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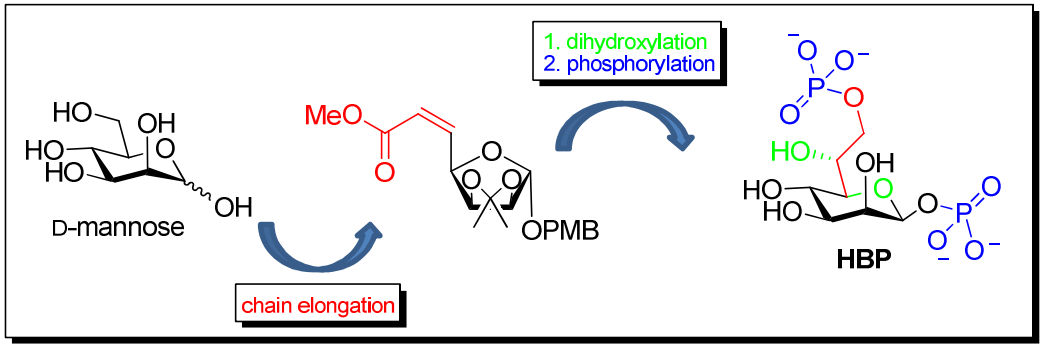

Alternate synthesis to D-glycero-β-D-manno-heptose 1,7-biphosphate

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Abstract:

D-glycero-β-D-manno-heptose 1,7-biphosphate (HBP) is an enzymatic intermediate in the biosynthesis of the heptose component of lipopolysaccharide (LPS), and was recently revealed to be a pathogen-associated molecular pattern (PAMP) that allows detection of Gram-negative bacteria by the mammalian immune system. Cellular detection of HBP depends upon its stimulation of a cascade that leads to the phosphorylation and assembly of the TRAF-interacting with forkhead-associated domain protein A (TIFA), which activates the transcription factor NF-κB. In this note, an alternate chemical synthesis of HBP is described and its biological activity is established, providing pure material for further assessing and exploiting the biological activity of this compound.

Keywords:

D-glycero-β-D-manno-heptose 1,7-biphosphate (HBP), nuclear factor-kappa B (NF-κB), pathogen-associated molecular pattern (PAMP), Wittig reaction, Traf-interacting with forkhead-associated domain protein A (TIFA)

1. Introduction

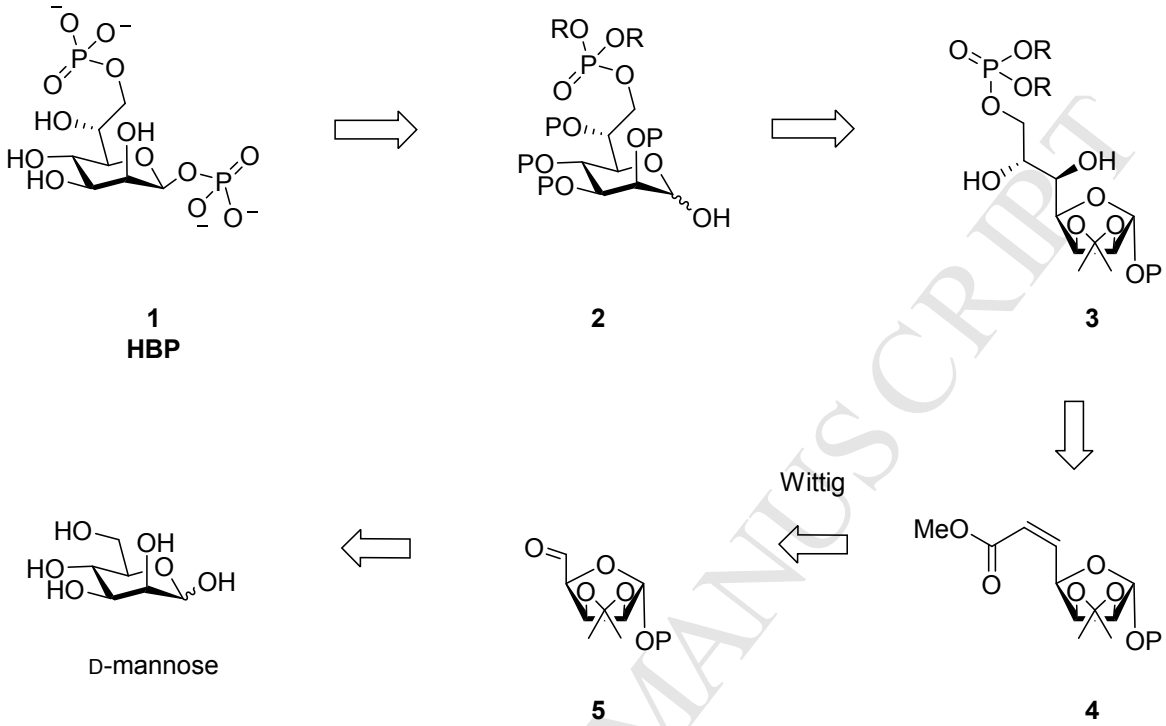
Bacterial-liberated D-glycero-β-D-manno-heptose 1,7-biphosphate (**HBP**) was revealed to function as a pathogen-associated molecular pattern (PAMP) by Gaudet *et al.*[1] Once transported into the mammalian cell cytoplasm, it promotes the alpha kinase 1 (ALPK1)-dependent phosphorylation and oligomerization of the TRAF-interacting forkhead-associated domain-containing protein A (TIFA), which leads to nuclear translocation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[1,2,3,4] Given the broad function of NF-κB in immune activation, this discovery may be exploited for a broad variety of

activities, ranging from the therapeutic stimulation of HIV from latency[3] to immune modulation for treatment of inflammatory diseases or cancer. A pure and plentiful supply of HBP would facilitate these developments, leading to this development of a robust synthesis of HBP for further biological testing.

Apart from the enzymatic biosynthesis of **HBP**,[5] there has been two reports of its chemical synthesis.[6,7] There has, however, been much research regarding D-glycero-D-heptose synthesis as well as the phosphorylated heptoses. A well-established method involves elongating a mannofuranose derivative using a Wittig methodology and performing an osmoylation to obtain the C-5, C-6 new stereocenters.[8,9,10,11,12,13] The isomerisation at C-6 from L-glycero-D-manno-heptose to D-glycero-D-manno-heptose provides a possible avenue to the synthesis of HBP and was recently reported in a 16-step synthesis with a 1.2% overall yield.[6] Another publication regarding the synthesis of HBP started from peracetylated D-glycero-D-manno-heptose, with an 11-step synthesis and overall yield of 9.7%.[7]

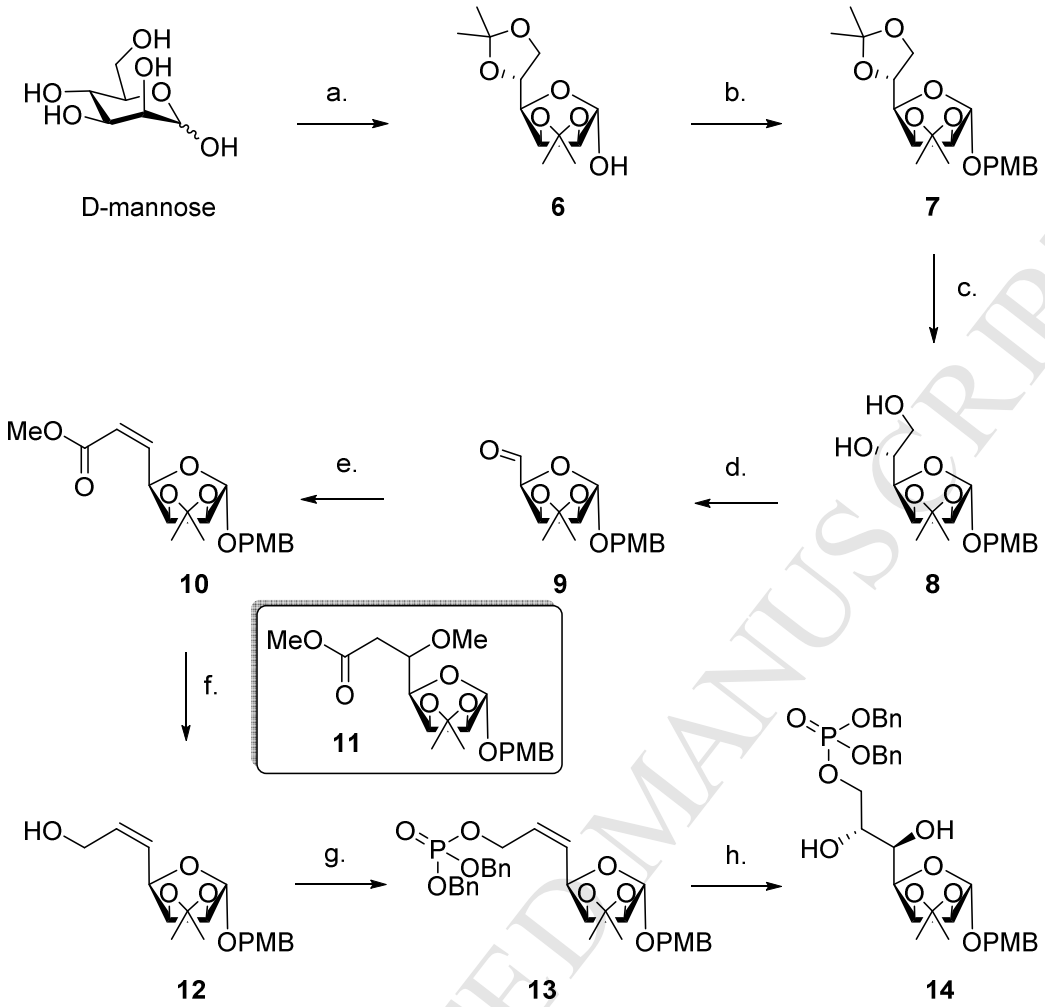
As Inuke *et al.* and Borio *et al.* [6,7] discussed, β-anomers are often regarded as being challenging to prepare, and reports of mannose phosphate β-anomers are sparse. Inuke *et al*.[6] developed a method utilizing dibenzylphosphate for this purpose, whereas another option for β-phosphorylation are chloro-phosphates.[7, 14, 15, 16, 17] In this note, an alternate synthesis to D-glycero-β-D-manno-heptose 1,7-biphosphate is described.

2. Results and discussion:



Scheme 1: Retrosynthesis of **HBP, 1**.

The retrosynthesis of **HBP** is described in Scheme 1. Here, **HBP** could be generated after phosphorylation and deprotection of intermediate **2**, this intermediate could then be made from furanose **3** after deprotection. Intermediate **3**, could be obtained from alkene **4** after subsequent ester reduction, osmoylation and phosphorylation. Elongated alkene **4** could be obtained after a Wittig reaction from aldehyde **5**. Aldehyde **5** could be obtained from D-mannose after protecting group manipulation, deprotection of the acetonide on 5ˈand 6ˈand sodium periodate cleavage.



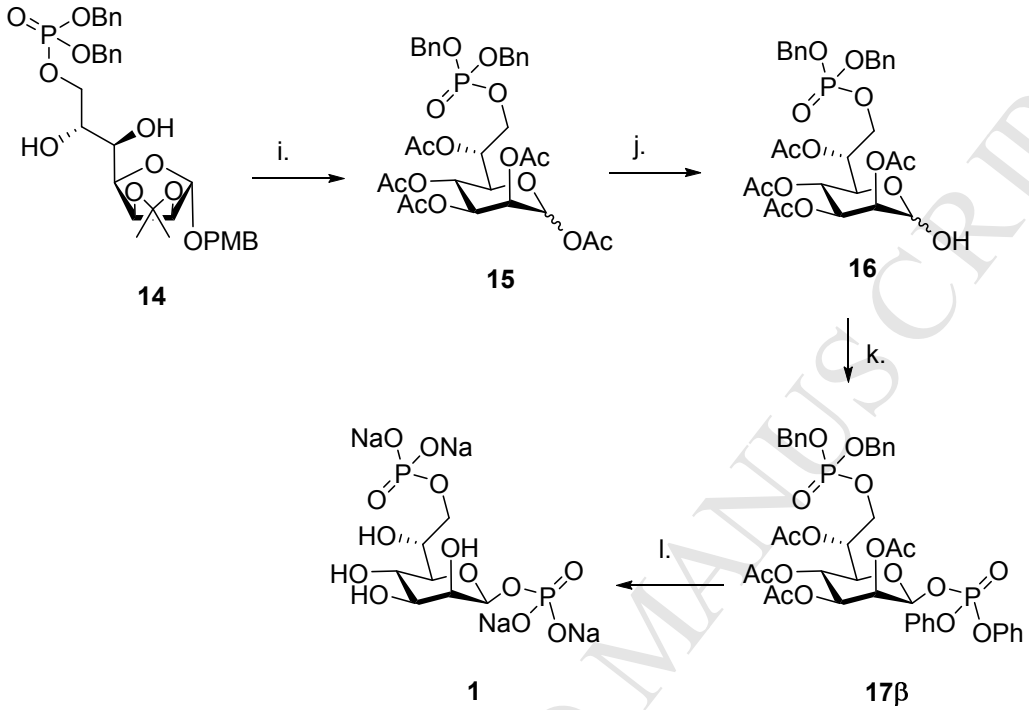
Scheme 2. Synthesis of phosphorylated derivative **14.** a. Acetone/$FeCl_3$, 94% b. NaH, PMBCl, DMF, 78% c. Acetic acid/water , 96% d. $NaIO_4$, Acetone, Water e. $Ph_3PCHCOOMe$, toluene, 60% over two steps f. DIBAL, DCM, 81% g. Tetrazole, Acetonitrile, Dibenzyl N,N-dimethylphosphoramidite, then *t*BuOOH, 67% h. OsO_4, NMMO, Dioxane, Water, 70%.

Diacetonide **6** was formed from D-mannose such as in Güzlek *et al.* and Brimacombe *et al.*[10,11,12] A benzyl group was first used as anomeric protecting group. The following steps including the 5',6' acetonide deprotection, oxidation, Wittig reaction and reduction of the ester went as reported by Brimacombe *et al.*. The phosphorylation using diphenylphosphoryl chloride was

also successful, however, hydrolysis of the phenyls in the oxidation step caused lesser yields. Another avenue which involved the protection of the C-7 hydroxyl with a TDBPS protecting group was thus attempted. This avenue was successful, however the challenge laid in deprotecting the acetonide and the benzyl group. At first, different hydrogenation catalysts were used such as Pd/C, $Pd(OH)_2$, $PdCl_2$ with or without acetic acid in presence of different solvents such as MeOH, EtOH, Ethyl acetate to no avail. Unsuccessfully, an acidic cleavage was attempted at different temperatures with different acids such as acetic acid, *p*TsOH, TFA and different acid concentrations.

It was theorised that a PMB group would be easier to cleave than a benzyl, intermediate **7** (Scheme 2) was synthesised. The deprotection of the acetonide (yield = 96%) and periodate cleavage generated satisfying yields, however, surprisingly ester **10** was obtained in only 40% yield over two steps. This low yield was caused by partial addition of methanol on the C-5 carbon preferentially to form intermediate **11**. NMR convincingly showed an additional methoxy group without any traces of characteristic alkene signals at 6 ppm in the proton NMR spectra, additionally a correlation in the HMBC spectra from H-5' to one of the methoxy carbon was key in determining that it was linked at C-5'. Mass-spectrometry data also agreed with this identification (m/z Calcd for $C_{20}H_{32}NO_8$= 414.2 found=414.0). Using toluene, improved the yield slightly over two steps (60%) and the product was reduced to form alcohol **12** (81% yield). After the phosphorylation of alcohol **12** (67% yield), the osmoylation yielded the expected diol **14** in a 92% yield. A mixture of the D-glycero-α- D-manno-heptofuranose and L-glycero-β-L-gulo-heptofuranose was obtained (3:1) and further purified with 70% yield. The new

stereocenters at C-5' and C-6' were assigned with confidence after comparing **14** with the benzylated intermediate described by Güzlek.[12]

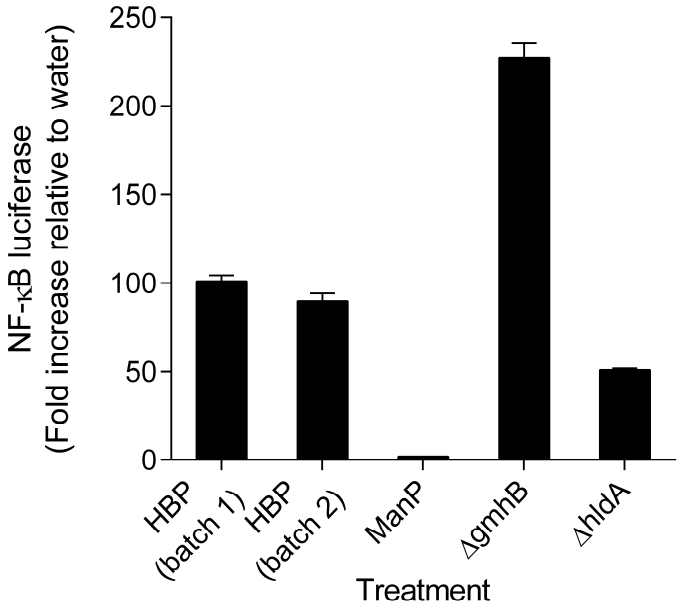


Scheme 3. Synthesis of D-glycero-β-D-manno-heptose 1,7-biphosphate i. TFA/Water/DCM then Acetic anhydride/Pyridine in DMF, 85%, j. DIPEA, Ammonium acetate in DMF, quant. k. DMAP, diphenyl phosphoryl chloride in DCM, 77% l. H_2, PtO_2 in MeOH then H_2, Pd/C in MeOH then Et_3N/Water/MeOH, 78% then NaCl treatment and desalting.

A two step one pot deprotection of acetonide and PMB protecting group followed by acetylation afforded peracetylated **15** in 85% yield over two steps (Scheme 3). The deacylation of the anomeric acetate was performed under slightly basic conditions, and yielded to intermediate **16** in quantitative yields. Phosphorylation afforded anomer **17**β in a 1:4 (α:β) ratio (77% yield). Key in this step is to add DMAP sparingly as well as adding the diphenyl phosphoryl chloride dropwise. Should one wish to obtain the alpha anomer preferentially, it is possible to add 5

equivalents of DMAP and introduce the phosphoryl chloride at a quicker rate. Both compounds **16** and **17β** NMR data were in accordance with previously published data.[7] Global deprotection of the purified β-anomer afforded **HBP, 1** in a 78% yield and an overall yield of 8.2% with 15 steps. For a representative evaluation of **HBP** biological function, endotoxin free water was used for the last purification step. It was also reported that 293T cells are refractory to LPS.[1]

The biological function of synthetic **HBP** was assessed based upon its ability to activate NF-κB in human cells, using the non-activating β-D-mannose phosphate as a negative control. In a first experiment, **HBP** was shown to activate an NF-κB-driven luciferase reporter construct in human embryonic kidney-derived (HEK 293) cells (Figure 1). In another experiment, the inflammatory cytokine IL-8 was produced by human colonic epithelial (HCT 116) cells exposed to **HBP** (Figure 2). Both batches of HBP induced a similar response in both assays, highlighting the reproducibility of our synthesis. Furthermore, the activity was comparable to that of our positive control – bacterial supernatant from a genetic mutant of *Neisseria meningitidis* that naturally expresses **HBP** (*ΔgmhB*), and was greater than supernatant from a mutant of *N. meningitidis* that does not produce **HBP** (*ΔhldA*).



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128 Figure 1. Effect of synthetic or naturally-produced HBP preparations on HEK 293T cells

129 encoding an NF-κB-driven luciferase reporter gene.

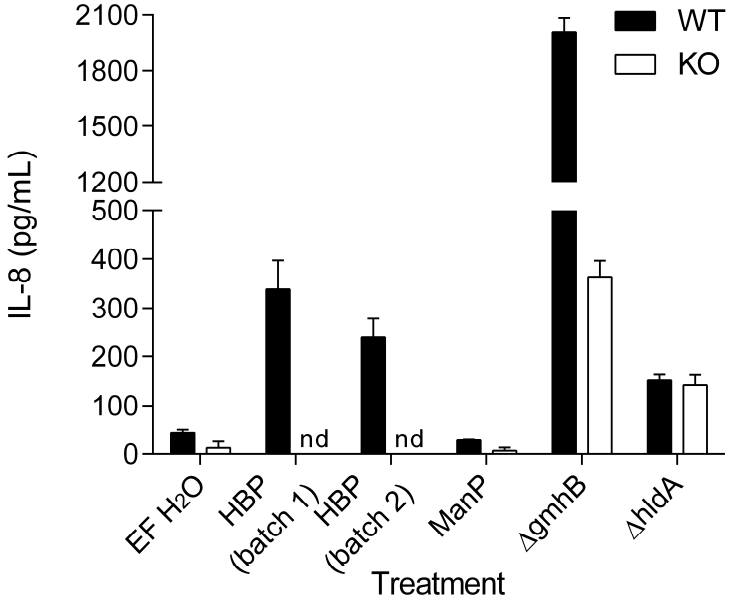

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Figure 2. Inflammatory response of human colonic epithelial cells exposed to synthetic or naturally-produced HBP preparations. EF H_2O, endotoxin-free water (solvent); nd, not detected. KO; TIFA knockout

3. Conclusion

In conclusion, we describe herein a method to obtain D-glycero-β-D-manno-heptose 1,7-biphosphate in 8.7% overall yield and 15 steps. In this synthesis, mannose is elongated using the Wittig methodology where an alkene is formed. After reduction of the ester and phosphorylation, oxidation of the alkene leads us to a protected furanose form of D-glycero-D-manno-heptose. HBP thus formed was tested for its activity and shown to activate NF-κB in HEK 293 cells and induce the TIFA- and NF-κB-dependent production of IL-8 in HCT 116 cells. This method is robust and will be useful to further determine how this molecule can be exploited for a variety of biological applications.

4. Experimental

4.1 General

All chemicals were purchased from Aldrich, Fisher Scientific or Alpha Aesar. They were used without further purification. Proton and Carbon NMR spectra were measured on either a Varian (^{1}H, 500 MHz), Bruker (^{1}H, 600 MHz, ^{13}C 150 MHz, ^{31}P 242 MHz) or Bruker (^{1}H, 400 MHz, ^{13}C 100 MHz, ^{31}P 162 MHz) spectrometer reported with the solvent residual signal ($CDCl_3$, 7.26 ppm for ^{1}H and 77.36 ppm for ^{13}C, D_2O, 4.79 ppm for ^{1}H and externally with dioxane (67.19 ppm) for ^{13}C). Compounds were purified using a CombiFlash RF system and RediSep RF silica columns. MS data were recorded on a SQ2 from waters and HRMS data were recorded on a Ultima from Waters with the LC/MS Calibrant Mix from Agilent as internal standard.

154 4.2 Biological Assays

155 Synthetic compounds were resuspended in endotoxin-free water (EF H_2O; Sigma, Oakville,
156 Ontario, Canada). HCT 116 cells were maintained in McCoy's 5A medium and HEK 293T cells
157 were maintained in a DMEM (Dulbecco's Modified Eagle's medium) buffer. All media was
158 supplemented with 10% FBS and 1% glutamax. *Neisseria meningitidis* supernatants were
159 purified as previously described.[1]

160 HEK 293T cells were transfected with a plasmid encoding an NF-κB-driven luciferase reporter.
161 After 24 hours, cells were stimulated for 20 minutes in permeabilization buffer (5 µg/mL
162 digitonin) in the presence of culture supernatant from *N. meningitidis* mutants with (*ΔgmhB*) or
163 without (*ΔhldA*) HBP, or with 20 µg/mL of synthetic compounds. Treatment was removed, and
164 cells were washed and incubated for 3.5 hours in complete medium Luciferase activity
165 determined using the Dual-Glo Luciferase Assay System (Promega) as previously described[1] and
166 luminescence was measured using a luminometer (Cytation 5). Results are expressed as fold
167 increase relative to transfected, mock-treated cells. The results are means ± standard error mean
168 of technical triplicates.

169 HCT 116 that were either wild type (WT) or deficient in TIFA protein expression (knockout,
170 KO)[18] were stimulated for 20 minutes in permeabilization buffer (5 µg/mL digitonin) in the
171 presence of culture supernatant from *N. meningitidis* mutants with (*ΔgmhB*) or without (*ΔhldA*)
172 HBP, or with 10 µg/mL of synthetic compound. Treatment was removed, and cells were washed,
173 then incubated for 6 hours in complete media. Quantitative measurement of IL-8 levels in the
174 culture supernatant was then performed using an ELISA kit from BD Biosciences. The results
175 are means ± standard error mean of technical triplicates.

4.3 Synthesis

4.3.1 1,2,3,4,6-penta-*O*-acetyl-7-*O*-[bis(benzyloxy)phosphoryl]-D-*glycero*- D-*manno*-heptopyranose) (**15**).

Compound **14** (120 mg, 190 µmol) was stirred with DCM (5 mL) and water (1 mL) at 0°C. TFA (5 mL) was then added and the reaction stirred for 1 hour. After few minutes a pink shade appears, this transforms slowly into a purple shade as the reaction warms up to RT and observed to be to completion on TLC (*Rf* = 0.14, ethyl acetate:hexane, 1:1, v:v). Concentration *in vacuo* of the reaction mixture and co-evaporation with toluene (6 times) gave a yellow oil. The mixture was dissolved in anhydrous DMF (200 µL), then anhydrous pyridine (1 mL) and acetic anhydride (1 mL) were added and stirred for 16 hours at RT. DCM was added and washed with $NaHCO_3$ (sat. aq.) until a neutral pH was reached and then washed with brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*, flash chromatography afforded compound **15** (1:0.6, α:β) in 85% yield (110 mg, 162 µmol). ^{1}H NMR (600 MHz, $CDCl_3$): δ= 7.4-7.3 (m, 10H, Bn), 6.03 (d, $J_{H1'\alpha,H2'\alpha}$ = 1.9Hz, 1H, H1'α), 5.80 (d, $J_{H1'\beta,H2'\beta}$ = 1.4Hz, 1H, H1'β), 5.41 (m, 1H, H2'β), 5.33-5.26 (m, 2H, H4'α and H3'α), 5.23-5.17 (m, 3H, H4' β, H6'β, H2'α), 5.13 (m, 1H, H6'α), 5.07-4.96 (m, 5H, CH_2Bn, H3'β), 4.34-4.16 (m, 2H, H7'α and β), 4.08 ($J_{H5'\alpha,H4'\alpha}$ = 9.0Hz, $J_{H5'\alpha,H6'\alpha}$ = 4.1Hz, dd, 1H, H5'α), 3.79 ($J_{H5'\beta,H4'\beta}$ = 9.1Hz, $J_{H5'\beta,H6'\beta}$ = 4.1Hz, dd, 1H, H5'β), 2.15-1.94 (m, 15H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$): δ= 170.33, 170.19, 170.10, 170.0, 169.9, 168.5, 168.3 (CH_3C(O)), 136.1, 136.0, 135.9 (CCH_2O, Bn), 128.9, 128.8 (CBn), 128.2, 128.1, 128.0 (CHBn), 90.3 (C1'α and β), 74.0 (C5'β), 71.4 (d, $J_{C6'\alpha,P}$ = 7.9 Hz, C6'α), 71.2 (C5'α), 71.1 (C6'β), 70.4 (C3'β), 69.7 and 69.6 (CH_2Ph), 69.0 (C3'α), 68.3 (C2'α), 67.8 (C2'β), 66.8 (C4'α), 66.3 (C4'β), 65.0 (d, $J_{C7,P}$ = 5.2 Hz, C7'), 21.1, 21.0, 20.9, 20.8 (CH_3C(O)); ESI-MS: *m/z* Calcd for $C_{31}H_{37}NaO_{15}P$ $[M+Na]^+$, 703.17; found, 703.17.

4.3.2 2,3,4,6-tetra-*O*-acetyl-7-*O*-[bis(benzyloxy)phosphoryl]- D -*glycero*- D-*manno*-heptopyranose (**16**).

Compound **15** (50 mg, 78 µmol) was dissolved into DMF (5 mL) and to this was added diisopropylethylamine (1 mL) and ammonium acetate (200 mg) under a nitrogen atmosphere. The reaction was stirred for 16 hours at RT after which TLC monitoring showed that the reaction was to completion (*Rf* = 0.8, ethyl acetate:hexane, 1:1, v:v). The reaction mixture was diluted with DCM, washed with $NaHCO_3$ (sat. aq.) and water. The DCM fraction was then dried with Na_2SO_4, filtered and concentrated *in vacuo*. Column chromatography afforded title compound **16** in quantitative yield (47 mg, 74 µmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ= 7.4-7.3 (m, 10H, Bn), 5.45 ($J_{H3',H2'}$ = 3.5Hz, $J_{H3',H4'}$ = 9. Hz, dd, 1H, H3'), 5.26 ($J_{H2',H1'}$ = 1.7Hz, $J_{H2',H3'}$ = 3.5Hz, dd, 1H, H2'), 5.22 ($J_{H4',H3'}$ = 9.8 Hz, $J_{H4',H5'}$ = 9.8 Hz, dd (apt), 1H, H4'), 5.12 (d, $J_{H1',H2'}$ = 1.4Hz, 1H, H1'), 5.06-5.00 (m, 5H, C$\underline{H}_2$Bn and H6'), 4.48 and 4.09 (m, 1H, H7'), 4.29 ($J_{H5',H6'}$ = 7.4Hz, $J_{H5',H4'}$ = 9.8Hz, dd, 1H, H5'), 2.11 (s, 3H, C$\underline{H}_3$COOCH2'), 2.03 (s, 3H, C$\underline{H}_3$COOCH4'), 2.01 and 2.00 (s, 3H, C$\underline{H}_3$COOCH3' and C$\underline{H}_3$COOCH6'). ^{13}C NMR (100 MHz, $CDCl_3$) δ= 170.3 (x2), 170.1 (x2) (C(O)), 135.8 (x2), 135.7 (x2) (CBn), 129.1, 129.0, 128.9 (x2) (CHBn), 92.7 (C1'), 73.1 ($J_{C6',P}$ = 5.8 Hz, d, C6'), 70.3 (C2'), 70.3-70.0 ($\underline{C}H_2$Bn), 69.3 (C3'), 68.7 (C4'), 66.1 (C5'), 65.7 ($J_{C6',P}$ = 5.7 Hz, C7'), 21.2-20.9 ($\underline{C}H_3$). ^{31}P NMR (161 MHz, $CDCl_3$) δ -1.0; HRMS *m/z* Calcd for $C_{29}H_{35}NaO_{14}P$ $[M+Na]^+$ 661.1662, found 661.1683.

4.3.3 Diphenyl (2,3,4,6-tetra-*O*-acetyl-[7-*O*-(bis[benzyloxy]phosphoryl)-D-glycero-β-D-manno-heptopyranosyl) phosphate (**17β**).

Compound **16** (29.3 mg, 43 µmol) was coevaporated using toluene and dried under vacuum overnight. After 16 hours, **16** was dissolved in anhydrous DCM (1 mL) and DMAP (5 mg, 43

µmol) was added. Phosphoryl chloride (90 µL, 430 µmol) was also coevaporated with toluene and was dissolved into DCM (1 mL) and using a syringe pump, dropped at a rate of 0.5 mL/h over 2 hours under N_2, the reaction was to completion after 5 hours. DCM was then added and the organic layer was washed with TEAB buffer (until a basic pH was reached), water and brine. The organic layer was then dried with Na_2SO_4, filtered and concentrated under reduced pressure. Flash chromatography using hexane: diethyl ether gave title compound **17β** in 77% yield (30 mg, 34 µmol, α:β, 1:4). ^{1}H NMR, (400 MHz, $CDCl_3$) δ= 7.4-7.1 (m, 2OH, Bn and Ph), 5.67 ($J_{H1',H2'}$ = 1.7Hz, $J_{H1',P}$ = 7.5Hz, dd, 1H, H1'), 5.40 ($J_{H2',H1'}$ = 1.7Hz, $J_{H2',H3'}$ = 3.1Hz, dd, 1H, H2'), 5.31 (m, 1H, H6'), 5.21 ($J_{H4',H3'}$ = $J_{H4',H5'}$ = 7.9Hz, dd (apt), 1H, H4'), 5.08 ($J_{H3',H2'}$ = 3.1Hz, $J_{H3',H4'}$ = 7.9Hz, dd, 1H, H3'), 5.08-5.01 (m, 4H, C$\underline{H_2}$Bn), 3.89 ($J_{H5',H6'}$ = 5.8Hz, $J_{H5',H4'}$ = 7.3Hz, dd, 1H, H5'), 4.24 (m, 2H, H7'), 2.06, 2.02, 2.00, 1.98 (CH_3). ^{13}C NMR (100MHz, $CDCl_3$) δ= 170.0, 169.9, 169.8, 169.7 (C(O)CH_3), 150.5, 150.4, 150.3, 150.2 (C, Ph and Bn), 136.0, 135.9, 135.9, 135.8 (CH, Ph and Bn), 130.14, 130.10 (CH, OBn), 128.8 (m, CH, OBn), 128.12 and 128.09 (CH, OPh), 126.0 and 128.9 (CH, OPh), 120.34 (m, CH, OPh), 94.84 ($^2J_{31P,C1'}$ = 4.6Hz, C1'), 73.3 (C5'), 70.7 ($^3J_{31P,C6'}$, = 7.2Hz, C6'), 69.5 (m, $\underline{C}H_2$Bn), 69.1 (C3'), 67.2 ($^3J_{31P,C2'}$, = 7.3Hz, C2'), 66.2 (C4'), 64.9 ($^2J_{31P,C7'}$ = 5.7Hz, C7'), 20.8, 20.7, 20.5 ($\underline{C}H_3$). ^{31}P NMR (161 MHz, $CDCl_3$) δ -0.36 and -13.27. HRMS *m/z* Calcd for $C_{41}H_{44}NaO_{17}P_2$ $[M+Na]^+$ 893.1951, found 893.1942.

4.3.4 D-*glycero*-D-*manno*-heptopyranose 1β,7-biphosphate (**1**, **HBP-β**).

Compound **17β** (6 mg, 6.9 µmol) was stirred in anhydrous methanol at RT with PtO_2 under balloon pressure for 48 hours. When activated, the brown PtO_2 turns to black. After filtration over celite and concentration, the compound was again dissolved in anhydrous methanol and Pd/C was added. After 48 hours of stirring under a H_2 atmosphere, filtration over celite and

concentration under reduced pressure, the compound was dissolved into methanol:endotoxin free water:triethylamine (7:3:1, v:v:v) at pH 11 for 4 hours after which it was concentrated and freeze dried over water. Purification using endotoxin free water on a desalting column (G-10) and exchanging the triethylamine ions for sodium ions gave compound **1, HBP-β** in 78% yield (2 mg, 5.4 µmol). NMR assignments are in agreement with literature data.[5,6] HRMS *m/z* Calcd for $C_7H_{16}NaO_{13}P_2$, 392.9964, found, 392.9941.

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Declaration of interest:

The authors, with the exception of Milan Basin, Itunuoluwa A. Adekoya and Cynthia X. Guo, have filed patents concerning the therapeutic potential of HBP.

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Highlights:

- Alternate synthesis to D-glycero-D-manno-heptose 1,7-diphosphate
- The synthesis starts from mannose and uses Wittig methodology and osmoylation as means to obtain D-glycero-D-manno heptose
- Cell-based assays validate that synthetic D-glycero-D-manno-heptose 1,7-diphosphate stimulates Nf-κB through a TIFA-dependent pathway