iodide (TBAI) as phase transfer catalyst gave the benzyl ether **17** in 94% yield. TBDPS group was removed with TBAF to give free 7-hydroxyl compound **18** in 92% yield. Phosphorylation of compound **18** in the presence of dibenzyl *N*,*N*-diisopropyl phosphoramidite and *1H*-tetrazole, followed by in situ *m*-CPBA oxidation gave the compound **19**.[22] The final deprotection of benzyl groups was achieved using Pearlman's catalyst ($Pd(OH)_2/C$) under H_2 to afford the desired D,D-heptose-7-phosphate **2** in 92% yield.

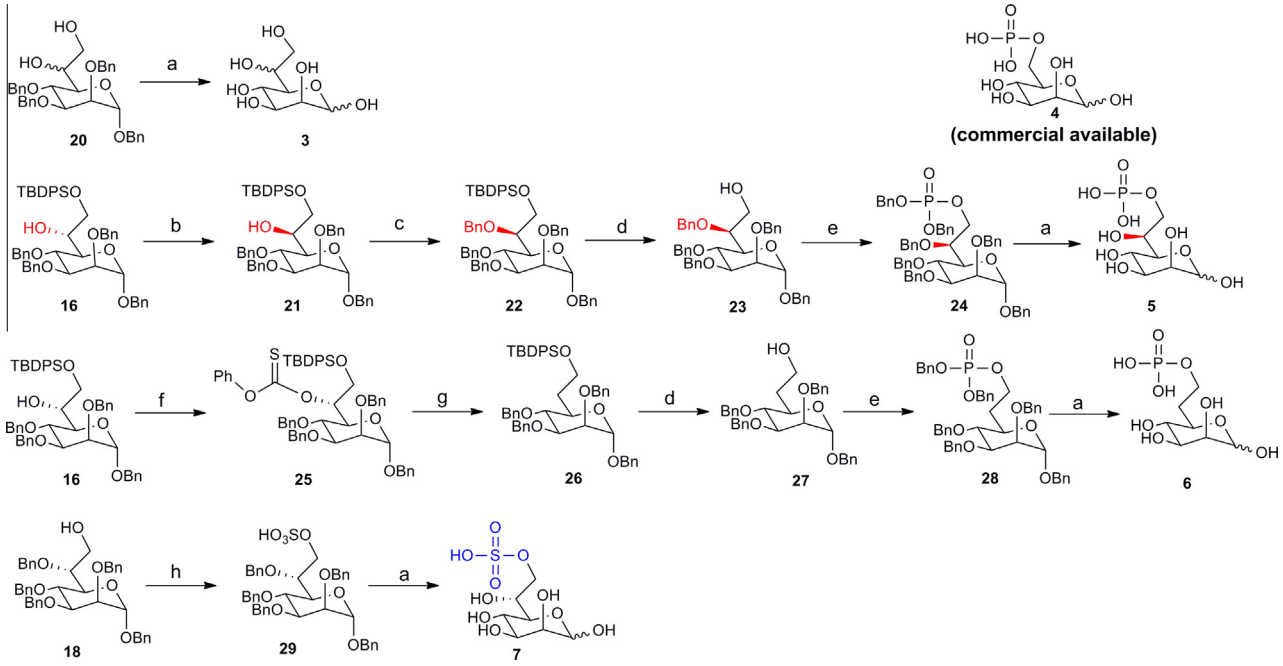
With the natural substrate (D, D-heptose-7-phosphate) of HldE in hand, the bifunctional enzyme (HldE) and phosphatase (GmhB) from *E. coli* were overexpressed, and purified as previously reported.[13,15] D, D-Heptose-7-phosphate was incubated with HldE, GmhB, inorganic pyrophosphatase and ATP, followed by inorganic pyrophosphatase. Inorganic pyrophosphatase, an enzyme (EC 3.6.1.1) that catalyzed the conversion of one molecule of pyrophosphate to two phosphate ions, contributed to promoting the chemical equilibrium of adenylylation for completion of the reaction. The resulting sugar nucleotide (ADP-D, D-heptose) can be separated from the other reaction components by Bio-Gel P-2 column with 42% separated yield. The NMR spectroscopic data of the product was in good agreement with chemically synthesized ADP-D, D-heptose by Paul Kosma and co-workers.[12]

2.2. Preparation of D, D-heptose-7-phosphate analogs

To determine the substrate specificity of the kinase activity of HldE, we designed and synthesized five D, D-heptose-7-phosphate analogs (Scheme 2). Similar to the synthesis of *N*-acetyl-hexosamine 1-phosphate using *N*-acetylhexosamine 1-kinase,[23,24] we hypothesized that the enzyme HldE would directly phosphorylate heptose diastereomers **3** at the anomeric carbon to give heptose 1-phosphate. Therefore, heptose diastereomers **3** were synthesized by hydrogenation of compound **20** in 92% yield. Based on the similarity of glycero-D-manno-heptose and regular D-mannose, commercially available mannose 6-phosphate **4** was hypothesized to be a potential substrate of HldE. In order to investigate the importance of the phosphate group on the C-7 of D, D-heptose-7-phosphate, and also the importance of the stereochemistry or group attached to C-6 of D, D-heptose-7-phosphate, analogs with functional group modifications on these positions were synthesized. Thus, compound **16** was subjected to Mitsunobu reaction in the presence of diisopropyl azodicarboxylate (DIAD), PPh_3 and *p*-nitrobenzoic acid to give *p*-nitrobenzyl ester, with inversion of configuration at the C-6, which was then treated with base (K_2CO_3) to afford compound **21**.[20] Compound **21** was benzylated at the 6-OH position to afford compound **22**. Conversion of compound **16** to the corresponding thionocarbonate **25** using phenyl chlorothionoformate and 4-(dimethylamino)-pyridine (DMAP), followed by radical deoxygenation in the presence of tributylstannane and 2, 2-azobisisobutyronitrile (AIBN) gave compound **28**.[24,25] Based on similar procedures, compounds **22** and **26** were subjected to deprotection of silylation (TBDPS), phosphorylation of free hydroxyl in C-7 and deprotection of benzyl groups by hydrogenation resulting in the desired analogs **5** and **6**. D, D-Heptose-7-sulfate **7** was prepared by a two-step synthesis: sulfonation of intermediate **18** with sulfur trioxide/pyridine complex gave compound **29**, followed by deprotection of benzyl groups by hydrogenation to afford **7**.[26–28]

2.3. Substrate specificity of HldE

With the D, D-heptose-7-phosphate analogs in hand, activity assays of HldE towards these analogs were performed. The resulting products were analyzed by ESI HRMS. Compound **2**, the natural substrate of HldE, was converted to D-glycero-D-manno-β-heptose 1, 7-bisphosphate. Treatment of the heptose diastereomers **3** with HldE yielded no products according to mass spectrometry analysis. This is most likely due to the absence of the C-7 phosphate group on the substrate. Compound **4**, mannose 6-phosphate, did not yield the hypothesized product of D-manno-β-heptose 1, 6-bisphosphate after HldE treatment because of missing the C-7. The desired products were not detected by treatment of HldE with L, D-heptose-7-phosphate **5** and D, D-heptose-7-sulfate **7**. Interestingly, 6-deoxy-glycero-D-manno-β-heptose 7-phosphate **6** showed weak reactivity with HldE (TLC analysis showed that the starting material **6** did not almost decrease and no desired product be found, but MS can detect the product with very low abundance compared with the starting material **6**). The lack of any functional group on C-6, but the presence of the C-7 phosphate group provided insight into the importance of the C-7 phosphate group for enzyme binding and function. Based on the reactivity of the substrate analogs assays, we concluded that the kinase action of HldE had exclusive substrate specificity towards structurally modified heptose-7-



Scheme 2. Synthesis of D, D-heptose-7-phosphate analogs. Reagents and conditions: (a) $Pd(OH)_2/C$, H_2, **3**: 91%; **5**: 90%; **6**: 92%; **7**: 92%; (b) DIAD, PPh_3, *p*-nitrobenzoic acid, THF; K_2CO_3, MeOH/DCM, 81% (two steps); (c) NaH, BnBr, TBAI, THF, 91%; (d) TBAF, THF, 92%; (e) dibenzyl *N*, *N*-diisopropyl phosphoramidite, *1H*-tetrazole, *t*-BuOOH, **24**: 81%; **28**: 82%; (f) phenyl chlorothionoformate, DMAP,CH_3CN, 60 °C, 72%; (g) $(C_4H_9)_3SnH$; AIBN, 60 °C, 63%; (h) SO_3/Pry, Pyridine, 89%.

phosphate analogs, especially, the stereo-configuration around the 6-hydroxyl and 7-phosphate group were showed to be critical for enzyme's functions.

3. Conclusion

In summary, an efficient chemoenzymatic synthesis of ADP-D, D-heptose was successfully performed using an improved chemical synthesis of the important intermediate D, D-heptose-7-phosphate and one-pot three enzymes reaction. This will contribute to studies of LPS assembly with heptosyltranferases. Moreover, the specificity study of the kinase action of HldE revealed that it had exclusive substrate specificity towards structurally modified heptose-7-phosphate analogs. This work will be important in our understanding of what substrates and functional groups play roles in recognition by enzyme, which in turn can aid in the development of inhibitors of the kinase for antimicrobial drug design.

4. Experimental

4.1. General procedures

All reagents were purchased from commercial sources and were used without further purification. All solvents were available with commercially dried or freshly dried and distilled prior to use. Reactions were monitored by Thin Layer Chromatography (TLC) using silica gel GF_{254} plates with detection by short wave UV fluorescence ($\lambda = 254$ nm) and staining with 10% phosphomolybdic acid in EtOH or *p*-anisaldehyde solution (ethanol/*p*-anisaldehyde/acetic acid/sulfuric acid 135:5:4:1.5), followed by heating on a hot plate. Column chromatography was conducted by silica gel (200–300 mesh) with ethyl acetate and hexane or ethyl acetate (or dichloromethane) and methanol as eluent. ^1H NMR and ^{13}C NMR were recorded with Bruker AV 400 spectrometer at 400 MHz (^1H NMR), 100 MHz (^{13}C NMR) using $CDCl_3$, CD_3OD and D_2O as solvents. Chemical shifts were reported in δ (ppm) from $CDCl_3$ (7.26 ppm for ^1H NMR, 77.00 ppm for ^{13}C NMR), CD_3OD (3.31 ppm for ^1H NMR, 49.00 ppm for ^{13}C NMR) and D_2O (4.79 ppm for ^1H NMR). Coupling constants were reported in hertz. High-resolution mass spectra (HRMS) were obtained on a Varian QFT-ESI mass spectrometer.

4.2. Synthesis

4.2.1. Benzyl α-D-mannopyranoside (10)

A solution of D-mannose **9** (10.0 g, 55.5 mmol) in 80 mL benzyl alcohol containing 4 mL acetyl chloride was heated at 50 °C for 2 h and then cooled to room temperature. Benzyl alcohol was removed under high vacuum pump at 75 °C. The residue was then titrated with ethyl acetate to form precipitate. The precipitate was collected by filtration and washed with ethyl acetate to give a white solid **10** (12.1 g, 81%). ^1H NMR (CD_3OD, 400 MHz): δ 3.61–3.66 (m, 2H), 3.71–3.75 (m, 2H), 3.84–3.87 (m, 2H), 4.52 (d, $J = 11.6$ Hz, 1H), 4.75 (d, $J = 11.6$ Hz, 1H), 4.84 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ 62.93, 68.63, 69.87, 72.19, 72.63, 74.86, 100.65, 128.76, 129.11, 129.38, 139.00. HRMS: *m*/*z* calcd for $C_{13}H_{19}O_6$ $[M+H]^+$ 271.1176, found 271.1173.

4.2.2. Benzyl 6-*O*-tetra-butyldiphenylsilyl-α-D-mannopyranoside (11)

Compound **10** (3.24 g, 12.0 mmol) was added to flask with imidazole (1.8 g, 26.4 mmol) and dissolved in DMF (10.0 mL) at 0 °C. TBDPSCl (3.74 mL, 14.4 mmol) was added to reaction mixture in a dropwise manner. The reaction mixture was then stirred at room temperature for 6 h. TLC analysis showed complete conversion of starting material to a major product (CH_2Cl_2/MeOH 10:1, $R_f = 0.67$). The reaction was quenched with MeOH and concentrated in vacuum. The residue was dissolved with CH_2Cl_2, and the organic layer was washed with 5% hydrochloric acid, followed by water, dried and filtrated. The filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 1:1) to afford a syrup **11** (5.0 g, 82%). ^1H NMR (CD_3OD, 400 MHz): δ 1.06 (s, 9H), 3.58 (t, $J = 9.6$ Hz, 1H), 3.73–3.80 (m, 2H), 3.84–3.88 (m, 2H), 4.07 (d, $J = 10.8$ Hz, 1H), 4.54 (d, $J = 10.8$ Hz, 1H), 4.81 (d, $J = 11.6$ Hz, 1H), 7.27–7.44 (m, 11H), 7.73–7.77 (m, 4H); ^{13}C

NMR (CD_3OD, 100 MHz): δ 20.13, 27.36, 65.41, 68.89, 69.49, 72.09, 72.86, 75.45, 100.29, 128.70, 128.72, 128.78, 129.19, 129.38, 130.76, 136.79, 138.85. HRMS: *m*/*z* calcd for $C_{29}H_{36}O_6SiNa$ $[M+Na]^+$ 531.2173, found 531.2159.

4.2.3. Benzyl 6-*O*-tetra-butyldiphenylsilyl-2,3,4-*O*-tribenzyl-α-D-mannopyranoside (12)

Compound **11** (3.9 g, 7.7 mmol) was dissolved in 10 mL DMF and benzyl bromide (5.5 mL, 46.2 mmol) was added quickly. Sodium hydride (60% dispersion in mineral oil, 1.85 g, 46.2 mmol) was then added slowly at 0 °C. The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 20:1, R_f = 0.23). The reaction was quenched with MeOH and concentrated in vacuum. The residue was dissolved with CH_2Cl_2, and the organic layer was washed with water, followed by drying and filtration. The filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 20:1) to afford a syrup **12** (5.58 g, 93%). ^1H NMR ($CDCl_3$, 400 MHz): δ 1.23 (s, 9H), 3.91 (dd, *J* = 3.6 Hz, *J* = 9.6 Hz, 1H), 3.99 (s, 1H), 4.07–4.16 (m, 3H), 4.24 (t, *J* = 9.6 Hz, 1H), 4.59 (d, *J* = 11.6 Hz, 1H), 4.73–4.85 (m, 5H), 4.92 (d, *J* = 12.4 Hz, 1H), 5.06–5.10 (m, 2H), 7.32–7.50 (m, 26H), 7.88–7.93 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.44, 26.91, 63.43, 68.61, 72.37, 72.79, 73.51, 74.99, 75.30, 75.40, 80.52, 96.86, 127.58, 127.65, 127.78, 128.06, 128.10, 128.37, 128.43, 128.47, 129.62, 135.76, 136.03, 137.45, 138.57, 138.63, 138.71; ESI HRMS: *m*/*z* calcd for $C_{50}H_{54}O_6NaSi$ $[M+Na]^+$ 801.3580, found 801.3574.

4.2.4. Benzyl 2,3,4-*O*-tribenzyl-α-D-mannopyranoside (13)

To a solution of **12** (4.0 g, 5.1 mmol) in THF (20 mL) was added 1 M TBAF in THF solution (10.2 mL, 10.2 mmol). The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 5:1, R_f = 0.11). The reaction mixture was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 4:1) to afford a syrup **13** (2.54 g, 92%). ^1H NMR ($CDCl_3$, 400 MHz): δ 2.47 (br, 1H), 3.86 (dd, *J* = 3.6 Hz, *J* = 8.8 Hz, 1H), 3.93–3.96 (m, 3H), 4.11–4.19 (m, 2H), 4.55 (d, *J* = 12.0 Hz, 1H), 4.73–4.81 (m, 5H), 4.88 (d, *J* = 12.4 Hz, 1H), 5.05–5.09 (m, 2H), 7.37–7.50 (m, 20H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 62.26, 69.10, 72.29, 72.58, 72.91, 74.89, 75.27, 80.20, 97.55, 127.58, 127.64, 127.72, 127.82, 127.87, 128.10, 128.38, 128.42, 128.45, 137.19, 138.21, 138.43, 138.50; ESI HRMS: *m*/*z* calcd for $C_{34}H_{36}O_6Na$ $[M+Na]^+$ 563.2410, found 563.2419.

4.2.5. Benzyl 2,3,4-*O*-tribenzyl-6,7-dideoxy-α-D-hept-6-enopyranoside (14)

To a solution of **13** (1.08 g, 2.0 mmol) in CH_2Cl_2 15 mL at 0 °C was added 0.3 M Dess–Martin reagent in CH_2Cl_2 solution (13.3 mL, 4.0 mmol). The reaction mixture was then stirred at room temperature for 40 min. The reaction was quenched with an aqueous solution of sodium sulfite (1 M, 10 mL), followed by a saturated solution sodium bicarbonate (10 mL). The reaction mixture was further stirred for 15 min and aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL), and the combined organic layers were dried and concentrated in vacuum to afford the crude aldehyde to directly use for next step without further purification. A solution of methyltriphenylphosphonium bromide (857 mg, 2.4 mmol) in 20 mL THF was cooled to −40 °C and *n*-BuLi (1.0 mL, 2.5 mmol, 2.5 M in hexane) was added. The reaction mixture was stirred at −40 °C for 30 min and a solution of the crude aldehyde in THF (5.0 mL) was added. The reaction mixture was stirred for an additional 1 h at −40 °C. Then the reaction was warmed up slowly to room temperature over a period of 4 h before it was quenched with a saturated solution of ammonium chloride (15 mL), and extracted with ethyl acetate. The organic layer was washed with water and brine, followed by drying and filtration. The filtrate was concentrated in vacuum to give a crude product, which was purified by silica gel column (hexane/ethyl acetate 20:1) to afford a syrup **14** (655 mg, 61%). ^1H NMR ($CDCl_3$, 400 MHz): δ 3.89–3.94 (m, 2H), 4.08 (dd, *J* = 2.8 Hz, *J* = 9.2 Hz, 1H), 4.24–4.27 (m, 1H), 4.55 (d, *J* = 12.0 Hz, 1H), 4.73–4.90 (m, 6H), 4.97 (d, *J* = 10.8 Hz, 1H), 5.05 (s, 1H), 5.42 (d, *J* = 10.4 Hz, 1H), 5.59 (d, *J* = 17.2 Hz, 1H), 6.13–6.22 (m, 1H), 7.38–7.46 (m, 20H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 69.03, 72.58, 72.88, 73.30, 75.08, 75.29, 78.93, 79.94, 97.42, 118.16, 127.59, 127.71, 127.84, 127.96, 128.17, 128.40, 128.48, 135.58, 137.38, 138.34, 138.53, 138.68; ESI HRMS: *m*/*z* calcd for $C_{35}H_{36}O_5Na$ $[M+Na]^+$ 559.2460, found 559.2466.

4.2.6. Benzyl 2,3,4-*O*-tri-benzyl-D-glycero-α-D-manno-heptopyranoside (15)

To a solution compound **14** (1.0 g, 1.86 mmol) in acetone/water (9:1, 20 mL) at 0 °C was added NMNO (435.8 mg, 3.72 mmol) and OsO_4 (12.7 mg, 0.05 mmol). The reaction mixture was then stirred at room temperature for 4 h. TLC analysis showed complete conversion of starting material to a major product **15** (hexane/ethyl acetate 1:1, R_f = 0.39, the diastereomer R_f = 0.30). The reaction was quenched with a saturated solution of $NaHSO_3$. After stirring for addition 15 min, the reaction mixture was extracted with ethyl acetate and the organic layer was washed with H_2O and brine, dried with anhydrous Na_2SO_4 and filtrated. The filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 3:2) to afford a syrup **15** (669 mg, 63%). ^1H NMR ($CDCl_3$, 400 MHz): δ 2.73 (br, 1H, –OH), 3.72 (d, *J* = 3.6 Hz, 1H), 3.78–3.81 (m, 1H), 3.91–3.98 (m, 3H), 4.08 (t, *J* = 3.6 Hz, 1H), 4.13 (dd, *J* = 2.8 Hz, *J* = 9.2 Hz, 1H), 4.20 (t, *J* = 9.2 Hz, 1H), 4.54 (d, *J* = 11.6 Hz, 1H), 4.68–4.86 (m, 6H), 5.00 (s, 1H), 5.15 (d, *J* = 10.4 Hz, 1H), 7.40–7.46 (m, 20H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 62.97, 69.00, 71.63, 71.97, 72.71, 72.85, 74.57, 75.00, 76.69, 80.46, 96.95, 127.70, 127.80, 127.87, 127.97, 128.19, 128.41, 128.52, 137.02, 137.70, 138.02, 138.11; ESI HRMS: *m*/*z* calcd for $C_{35}H_{42}NO_7$ $[M+NH_4]^+$ 588.2961, found 588.2939.

4.2.7. Benzyl 7-*O*-tetra-butyldiphenylsilyl-2,3,4-*O*-tri-benzyl-D-glycero-α-D-manno-heptopyrano-side (16)

To a solution of **15** (467 mg, 0.82 mmol) and imidazole (55.8 mg, 1.8 mmol) in 10 mL DMF was added TBDPSCl (0.25 mL, 0.98 mmol) at 0 °C. The reaction mixture was then stirred at room temperature for 6 h. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.22). The reaction was quenched with MeOH and concentrated in vacuum. The residue was added DCM, and the organic layer was washed with water, dried and filtrated. The filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 8:1) to afford a syrup **16** (491 mg, 74%). ^1H NMR ($CDCl_3$, 400 MHz): δ 1.18 (s, 9H), 3.01 (d, *J* = 3.6 Hz, 1 H, -OH), 3.88 (m, 1H), 3.94–3.99 (m, 2H), 4.03–4.14 (m, 3H), 4.22 (dd, *J* = 3.6 Hz, *J* = 6.8 Hz, 1H), 4.50 (t, *J* = 12.0 Hz, 1H), 4.62 (d, *J* = 10.4 Hz, 1H), 4.69–4.80 (m, 5H), 4.97–5.00 (m, 2H), 7.27–7.50 (m, 26H), 7.77–7.82 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.34, 27.00, 64.88, 69.04, 71.88, 72.21, 72.66, 73.40, 74.63, 74.81, 75.63, 80.39, 97.06, 127.60, 127.67, 127.78, 127.91, 128.39, 129.76, 133.48, 133.57, 135.71, 137.30, 138.32, 138.43; ESI HRMS: *m*/*z* calcd for $C_{51}H_{56}O_7NaSi$ $[M+Na]^+$ 831.3693, found 831.3671.

4.2.8. Benzyl 7-*O*-tetra-butyldiphenylsilyl-2,3,4,6-*O*-tetra-benzyl-D-glycero-α-D-manno-heptopyranoside (17)

To a solution **16** (447 mg, 0.55 mmol), TBAI (20 mg, 0.055 mmol) and BnBr (0.13 mL, 1.1 mmol) in 10 mL THF at 0 °C was slowly added NaH (60% dispersion in mineral oil, 44 mg,

1.1 mmol). The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.53). The reaction mixture was filtered, the filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 15:1) to afford a syrup **17** (465 mg, 94%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.27 (s, 9H), 4.00 (s, 1H), 4.12–4.22 (m, 5H), 4.35 (t, J = 9.2 Hz, 1H), 4.61 (d, J = 11.6 Hz, 1H), 4.70 (d, J = 10.8 Hz, 1H), 4.81–4.83 (m, 3H), 4.87–5.13 (m, 6H), 7.26–7.27 (m, 2H), 7.38–7.57 (m, 29H), 7.85–7.91 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.24, 27.00, 65.14, 68.90, 72.31, 72.70, 72.85, 73.15, 74.75, 74.94, 75.29, 80.61, 81.36, 97.10, 127.30, 127.40, 127.56, 127.65, 127.72, 127.78, 127.86, 127.94, 128.24, 128.28, 128.35, 128.40, 128.44, 129.62, 133.71, 135.71, 135.82, 137.50, 138.54, 138.61, 139.24; ESI HRMS: m/z calcd for $C_{58}H_{66}O_7Si$ $[M+NH_4]^+$ 916.4609, found 916.4592.

4.2.9. Benzyl 2,3,4,6-*O*-tetra-benzyl-D-glycero-α-D-manno-heptopyranoside (18)

To a solution of **17** (360 mg, 0.4 mmol) in 10 mL THF was added 1 M TBAF in THF solution (0.6 mL, 0.6 mmol). The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 3:1, R_f = 0.27). The reaction mixture was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 3:1) to afford a syrup **18** (243 mg, 92%).^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.45 (br, 1 H, -OH), 3.77–3.82 (m, 1H), 3.91–3.94 (m, 1H), 4.06–4.17 (m, 3H), 4.54 (d, J = 12.0 Hz, 1H), 4.66–4.74 (m, 5H), 4.80–4.89 (m, 3H), 5.02–5.04 (m, 2H), 7.31–7.44 (m, 25H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 62.16, 69.08, 72.04, 72.23, 72.72, 72.80, 74.81, 74.93, 78.81, 80.47, 97.06, 127.71, 127.78, 127.81, 127.92, 128.12, 128.43, 128.49, 137.31, 138.23, 138.25, 138.37, 138.43; ESI HRMS: m/z calcd for $C_{42}H_{44}O_7Na$ $[M+Na]^+$ 683.2985, found 683.3006.

4.2.10. Benzyl 7-*O*-dibenzyl-phosphono-2,3,4,6-*O*-tetra-benzyl-D-glycero-α-D-manno-heptopyranoside (19)

A mixture of compound **18** (220 mg, 0.33 mmol), 4 Å molecular sieves (200 mg) and 1*H*-tetrazole (3.7 mL, 0.45 M in CH_3CN, 1.67 mmol) in anhydrous DCM (10 mL) was stirred 30 min at room temperature. Then $(BnO)_2PN(i\text{-}Pr)_2$ (330 μL, 1.0 mmol) was added. The reaction mixture was stirred at room temperature for another 2 h. Subsequently, the reaction mixture was cooled −20 °C, *m*-CPBA (374.3 mg 1.67 mmol) was added, and slowly warmed up room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 3:1, R_f = 0.23). The reaction was quenched by addition of 0.2 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel flash chromatography (hexane/ethyl acetate 4:1) to afford a syrup **19** (249 mg, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.90 (m, 1H), 4.01 (d, J = 10.0 Hz, 1H), 4.06–4.19 (m, 3H), 4.39–4.44 (m, 1H), 4.52 (d, J = 12.0 Hz, 1H), 4.67–4.88 (m, 8H), 4.97 (d, J = 10.4 Hz, 1H), 5.02–5.12 (m, 6H), 7.29–7.42 (m, 35H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 67.96 (d, J_{C-P} = 5.3 Hz), 68.95, 69.11 72.17, 72.30, 72.68, 72.73, 74.62, 74.84, 78.40, 78.44 (d, J_{C-P} = 7.0 Hz), 80.44, 97.00, 127.43, 127.50, 127.59, 127.71, 127.75, 127.78, 127.85, 127.88, 128.21, 128.33, 128.40, 128.47, 137.22, 138.25, 138.28, 138.36; ESI HRMS: m/z calcd for $C_{56}H_{57}O_{10}NaP$ $[M+Na]^+$ 943.3587, found 943.3586.

4.2.11. 7-Phosphate-D-glycero-β-D-manno-heptose (2)

To a solution of **19** (184 mg, 0.2 mmol) in MeOH (4 mL) was added $Pd(OH)_2$ on carbon (100 mg). The reaction was stirred in H_2 atmosphere (50 psi) for 4 h. The reaction mixture was filtered and washed with MeOH. The filtrate was concentrated to give a syrup **2** (53 mg, 92%).^{1}H NMR (CD_3OD, 400 MHz): 3.35–3.38 (m, 1H), 3.77–3.80 (m, 2H), 4.06–4.12 (m, 2H), 4.19–4.23 (m, 2H), 5.06 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ 68.93 (d, J_{C-P} = 5.3 Hz), 69.88, 72.30, 72.58, 73.02, 73.14 (d, J_{C-P} = 7.7 Hz), 95.78; HRMS: m/z calcd for $C_7H_{14}O_{10}P$ $[M-H]^-$ 289.0325, found 289.0328.

4.2.12. Adenosine 5′-diphosphate-D-glycero-β-D-manno-heptopyranose (Tris-salt) (1)

A solution (7.0 mL) containing 10 mM D, D-heptose-7-phosphate **2** (20 mg, 0.07 mmol), 25 mM ATP (0.175 mmol), 5 mM $MgCl_2$ (0.15 mmol) and 20 mM Tris–HCl buffer (pH 8.0), and the pH of the solution was adjusted to 8.0 by the addition of 1 M NaOH, followed by adding 3.0 mg HldE, 3.0 mg phosphatese GmhB, and 3.0 mg inorganic pyrophosphatase into the reaction mixture, incubating for 12 h at 37 °C. The reaction mixture was removed the proteins using Millipore (Amicon Ultra-4, 10 000 MWCO). Then the reaction mixture was added alkaline phosphatase (100 units) incubating for 12 h at 37 °C. The resulting solution was purified by Bio-Gel P-2 column. The product fractions were lyophilized to give ADP-D, D-heptose **1** as white powder (18 mg, 42%). ^{1}H NMR (D_2O, 400 MHz): δ 3.35 (dd, J = 2.8 Hz, J = 9.6 Hz, 1H), 3.65–3.70 (m, 4H), 3.91–3.92 (m, 1H), 3.98 (m, 1H), 4.15–4.16 (m, 2H), 4.32 (m, 1H), 4.44 (dd, J = 3.6 Hz, J = 4.4 Hz, 1H), 4.74 (m, 1H), 5.11 (d, J = 8.8 Hz, 1H), 6.05 (d, J = 5.6 Hz, 1H), 8.15 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (D_2O, 100 MHz): δ 62.75, 66.37 (d, J_{C-P} = 5.2 Hz), 68.18, 71.45, 71.62, 72.77, 73.73, 75.38, 78.05, 84.90 (d, J_{C-P} = 8.9 Hz), 88.00, 96.94, 119.69, 140.94, 150.16, 153.90, 156.64; ESI HRMS: m/z calcd for $C_{17}H_{26}N_5O_{16}P_2$ $[M-H]^-$ 618.0850, found 618.0845.

4.2.13. D, L-glycero-D-manno-heptose (3)

To a solution of **20** (114 mg, 0.2 mmol) in MeOH (4.0 mL) was added $Pd(OH)_2$ on carbon (100 mg). The reaction was stirred in H_2 atmosphere (50 psi) for 4 h. The reaction mixture was filtered and washed MeOH. The filtrate was concentrated to give a syrup **3** (38 mg, 90%). ESI HRMS: m/z calcd for $C_7H_{14}O_7Na$ $[M+Na]^+$ 233.0637, found 233.0637.

4.2.14. Benzyl 7-*O*-tetra-butyldiphenylsilyl-2,3,4-*O*-tri-benzyl-L-glycero-α-D-manno-heptopyranoside (21)

To a solution **16** (435 mg, 0.538 mmol), PPh_3 (283 mg, 1.08 mmol) and *p*-$NO_2C_6H_4COOH$ in THF (20 mL) was added DIAD (213 μL, 1.08 mmol). The reaction mixture was then stirred at room temperature for 5 h. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.36). The reaction mixture was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 15:1) to afford a syrup (474 mg). Then the solution of the syrup (474 mg, 0.49 mmol) and K_2CO_3 (82 mg, 0.59 mmol) in 11 mL MeOH/DCM (10:1) was stirred at room temperature for 4 h. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 4:1, R_f = 0.30). The reaction mixture was concentrated in vacuum, and then the crude was dissolved with ethyl acetate, and washed with water and brine. The organic layer was dried with Na_2SO_4, followed by filtration, the filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 5:1) to afford a syrup (351 mg, 81%, two steps). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.18 (s, 9H), 2.47 (d, J = 8.4 Hz, 1 H, -OH), 3.88 (dd, J = 7.6 Hz, J = 9.6 Hz, 1H), 3.95–3.98 (m, 2H), 4.02 (d, J = 9.6 Hz, 1H), 4.14 (dd, J = 1.2 Hz, J = 9.2 Hz, 1H), 4.21 (dd, J = 7.6 Hz, J = 14.4 Hz, 1H), 4.36 (t, J = 9.6 Hz, 1H), 4.48 (d, J = 12.0 Hz, 1H), 4.73–4.82 (m, 4H), 4.86–4.89 (m, 2H), 5.02 (s, 1H), 5.13 (d, J = 11.6 Hz, 1H), 7.27–7.29 (m, 2H), 7.37–7.53 (m, 24H), 7.78–7.79 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.13, 26.78, 64.58, 68.62, 69.43, 70.55, 72.25, 72.69, 74.40, 74.60, 75.13, 80.28, 96.97, 127.43, 127.51, 127.64, 127.74, 128.24, 128.30, 129.63, 133.18, 133.45, 135.42, 135.50, 136.87, 138.17,

138.51, 138.68; ESI HRMS: *m*/*z* calcd for $C_{51}H_{57}O_7Si$ $[M+H]^+$ 809.3868, found 809.3861.

4.2.15. Benzyl 7-*O*-tetra-butyldiphenylsilyl-2,3,4,6-*O*-tetra-benzyl-L-glycero-α-D-manno-heptopyranoside (22)

To a solution **21** (160 mg, 0.2 mmol), TBAI (3.7 mg, 0.01 mmol) and BnBr (47 μL, 0.4 mmol) in THF (10 mL) at 0 °C was slowly added NaH (60% dispersion in mineral oil, 16 mg, 0.4 mmol). The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.38). The reaction mixture was filtered, the filtrate was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 15:1) to afford a syrup **22** (164 mg, 91%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.19 (s, 9H), 3.95 (m, 1H), 4.10–4.16 (m, 5H), 4.33 (t, *J* = 9.6 Hz, 1H), 4.35–4.45 (m, 2H), 4.52 (d, *J* = 12.0 Hz, 1H), 4.72 (s, 2H), 4.76–4.86 (m, 4H), 5.02 (d, *J* = 11.6 Hz, 1H), 5.15 (s, 1H), 7.30–7.55 (m, 31H), 7.71–7.82 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.05, 26.77, 62.78, 68.48, 70.85, 71.95, 72.21, 72.56, 74.19, 74.24, 74.28, 76.75, 80.68, 96.46, 127.21, 127.24, 127.27, 127.34, 127.41, 127.62, 127.66, 127.72, 127.78, 128.13, 128.21, 128.25, 129.63, 129.67, 133.23, 133.33, 135.44, 135.59, 137.01, 138.21, 138.37, 138.48, 138.95; ESI HRMS: *m*/*z* calcd for $C_{58}H_{63}O_7Si$ $[M+H]^+$ 899.4338, found 899.4320.

4.2.16. Benzyl 2,3,4,6-*O*-tetra-benzyl-L-glycero-α-D-manno-heptopyranoside (23)

To a solution of **22** (158 mg, 0.176 mmol) in THF 10 mL was added 1 M TBAF in THF solution (352 μL, 0.352 mmol). The reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 2:1, R_f = 0.43). The reaction mixture was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 3:1) to afford a syrup **23** (107 mg, 92%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.39 (br s, 1H, –OH), 3.92–3.97 (m, 4H), 4.03–4.09 (m, 2H), 4.33 (t, *J* = 9.6 Hz, 1H), 4.50–4.55 (m, 2H), 4.64–4.71 (m, 3H), 4.75–4.79 (m, 3H), 4.87 (d, *J* = 11.6 Hz, 1H), 5.02 (d, *J* = 11.2 Hz, 1H), 5.09 (s, 1H), 7.28–7.46 (m, 25H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 62.29, 69.01, 71.92, 72.02, 72.39, 73.20, 74.33, 74.54, 75.93, 80.31, 97.04, 127.44, 127.59, 127.65, 127.75, 128.17, 128.24, 128.36, 136.99, 138.12, 138.19, 138.32, 138.47; ESI HRMS: *m*/*z* calcd for $C_{42}H_{44}O_7Na$ $[M+Na]^+$ 683.2979, found 683.2969.

4.2.17. Benzyl 7-*O*-dibenzyl-phosphono-2,3,4,6-*O*-tetra-benzyl-L-glycero-α-D-manno-heptopyranoside (24)

A mixture of compound **23** (108 mg, 0.163 mmol), 4 Å molecular sieves (200 mg) and 1*H*-tetrazole (1.8 mL, 0.45 M in CH_3CN, 0.815 mmol) in anhydrous DCM (10 mL) was stirred 30 min at room temperature. Then $(BnO)_2PN(i\text{-}Pr)_2$ (161 μL, 0.489 mmol) was added. The reaction mixture was stirred at room temperature for another 2 h. Subsequently, the reaction mixture was cooled −20 °C, *t*-BuOOH (163 μL, 5.0 M in decane, 0.815 mmol) was added, slowly warmed up room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 2:1, R_f = 0.36). The reaction was quenched by addition of 0.2 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel flash chromatography (hexane/ethyl acetate 4:1) to afford a syrup **24** (121 mg, 81%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.86–3.88 (m, 2H), 4.03 (dd, *J* = 2.8 Hz, *J* = 9.2 Hz, 1H), 4.15 (t, *J* = 6.8 Hz, 1H), 4.24–4.30 (m, 2H), 4.37–4.42 (m, 3H), 4.50 (d, *J* = 11.6 Hz, 1H), 4.63–4.70 (m, 3H), 4.75–4.78 (m, 3H), 4.96 (d, *J* = 11.2 Hz, 1H), 5.02–5.12 (m, 5H), 7.25–7.37 (m, 35H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 65.66 (d, J_{C-P} = 6.2 Hz), 68.86, 69.23 (d, J_{C-P} = 3.4 Hz), 69.29 (J_{C-P} = 3.1 Hz), 70.77, 71.91, 72.37, 72.78, 73.90, 74.16, 74.50, 74.83 (d, J_{C-P} = 8.7 Hz), 80.51, 96.86, 127.40, 127.44, 127.48, 127.51, 127.68, 127.74, 127.81, 127.84, 127.87, 128.19, 128.23, 128.25, 128.32, 128.45, 128.48, 135.60, 135.67, 137.61, 137.99, 138.14, 138.20, 138.63; ESI HRMS: *m*/*z* calcd for $C_{56}H_{58}O_{10}P$ $[M+H]^+$ 921.3762, found 921.3750.

4.2.18. 7-*O*-Phosphono-L-glycero-D-manno-heptopyranosyl phosphate (5)

To a solution of **24** (80 mg, 0.087 mmol) in MeOH (4.0 mL) was added $Pd(OH)_2$ on carbon (100 mg). The reaction was stirred in H_2 atmosphere (50 pis) for 4 h. The reaction mixture was filtered and washed with MeOH. The filtrate was concentrated to give a syrup **5** (22 mg, 90%). ^{1}H NMR (D_2O, 400 MHz): δ 3.30–3.34 (m, 1H), 3.73–3.81 (m, 2H), 3.87 (m, 1H), 3.94–4.00 (m, 2H), 4.10–4.16 (m, 1H), 5.13 (s, 1H); ^{13}C NMR (D_2O, 100 MHz): δ 65.23, 66.44 (d, J_{C-P} = 7.7 - Hz), 69.66 (d, J_{C-P} = 6.4 Hz), 70.30, 72.35, 73.26, 93.32; HRMS: *m*/*z* calcd for $C_7H_{14}O_{10}P$ $[M-H]^-$ 289.0330, found 289.0333.

4.2.19. Benzyl-7-*O*-tetra-butyldiphenylsilyl-6-*O*-(phenorythiocarbonyl)-2,3,4-*O*-tri-benzyl-D-glycero-α-D-manno-heptopyranoside (25)

To the mixture of compound **16** (380 mg, 0.47 mmol), DMAP (103.8 mg, 0.85 mmol) and anhydrous acetonitrile (8 mL) was added phenyl chlorothionoformate (98 μL, 0.71 mmol), and the solution was stirred at 60 °C for 5 h at Ar atmosphere. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 4:1, R_f = 0.46). Then the reaction mixture was cooled room temperature and filtered. The precipitate was washed with ethyl acetate. The filtrate was concentrated in vacuum, and purified by silica gel flash chromatography (hexane/ethyl acetate 15:1) to afford a syrup **25** (320 mg, 72%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.23 (s, 9H), 3.93 (m, 1H), 4.09 (d, *J* = 8.8 Hz, 1H), 4.17 (t, *J* = 9.6 Hz, 1H), 4.27–4.29 (m, 2H), 4.36 (dd, *J* = 8.0 Hz, *J* = 10.8 Hz, 1H), 4.53 (d, *J* = 12.0 Hz, 1H), 4.67 (d, *J* = 10.8 Hz, 1H), 4.73–4.77 (m, 3H), 4.82–4.85 (m, 2H), 4.98 (d, *J* = 10.8 Hz, 1H), 5.04 (s, 1H), 6.33 (m, 1H), 7.13–7.15 (m, 2H), 7.38–7.51 (m, 27H), 7.84–7.89 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.13, 26.74, 61.75, 68.83, 71.52, 72.16, 72.56, 74.83, 74.87, 74.96, 80.25, 84.51, 96.76, 121.91, 126.27, 127.51, 127.60, 127.63, 127.66, 127.76, 127.90, 127.94, 128.23, 128.36, 129.34, 129.54, 129.61, 129.64, 134.71, 135.57, 135.60, 136.92, 138.02, 138.16, 138.27, 153.39, 194.59; ESI HRMS: *m*/*z* calcd for $C_{58}H_{60}O_8SSiNa$ $[M+Na]^+$ 967.3670, found 967.3649.

4.2.20. Benzyl-7-*O*-tetra-butyldiphenylsilyl-2,3,4-*O*-tri-benzyl-6-deoxy-glycero-α-D-manno-heptopyranoside (26)

A solution of **25** (284 mg, 0.3 mmol), Tributylstannane (1.2 mL, 1.0 M in hexane, 1.2 mmol) and AIBN (24.6 mg, 0.15 mmol) in dry toluene (10 mL) was stirred at 60 °C for 4 h in Ar atmosphere. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.50). Then the reaction mixture was cooled room temperature and concentrated in vacuum, and purified by silica gel flash chromatography (hexane/ethyl acetate 20:1) to afford a syrup **26** (150 mg, 63%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.18 (s, 9H), 1.89–1.94 (m, 1H), 2.36–2.43 (m, 1H), 3.86 (t, *J* = 9.2 Hz, 1H), 3.97–3.99 (m, 2H), 4.07–4.13 (m, 3H), 4.52 (d, *J* = 12.0 Hz, 1H), 4.73–4.88 (m, 6H), 4.98 (s, 1H), 5.10 (d, *J* = 11.6 Hz, 1H), 7.39–7.50 (m, 25H), 7.80–7.84 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.14, 26.83, 35.01, 60.45, 68.41, 72.18, 72.70, 74.72, 75.08, 79.04, 80.38, 96.59, 127.55, 127.72, 127.76, 127.83, 128.25, 129.45, 129.54, 135.46, 135.52, 137.15, 138.19, 138.53, 138.64; ESI HRMS: *m*/*z* calcd for $C_{51}H_{57}O_6Si$ $[M+H]^+$ 793.3919, found 793.3896.

4.2.21. Benzyl 2,3,4,6-*O*-tri-benzyl-6-deoxy-glycero-α-D-manno-heptopyranoside (27)

To a solution of **26** (172 mg, 0.217 mmol) in THF 10 mL was added 1 M TBAF in THF solution (434 μL, 0.434 mmol). The

reaction mixture was then stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 4:1, R_f = 0.65). The reaction mixture was concentrated in vacuum and purified by silica gel column (hexane/ethyl acetate 8:1) to afford a syrup **27** (111 mg, 92%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.87–1.96 (m, 1H), 2.18–2.21 (m, 1H), 2.44 (br s, 1H), 3.82–3.96 (m, 5H), 4.01 (dd, J = 2.4 Hz, J = 9.2 Hz, 1H), 4.48 (d, J = 12.0 Hz, 1H), 4.68–4.83 (m, 6H), 4.92 (s, 1H), 5.03 (d, J = 11.2 Hz, 1H), 7.35–7.40 (m, 20H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 33.80, 60.77, 68.90, 71.43, 72.08, 72.78, 74.66, 75.26, 78.22, 80.07, 96.98, 127.49, 127.57, 127.62, 127.77, 127.97, 128.28, 128.37, 136.97, 138.06, 138.25, 138.31; ESI HRMS: m/z calcd for $C_{51}H_{57}O_7Si$ $[M+H]^+$ 555.2741, found 555.2738.

4.2.22. Benzyl-7-*O*-dibenzyl-phosphono-2,3,4-*O*-tri-benzyl-6-deoxy-glycero-α-D-manno-heptopyranoside (28)

A mixture of compound **27** (95 mg, 0.17 mmol), 4 Å molecular sieves (100 mg) and 1*H*-tetrazole (1.9 mL, 0.45 M in CH_3CN, 0.85 mmol) in anhydrous DCM (10 mL) was stirred 30 min at room temperature. Then $(BnO)_2PN(i\text{-}Pr)_2$ (168 μL, 0.51 mmol) was added. The reaction mixture was stirred at room temperature for another 2 h. Subsequently, the reaction mixture was cooled −20 °C, *t*-BuOOH (170 μL, 5.0 M in decane, 0.85 mmol) was added, slowly warmed up room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 2:1, R_f = 0.34). The reaction was quenched by addition of 0.2 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel flash chromatography (hexane/ethyl acetate 4:1) to afford a syrup **28** (114 mg, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.84–1.89 (m, 1H), 2.32–2.40 (m, 1H), 3.75 (t, J = 9.6 Hz, 1H), 3.83–3.87 (m, 2H), 3.98 (dd, J = 2.4 Hz, J = 9.2 Hz, 1H), 4.25–4.30 (m, 2H), 4.38 (d, J = 12.0 Hz, 1H), 4.66–4.80 (m, 6H), 4.88 (s, 1H), 4.98–5.04 (m, 5H), 7.28–7.40 (m, 30H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 32.54 (d, J_{C-P} = 7.4 Hz), 64.42 (d, J_{C-P} = 5.8 Hz), 67.89, 68.79, 69.17, 69.23, 72.25, 72.93, 74.68, 75.36, 78.67, 80.39, 96.99, 127.64, 127.71, 127.77, 127.78, 127.89, 127.93, 127.99, 128.07, 128.42, 128.44, 128.46, 128.56, 135.81, 135.96, 137.21, 138.19, 138.44, 138.47; ESI HRMS: m/z calcd for $C_{49}H_{52}O_9P$ $[M+H]^+$ 815.3344, found 815.3328.

4.2.23. 7-*O*-Phosphono-6-deoxy-glycero-D-manno-heptopyranosyl phosphate (6)

To a solution of **28** (100 mg, 0.123 mmol) in MeOH (4.0 mL) was added $Pd(OH)_2$ on carbon (100 mg). The reaction was stirred in H_2 atmosphere (50 psi) for 4 h. The reaction mixture was filtered and washed with MeOH. The filtrate was concentrated to give a syrup **6** (31 mg, 92%). ^{1}H NMR (D_2O, 400 MHz): 1.68–1.74 (m, 1H), 2.13–2.20 (m, 1H), 3.31–3.38 (m, 1H), 3.42–3.47 (m, 1H), 3.72–3.86 (m, 2H), 3.99–4.04 (m, 2H), 5.05 (s, 1H); ^{13}C NMR (D_2O, 100 MHz): δ 31.60 (d, J_{C-P} = 7.4 Hz), 62.96 (d, J_{C-P} = 5.2 Hz), 68.12, 70.36, 71.09, 72.93, 93.95; HRMS: m/z calcd for $C_7H_{14}O_9P$ $[M-H]^-$ 273.0381, found 273.0380.

4.2.24. Benzyl 7-*O*-sulfo-2,3,4,6-*O*-tetra-benzyl-D-glycero-α-D-manno-heptopyranoside (29)

A solution of **18** (186 mg, 0.28 mmol) in 8.0 mL pyridine was added SO_3·Pry (89 mg, 0.56 mmol), and the reaction was stirred at room temperature for 6 h. TLC analysis showed complete conversion of starting material to a major product (methanol/ethyl acetate 1:10, R_f = 0.20). Then the reaction was neutralized with $Na_2CO_{3(aq)}$ (1.0 M, 8.0 mL) and concentrated under reduced pressure. The salts were washed with 40 mL methanol/dichloromethane 1:1, and filtered. The filtrate was concentrated under in vacuum and purified by silica gel flash chromatography (methanol/ethyl acetate 1:20) to afford a syrup **29** (190 mg, 89%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.73–3.76 (m, 2H), 3.90–4.08 (m, 4H), 4.39 (d, J = 11.6 Hz, 1H), 4.50–4.56 (m, 5H), 4.62–4.80 (m, 5H), 5.06 (s, 1H), 7.11–7.32 (m, 25H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 68.96, 71.97, 72.12, 72.53, 73.61, 74.55, 74.76, 80.20, 97.04, 127.43, 127.66, 127.85, 128.06, 128.21, 128.24, 128.35, 137.38, 137.76, 138.23; HRMS: m/z calcd for $C_{42}H_{43}O_{10}S$ $[M-H]^-$ 739.2582, found 739.2561.

4.2.25. 7-*O*-Sulfo-D-glycero-D-manno-heptopyranoside (7)

To a solution of **29** (150 mg, 0.2 mmol) in 6.0 mL *t*-BuOH/H_2O (5:1) was added $Pd(OH)_2$/C on carbon (100 mg). The reaction was stirred in H_2 atmosphere (50 psi) for 4 h. The reaction mixture was filtered and washed with *t*-BuOH/H_2O (1:2). The filtrate was concentrated to give a syrup **7** (54 mg, 92%). ^{1}H NMR (D_2O, 400 MHz): δ 3.67 (dd, J = 5.2 Hz, J = 10.8 Hz, 1H), 3.80–3.89 (m, 2H), 3.94 (t, J = 8.4 Hz, 1H), 4.16–4.17 (m, 2H), 4.25–4.26 (m, 1H), 5.18 (s, 1H); ^{13}C NMR (D_2O, 100 MHz): δ 68.30, 68.63, 68.96, 71.19, 72.32, 74.92, 93.14. ESI HRMS: m/z calcd for $C_7H_{13}O_{10}S$ $[M-H]^-$ 289.0235, found 289.0238.

4.3. HldE assays for D, D-heptose-7-phosphate analogs

A mixture containing 10 mM D, D-heptose-7-phosphate **2** and its analogues (**3**,**4**,**5**,**6**,**7**), 15 mM ATP, 10 mM $MgCl_2$ and 1 mg/mL HldE in 20 mM Tris–HCl buffer (pH 8.0), and the pH of the solution was adjusted to 8.0 by the addition of 1 M NaOH. After incubation at 37 °C for 12 h, the mixture was briefly boiled and centrifuged to remove protein. The supernatant was subjected to analysis by ESI HRMS.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.019.

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