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Chemical synthesis of the innate immune modulator – bacterial D-*glycero*-β-D-*manno*-heptose-1,7-bisphosphate (HBP)

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ABSTRACT

The bacterial metabolite and potent innate immune modulator D-*glycero*-β-D-*manno*-heptose-1,7-bisphosphate (HBP) and its α-configured counterpart D-*glycero*-α-D-*manno*-heptose-1,7-bisphosphate were synthesized via stereoselective anomeric phosphorylation of the peracetylated D,D-heptose 7-dibenzylphosphate by exploiting different nucleophilicity of equatorial and axial lactols in the D-*manno*-series. We also report a novel approach for anomeric phosphorylation using modified Mitsunobu reaction conditions and provide the first full structural characterization of HBP. The first chemical synthesis of HBP offers access to an anomerically pure structurally defined probe for biological studies and to a lead compound operating as a powerful stimulator of intracellular signaling for possible therapeutic immunomodulation.

Introduction

The innate immune response is the "first line of defense" against microbial challenge and is engaged to recognize highly conserved pathogen motifs, the so-called Pathogen Associated Molecular Patterns (PAMPs) which are typically represented by microbial surface antigens. For example, lipopolysaccharide (LPS), a major constituent of the outer membrane of the cell-wall of Gram-negative bacteria, can potently stimulate the mammalian innate immune system *via* interaction with a transmembrane Toll-like Receptor 4 (TLR4) complex which results in the triggering of different intracellular signaling pathways leading to production of pro-inflammatory mediators (cytokines and interleukins).[1–3]

In addition, a number of severe inflammatory effects are induced by LPS-mediated activation of an atypical inflammasome, a protein complex that is assembled in the cytosol of macrophages in response to extracellular stimuli. Inflammasome promotes the processing and secretion of inflammatory cytokines of the interleukin-1 (IL-1) family which are critical for anti-bacterial defense.[4,5] The assembly and activation of the nucleotide oligomerization domain-like (NOD) receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome constitutes a principal mechanism by which persistent inflammatory stress leads to induction of the IL-1β pathway and programmed cell death by pyroptosis. The downstream signaling induced by NLRP3 inflammasome is associated with a number of acute and chronic inflammatory disorders such as sepsis syndrome, diabetes, atherosclerosis and Alzheimer disease related pathology.[6–8] It has been recently shown that NLRP3 inflammasome can be assembled and efficiently activated by TNF-α receptor-associated factor-interacting protein with a forkhead-associated domain (TIFA).[9]

Recent reports disclosed that TIFA-dependent NFκB activation resulting in the expression of cytokines is induced by the Gram-negative bacterial metabolite D-*glycero*-β-D-*manno*-heptose 1,7-bisphosphate (HBP).[10,11] *Glycero-manno*-heptose, a seven carbon sugar of purely nonself origin, is an integral component of the inner core region of LPS.[12] Heptoses can be also sometimes found in LPS outer core regions, in O-antigens and as constituents of capsular polysaccharides and S-layer glycoproteins.[13] The α-anomer of HBP, D-*glycero*-α-D-*manno*-heptose 1,7-bisphosphate, is formed as an intermediate in the GDP-D-*glycero*-α-D-*manno*-heptose pathway during S-layer glycoprotein and capsular polysaccharide biosynthesis, whereas its counterpart having an anomeric phosphate group in the β-configuration, D-*glycero*-β-D-*manno*-heptose 1,7-bisphosphate, is generated during the ADP-L-*glycero*-β-D-*manno*-heptose biosynthetic pathway involved in the assembly of LPS.[14–16]

HBP is currently available only in limited quantities as a heterogeneous enzymatic preparation which is poorly characterized.[17] In order to provide well-defined and pure material for biological studies and to ascertain a reliable approach to HBP as an immuno-modulator for potential pharmaceutical application, we

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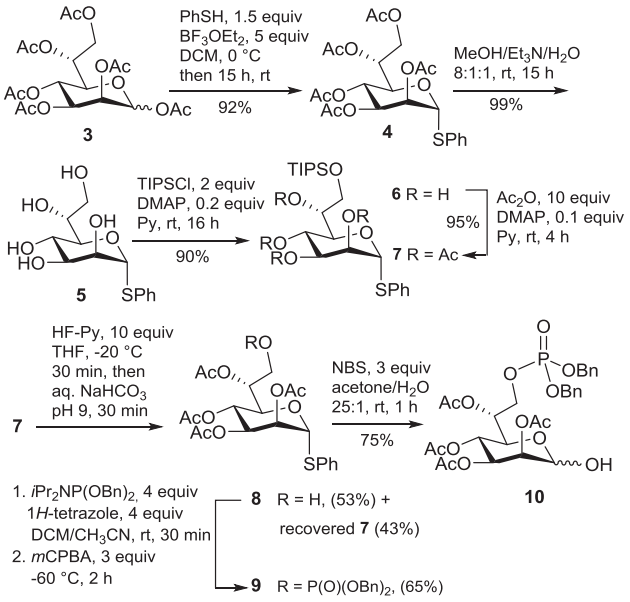
have developed a facile chemical synthesis of D-*glycero*-β-D-*manno*-heptose 1,7-bisphosphate **1** and its α-configured counterpart **2** (Fig. 1).

The synthesis of anomeric phosphates is challenging with respect to the requirements for strict anomeric stereocontrol and the intrinsic instability of the intermediate glycosyl phosphotriesters, representing perfect leaving groups, which renders the option of anomeric phosphorylation under glycosylation conditions unreliable. Two major types of phosphorus compounds could serve as intermediates in the synthesis of anomeric phosphates, namely P(III)-based reagents such as phosphoramidites or P(V)-derived chloroanhydrides of protected phosphoric acid. Upon application of reagents of these types, the stereoselectivity of anomeric phosphitylation/phosphorylation reflects the initial α/β ratio in the starting hemiacetal, unless the anomeric ratio is manipulated *in situ*. The advantage of the phosphoramidite procedure lies in the mildness of the phosphitylation conditions, though an additional oxidation step is needed to produce a P(V)-phosphate. The reaction of a nucleophilic component with a phosphoramidite is catalyzed by 1*H*-tetrazole which generates an extremely unstable P-N tetrazolide species that instantaneously reacts with nucleophiles (e.g. alcohols, lactols) making the slow process of *in-situ* anomerisation unsuitable. The merit of the P(V) phosphotriester methodology involves bypassing of the additional oxidation step along with relative stability and lower reactivity of protected chlorophosphates which could allow for *in-situ* anomerisation. Harsher reaction conditions and a necessity to use a strong base count as drawbacks.

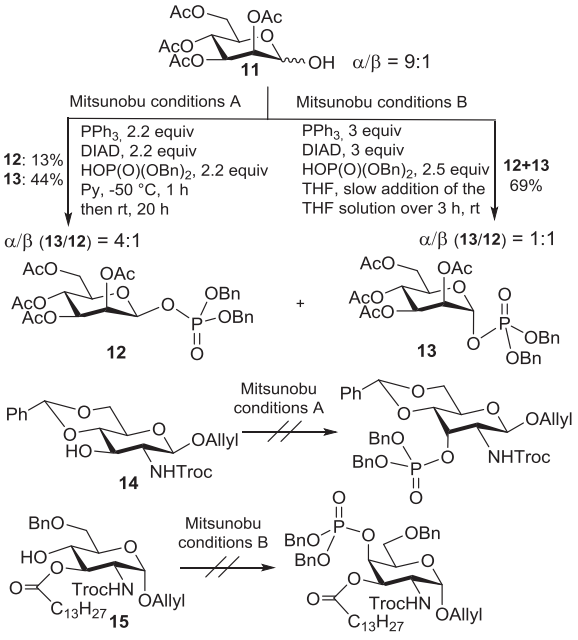
In protected synthetic heptose hemiacetal intermediates having *manno*-configuration, the anomeric ratio is strongly shifted in favor of the α-lactol such that the synthesis of the β-configured glycosyl phosphate requires either *in-situ* anomerisation or inversion of configuration. We considered the latter option particularly attractive and turned our attention to modified Mitsunobu reaction conditions which entail application of phosphoric acid derivatives instead of the classic carboxylic acid. Though such conditions were successfully applied for the synthesis of sugar phosphates, only primary hydroxyl groups were examined.[18–21]

Our synthesis commenced with the preparation of peracetylated thiophenyl glycoside **4** by the Lewis acid catalysed reaction of the D,D-heptose peracetate **3** with thiophenol which gave a mixture of anomeric products (92%, α/β = 12:1) followed by chromatographic separation of the α-thioglycoside **4** (Scheme 1). Deacetylation with aqueous methanolic Et_3N provided **5** in quantitative yield. Next, the primary OH group in position 7 was regioselectively silylated by reaction with triisopropylsilyl chloride in pyridine in the presence of 4,4′-dimethylaminopyridine (DMAP) to give **6** which was peracetylated to furnish **7**. Cleavage of the TIPS protecting group was performed under mild conditions with a diluted solution of HF in pyridine at −20 °C to suppress acetate group migration. The reaction was stopped prior to appearance of the migration by-products (30 min); the target primary alcohol **8** and unreacted **7** were separated by flash column chromatography on silica gel in 53% and 43% yield, respectively. To avoid acetyl migration, heptopyranoside **8** was immediately subjected to further reaction with *N*,*N*-diisopropyl-bisbenzyl phosphoramidite in the presence of 1*H*-tetrazole followed by *in-situ* oxidation with *meta*-chloroperbenzoic (*m*-CPBA) acid at −60 °C to provide protected 7-*O*-phosphate **9** in 65% yield. Thiophenyl glycoside **9** was anomerically deprotected with *N*-bromosuccinimide in aqueous acetone to furnish lactol **10** (α/β = 9:1).



Scheme 1. Synthesis of protected D,D-heptose 7-phosphate **10**.

To explore the stereoselectivity and efficiency of the anomeric phosphorylation under Mitsunobu reaction conditions, we applied peracetylated mannose hemiacetal **11** as a model compound. When the transformation was performed under "classic conditions" using diethyl azodicarboxylate (DEAD) and PPh_3 in THF at reduced temperature (−30 to 0 °C) with dibenzylphosphate as an



Scheme 2. Anomeric phosphorylation under modified Mitsunobu reaction conditions.

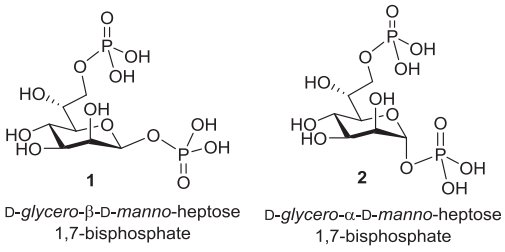
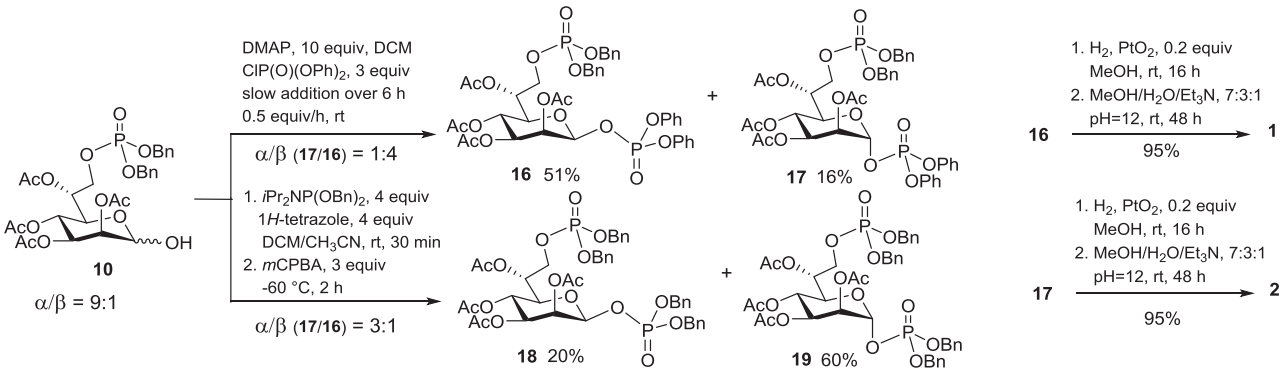


Fig. 1. Structure of D-*glycero*-β-D-*manno*-heptose 1,7-bisphosphate 1 (HBP) and its α-configured counterpart 2.



Scheme 3. Stereoselective synthesis of D-β-D-heptose 1,7-bisphosphate 1 and D-α-D-heptose 1,7-bisphosphate **2**.

acid component, only minor reaction progress could be detected (Scheme 2). Using diisopropyl azodicarboxylate (DIAD) and pyridine to accelerate deprotonation of the nucleophile (lactol **11**) improved the overall outcome resulting in full conversion, though with unsatisfactory stereoselectivity **13**/**12** (α/β) = 4:1. The prevalence of the α-phosphate **13** insinuated participation of a different reaction mechanism, presumably, an acylation with the activated dibenzylphosphate leading to phosphorylation without inversion of configuration. Accordingly, to circumvent the latter event, we avoided using pyridine and performed further trials using THF as the reaction solvent.[22] The best reaction outcome was achieved by pre-forming the betaine (PPh_3 was mixed with DIAD in THF for 10 min) followed by addition of the nucleophilic component lactol **11** and gradual addition (over 3 h) of dibenzylphosphate. Following the reaction progress by ^1H and ^{31}P NMR revealed that the β-phosphate **12** was formed as a major product in the first 20 min of reaction (*ca.* 40% overall conversion) followed by ensuing slow formation of the α-phosphate **13** indicating a switch from the Mitsunobu reaction mechanism to other competing processes (apparently, S_N1 reaction or concurrent formation of an oxocarbenium ion which is then trapped by the nucleophile dibenzylphosphate). To gain deeper insight into the mechanistic backgrounds for the poor stereoselective outcome, we applied the conditions described above for phosphorylation of the secondary OH groups in GlcN derivatives **14** and **15** which was anticipated to proceed with inversion of configuration. The expected products could not be detected, demonstrating the involvement of a different reaction mechanism in the anomeric β-*manno*-phosphorylation under modified Mitsunobu reaction conditions. Because of unsatisfactory stereoselectivity **13**/**12** (α/β) = 1:1, the Mitsunobu reaction conditions were no longer pursued.

Thus, our stereoselective synthesis of D-*glycero*-β-D-*manno*-heptose 1,7-bisphosphate **1** was based on our previous finding disclosing that the anomeric ratio in the synthesis of D-*manno*-heptosyl-1-phosphate from the relevant lactol can be manipulated by the rate of addition of phosphorylating reagent.[23,24] The higher nucleophilicity of the β-*manno*-configured lactol ensures its higher reaction rates with electrophiles (P-activated phosphates or phosphites). As soon as the faster reacting β-lactol is consumed by a limited amount of the gradually added phosphorylating reagent, the anomerisation process toward β-anomer supported by an excess of DMAP sets in. In this manner, different β-configured D-*manno*-1-phosphates could be prepared in exceptionally high yields.[24–26] Along these lines, the *in-situ* anomerisation of lactol **10** in favor of the more nucleophilic β-configured anomer was achieved by treatment with excess of DMAP. Slow continuous addition of a diluted DCM solution of diphenyl phosphorochloridate (DPCP, 0.5 equiv/h) to a solution of **10** resulted in a preferential formation of a kinetically controlled product, β-heptosyl phosphotriester **16** (α/β = 1:4), which was isolated in 51% yield (Scheme 3). The somewhat lower yield compared to the β-*manno*-phosphorylation outcome (85%) in our previous synthesis of ADP-D-*glycero*-β-D-*manno*-heptose[24] could be explained by a remote influence of the bulky 7-*O*-dibenzylphosphate group which shields the anomeric position, hindering access from the β-face. Also the separation of **16** from the residual α-anomer **17** was less efficient due to a smaller difference in R_f values inflicted by the 7-phospate substituent. By using the phosphoramidite approach for the phosphitylation of **10**, the equilibrium could be shifted toward formation of the thermodynamically preferred α-phosphite, which, after oxidation with *m*-CPBA and separation by column chromatography, furnished α-heptosyl phosphotriester **19** as a major product. Phenyl protecting groups at phosphorus in diphosphates **16** and **17** were readily cleaved by catalytic hydrogenation in the presence of PtO_2, followed by complete deacetylation with MeOH/H_2O/Et_3N (7:3:1) which afforded D-β-D-heptose 1,7-bisphosphate **1** and D-α-D-heptose 1,7-bisphosphate **2**. The assignment of the anomeric configuration in the

Table 1
^1H and ^{13}C NMR data for HBP **1** and **2**.

Proton/carbon	H-1 ppm/^3J Hz C-1 ppm/^2J Hz	H-2 ppm/^3J Hz C-2 ppm/^2J Hz	H-3 ppm/^3J Hz C-3 ppm/^2J Hz	H-4 ppm/^3J Hz C-4 ppm/^2J Hz	H-5 ppm/^3J Hz C-5 ppm/^2J Hz	H-6 ppm/^3J Hz C-6 ppm/^2J Hz	H-7a ppm/^3J Hz C-7 ppm/^2J Hz	H-7b ppm
1	5.05 (d)	3.91 (m)	3.60(dd)	3.67 (t)	3.44 (dd)	4.11 (dt)	4.02 (ddd)	3.92 (m)
^1H NMR	$^3J_{1,P}$ = 8.5		$^3J_{2,3}$ = 3.2 $^3J_{3,4}$ = 9.8	$^3J_{4,5}$ = 9.8	$^3J_{5,6}$ = 3.1	$^3J_{7b,6}$ = 7.4 $^3J_{7a,6}$ = 3.5	$^2J_{7a,7b}$ = 11.1 $^3J_{7a,P}$ = 6.6	
1	95.54	70.57	72.84	67.06	76.73	70.89	65.41	
^{13}C NMR	$^2J_{1,P}$ = 4.4	$^3J_{2,P}$ = 7.7				$^3J_{6,P}$ = 6.6	$^2J_{7,P}$ = 5.5	
2	5.36 (dd)	3.93 (m)	3.85 (dd)	3.79 (t)	3.88 (dd)	4.14 (dt)	4.05 (ddd)	3.93 (m)
^1H NMR	$^3J_{1,2}$ = 1.9 $^3J_{1,P}$ = 8.0		$^3J_{2,3}$ = 3.3 $^3J_{3,4}$ = 9.5	$^3J_{4,5}$ = 9.8	$^3J_{5,6}$ = 2.9	$^3J_{7a,6}$ = 7.6 $^3J_{5,6}$ = $^3J_{7b,6}$ = 3.3	$^2J_{7a,7b}$ = 10.9 $^3J_{7a,P}$ = 6.5	
2	96.36	71.02	70.79	67.62	74.00	71.45	66.25	
^{13}C NMR	$^2J_{1,P}$ = 5.2	$^3J_{2,P}$ = 8.8				$^3J_{6,P}$ = 7.7	$^2J_{7,P}$ = 4.4	

D-*manno*-heptopyranosides was corroborated on the basis of characteristic upfield chemical shifts of H-1, H-3 and H-5 for the β-anomer compared to the α-counterpart as well as by comparing the coupling constants $^{3}J_{1,2}$ which is smaller (<0.5 Hz) for the β-anomer **1** (Table 1).[24]

In conclusion, we describe the first chemical synthesis of the innate immune modulator, D-β-D-heptose 1,7-bisphosphate (HBP), which was efficiently prepared by application of stereoselective β-*manno*-phosphorylation and isolated in anomerically pure form. We show that anomeric phosphorylation under modified Mitsunobu reaction conditions proceeds with only partial inversion of configuration and undergoes different reaction mechanism which needs to be further investigated. Biological studies applying D-β-D- and D-α-D-heptose 1,7-bisphosphates **1** and **2** will be published elsewhere.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2017.06.014.

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