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Non-hydrolyzable heptose bis- and monophosphate analogues modulate the pro-inflammatory TIFA-NF-κB signalling

Authors

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Abstract –

D-*glycero*-D-*manno*-heptose-1β,7-bisphosphate (HBP) and D-*glycero*-D-*manno*-heptose-1β-phosphate (H1P) are bacterial metabolites that were recently shown to stimulate inflammatory responses in host cells through the activation of the TIFA-dependent NF-*k*B pathway. To better understand the structure-based activity in relation to this process, a family of non-hydrolyzable phosphonate analogues of HBP and H1P was synthesized. The inflammation modulation by which these molecules induce the TIFA-NF-κB signal axis was evaluated *in vivo* at a low-nanomolar concentration (6 nM) and compared to the natural metabolites. Our data showed that three phosphonate analogues resembled the stimulatory activity of HBP, while two phosphonates antagonized the HBP-induced TIFA-NF-κB signalling. These results open new horizons for the design of pro-inflammatory and innate immune modulators that could be used as vaccine adjuvant.

Introduction –

Lipopolysaccharide (LPS) is a major, and most often essential, component of Gram-negative bacteria's outer membrane. It has several important biological functions and it is also a major virulence factor of pathogenic bacteria.[1] D-*glycero*-D-*manno*-heptose-1β,7-bisphosphate (HBP) **1** represented in Figure 1 is a central intermediate of the bacterial LPS biosynthesis. The *glycero*-D-*manno*-heptose core structure of HBP is exclusively found in bacteria, either with the 6-D configuration as in HBP **1** or with a 6-L configuration as in ADP-L-β-D-heptose **3**. HBP is transformed into its activated form **3** by three enzymatic steps. ADP-heptose **3** then serves as a donor substrate of heptosyltansferases for the LPS construction within the bacterial

cytoplasm.[2] Although the enzymes involved in the bacterial heptose biosynthesis are not essential for Gram-negative bacteria, lack of heptose yields a phenotypic change that is not virulent any longer and that dramatically fragilizes the microorganism.[1b, 3] These enzymes thus represent potential targets for the development of anti-virulence agents.[4]

Recently, the team of Gray-Owen discovered that HBP **1** has another major biological function: HBP can activate the innate immune response of the host cell through a TIFA-dependent pathway,[5] where TIFA is an adapter molecule that promotes the activation of pro-inflammatory NF-*κ*B signalling.[6] The effect of HBP was also dependent on the phosphorylation of TIFA, which was shown to occur at Thr9[6a] and mediated by Aurora A kinase.[7] Such a novel function for HBP was also evidenced for several important bacterial pathogens. Recently, D-heptose-1-phosphate **2** (H1P), the following intermediate in the heptose biosynthetic pathway, was also found to activate the TIFA-dependent NF-*κ*B inflammatory response.[8]

Even more recently, ADP-L-β-D-heptose **3** and its epimer at C-6, ADP-D-β-D-heptose, were also identified as a pathogen-associated molecular pattern (PAMP) to trigger innate immunity through binding with ALPK1 (alpha kinase 1) and activating downstream NF-*κ*B signalling.[9] These major observations were also discovered in parallel in the case of *Shigella flexneri*[10] and *Helicobacter pylori* infections.[6d, 11] Importantly, the latter studies also clearly established that 6-L or 6-D configured ADP-heptose are much more efficient promotor of inflammation responses, and showed that HBP **1** could be processed by AMP transfer in the host cell to yield the real promotor of inflammation. All these results point to these bacterial metabolites as critical activators of innate immunity.

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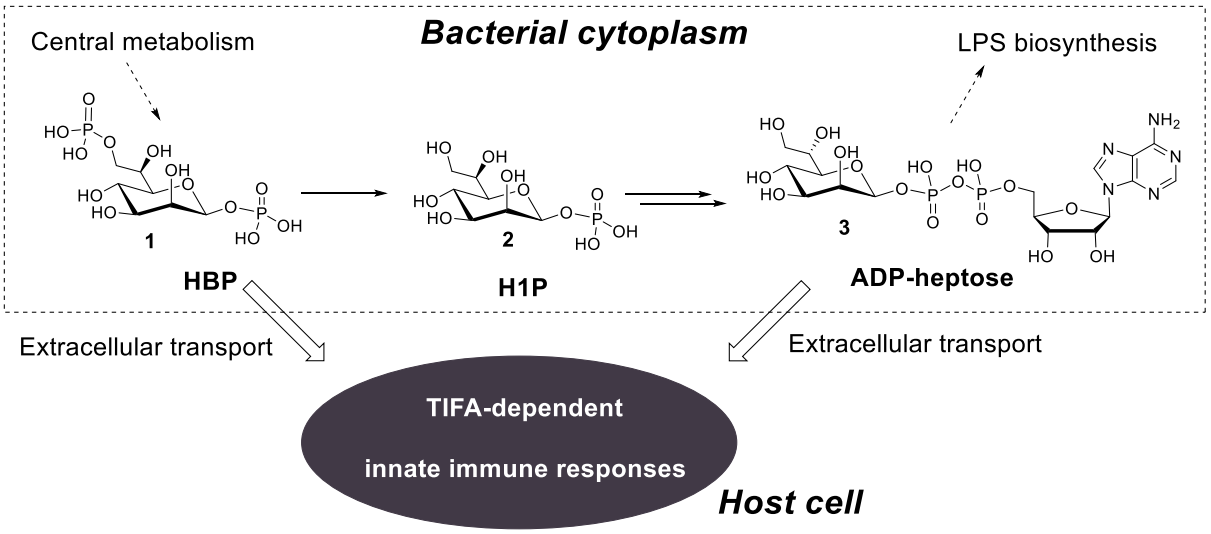

Figure 1 – Key intermediates of the biosynthesis of bacterial heptoses.

These major discoveries stimulated the development of innovative synthetic pathways of HBP itself, with a particular focus on the control of the stereoselectivity at both the 6- and the anomeric positions.[12] These and additional synthetic analogues of HBP and related molecules such as H1P and ADP-heptose could be used to address the important questions mentioned above, and also find, for instance, interesting applications to develop immunomodulators or vaccine adjuvants.[4e, 13]

Objectives of the study - The newly discovered role of HBP **1** as an activator of innate immune system opens new avenues in the exploration of heptose glycomimetics. Indeed, it remains to be evaluated how HBP analogues would interact with the innate immunity machinery. The present study aims at: 1) synthesizing HBP and H1P stable phosphonates analogues, 2) evaluating their ability to trigger TIFA-NF-*k*B signaling *in vivo* in comparison with natural HBP, and 3) evaluate their ability to antagonize HBP activity.

Design of the molecules - As a bis-phosphate, HBP is prone to *in vivo* enzymatic or chemical hydrolysis. Our first purpose was to explore the synthesis and biological properties of non-hydrolyzable HBP analogues (Figure 2). Due to its particularly sensitive acetal nature, we focused our attention on the development of anomeric phosphonate analogues. Indeed, anomeric phosphates are very common leaving groups *in vivo*, for instance for enzymatic glycosylations, and also in the installation of nucleobases in nucleoside biosynthesis. Chemists have also exploited the reactivity

of anomeric phosphates to develop glycosylation techniques.[14] Furthermore, many phosphatases have been shown to hydrolyse anomeric phosphates. Anomeric phosphonates thus represent ideal tools for investigating the pro-inflammatory response of heptosides using *in vitro* cell-based assays. It is worth mentioning that phosphonates have been used as phosphate analogues for many classes of natural molecules (nucleotides, lipids, peptides etc…) and drugs to increase their metabolic stability.[15]

This report describes the synthesis of HBP analogues **4**-**7** (Figure 2) as well their heptose-1-phosphate H1P counterparts **8**-**10** and natural product H1P **2** in order to assess: 1) the impact of the presence of an anomeric phosphonate and 2) the effect of the absence of the second phosphate at the 7-position. We introduced a fluorine atom in **7** and **10** to modulate both the structure and the pKa of the phosphonate functional group. In this study, we aimed to compare these molecules in a systematic way: 1) All *C*-glycosidic analogues of HBP have their H1P counterparts. 2) A single structural transformation is provided from one molecule to the next one: a methylene groups CH_2 distinguishes natural HBP and **5** (or H1P and **8**); an α-hydroxyl group then decorates **6** (or **9**); then a fluorine atom completes the structure in **7** (or **10**).

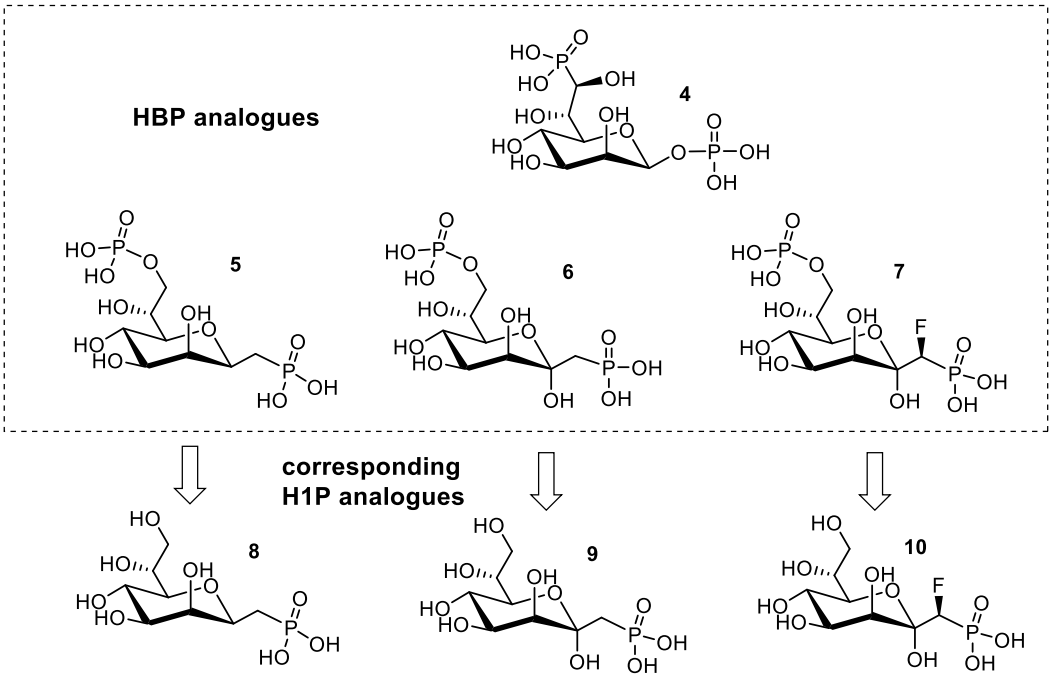

Figure 2 – Targeted H1P and HBP analogues.

Synthesis –

The key steps of the synthesis of all targets **2**, **4**-**10** are the stereoselective homologation of a hexose into a heptose and the installation of the anomeric β-configured phosphonate.

Our group has been involved in the synthesis of both L-[16] and D-heptose[4b, 12a] scaffolds in the context of the inhibition of the enzymes of the bacterial heptose biosynthetic pathway.[17] HBP being 6-D configured, we decided to follow the well-established methodology based on the dihydroxylation of intermediate alkene **12** easily obtained, in large scale, by a Wittig methylenation of the corresponding aldehyde derived from commercial D-mannoside **11** in four steps.[4b, 12a] A D-selective dihydroxylation followed by protective groups installation and acetolysis furnished intermediate **13**. From this central intermediate, we have developed a β-stereoselective concomitant phosphorylations at the 1- and 7-position yielding HBP **1** after deprotection.[12a] H1P **2** could then be obtained in a similar manner from **13**: The Mitsunobu phosphorylation of lactol **14,** that was obtained from **13** in the presence of DMAPA,[18] selectively gave the protected β-phosphate **15** in 49% yield. The β configuration was ascertained by NMR techniques. NOE correlations between H1, H-3 and H-5 confirmed this assignment. In addition, the ^{1}J(C1-H1) was found to be 160.80 Hz, which is consistent with a β configuration.[19] A sequence of hydrogenolysis followed by a saponification provided H1P **2** as a triethylammonium salt in 95% yield. (Scheme 1). For compounds **1** and **2**, we utilized two different counter ions because of solubility issues. Compound **1** was converted into its sodium salt because the solubility of its triethylammonium salt was not good enough in water for NMR analyses.

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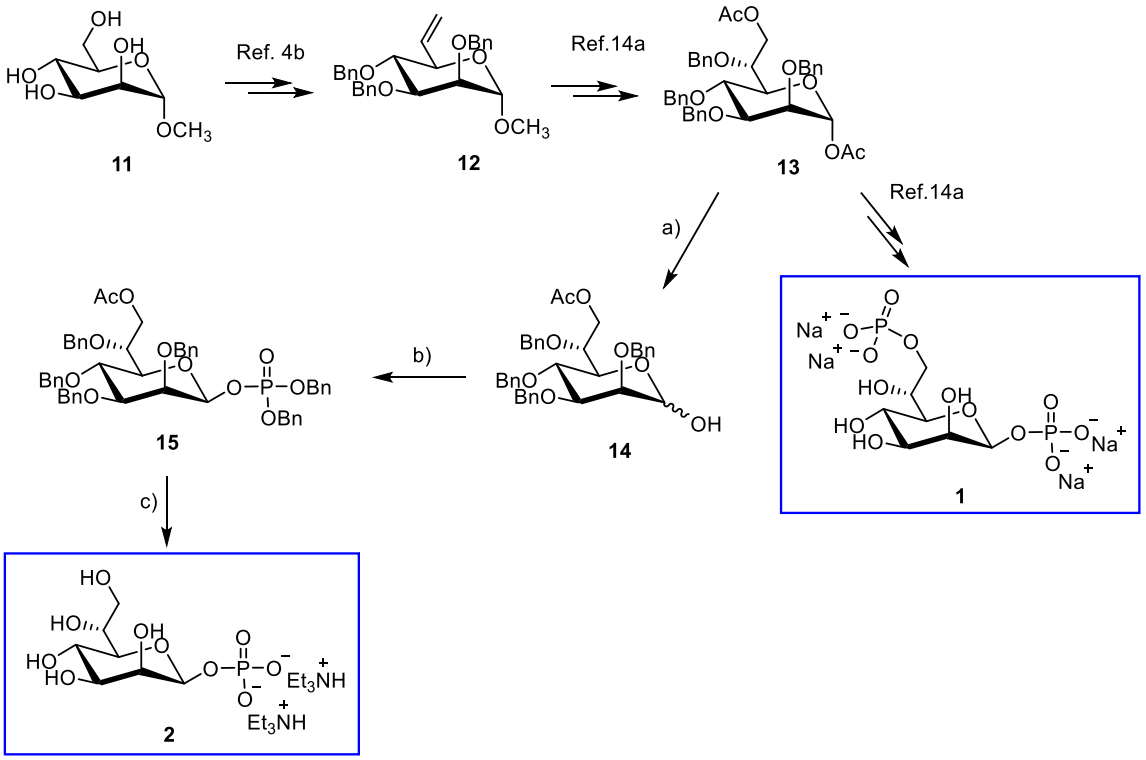
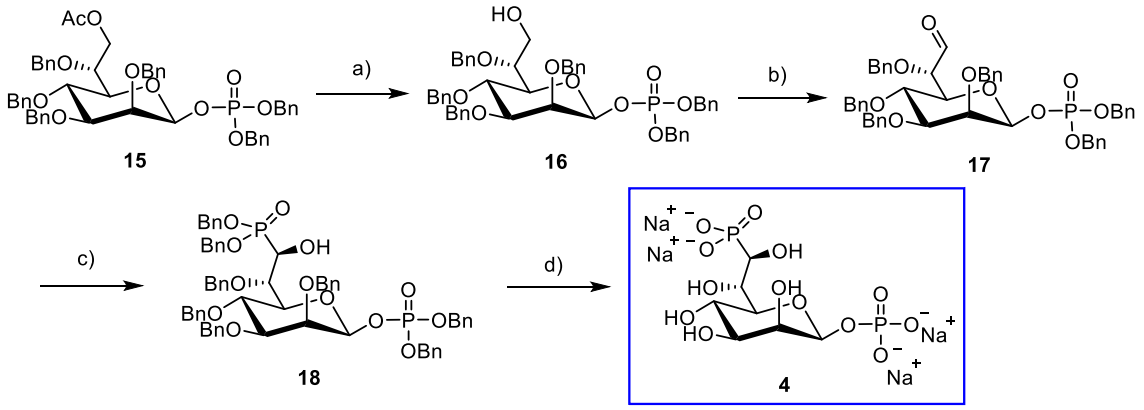


Scheme 1 – Synthesis of natural HBP **1** and H1P **2** bacterial metabolites: a) 3-(Dimethylamino)-1-propylamine, THF, r.t, overnight, 95%; b) dibenzylphosphate, PPh_3, DIAD, Et_3N, r.t. overnight, 49% (isolated yield, α/β 3:7); c) H_2, $Pd(OH)_2/C$, dioxane/H_2O; then Et_3N, CH_3OH/H_2O, 95%.

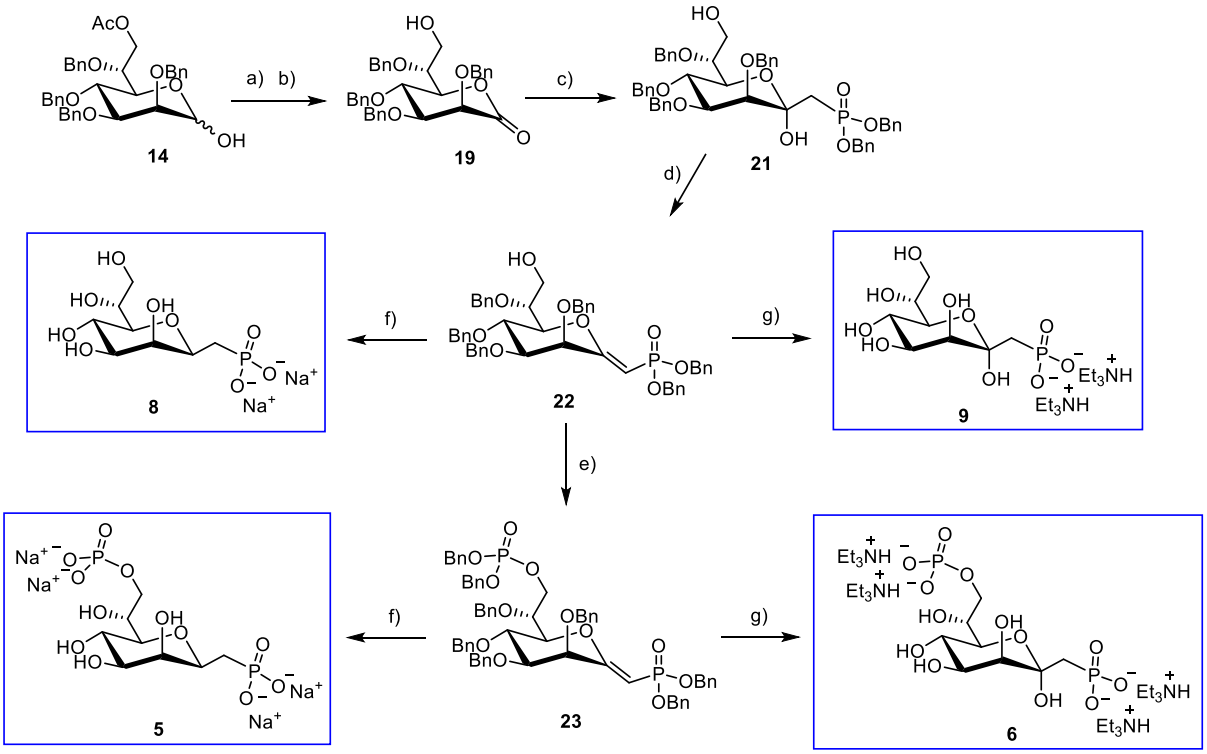
In order to have a better comparison between HBP and H1P in the biological assays, we designed and synthesized analogue **4** from intermediate **15**, as detailed in Scheme 2. Selective deacetylation of **15** followed by a Dess-Martin oxidation provided aldehyde **17**. Condensation of the latter with dibenzyl phosphite yielded α-hydroxy phosphonate **18** as a single stereoisomer, as attested by ^{1}H, ^{13}C and ^{31}P NMR data. The absolute configuration of C*7-OH of **18** was evaluated by vibrational circular dichroism (VCD) measurements and correlation with computed spectra (see supplementary information).[20] The actual measurements indicate that the absolute configuration of C-7 is (R). Hydrogenolysis of the eight benzyl groups provided the targeted phosphonate **4** in 80% yield. Considering its limited solubility, the final product was converted into a sodium salt using a cation exchange resin (Dowex 50-WX8, Na^+-form).



Scheme 2 – Synthesis of α-hydroxy 7-phosphono analogue of HBP **1**: a) Cs_2CO_3, MeOH, 67%; b) DMP, DCM, overnight, 98%; c) dibenzylphosphite, LiHMDS, THF, -78°C, 64%; d) $Pd(OH)_2/C$, H_2, 1,4-dioxane/H_2O; then Dowex 50-WX8 (Na^+-form), 80%.

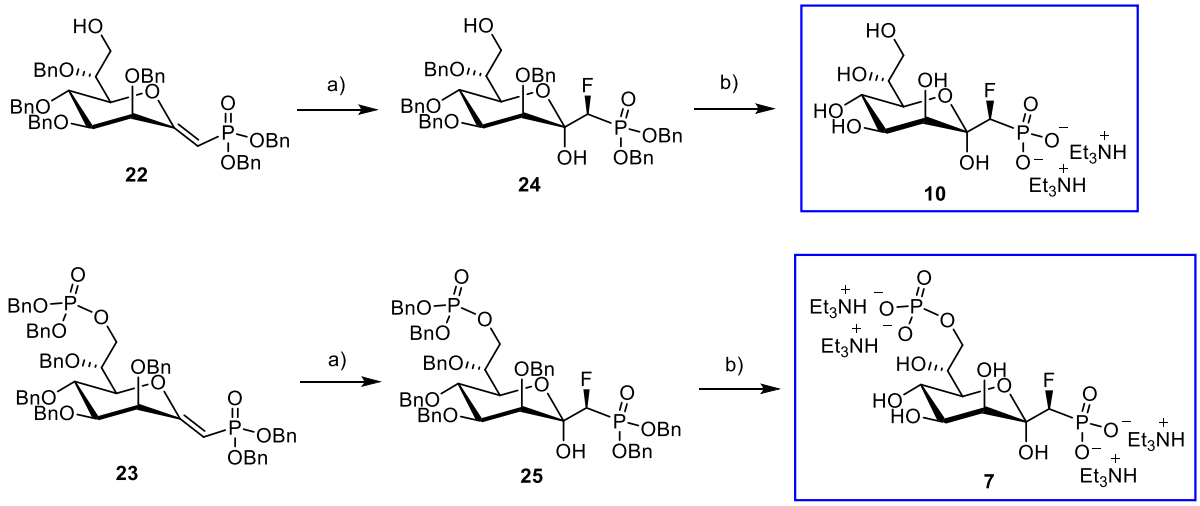
Then, we exploited intermediate **14**, that was originally used in the synthesis of H1P (scheme 1) to prepare 1-phosphonate analogues **5**, **6**, **8** and **9** (Scheme 3). A PCC oxidation of lactol **14** followed by a deacetylation provided lactone **19** that was condensed with the lithium anion of methyl dibenzylphosphonate to give phosphonate **21** in 77% yield. The subsequent elimination was achieved by treating **21** with pyridine and trifluoroacetic anhydride (TFAA) to give a mixture of *exo*-glycal **22** as a single stereoisomer and the corresponding 7-*O*-trifluoroacetyl *exo*-glycal. The removal of the trifluoroacetyl group could be realized *in situ* under basic condition. The structure of **22** was ascertained by NMR spectroscopic techniques. Moreover, the NOE correlations between the proton adjacent to the phosphonate group and the H-2 proton of the heptose ring confirmed the Z-configuration.[21] Mitsunobu phosphorylation at the 7- position of **22** gave *exo*-glycal **23**. Hydrogenolysis of *exo*-glycal **22** and **23** gave **8** and **5** (sodium form) in 83% and 82% yield, respectively. The structure of **8** and **5** were demonstrated by NMR techniques. The NOE correlations between the H-1 and H-3, H-5 of heptose ring could be observed and ascertained the "β" configuration. Such a high β-selectivity had been previously observed in the literature with simpler exoglycals.[22] The deprotection of **22** and **23** carried out with BCl_3 in dry DCM generated phosphonates **9** and **6,** in 85% and 86% yield, respectively. The coupling constant between proton H-3 and H-4, as well as proton H-4 and H-5 are around 9.7 Hz, which strongly suggested they are both in a $^4C_1$ chair conformation. There was no NOE between the methylenic protons of the phosphonate and the H-3 proton of the heptose rings. All these NMR data strongly

suggest that the anomeric OH in **6** and **9** is axial, which is the expected configuration based on the anomeric effect. Three parameters explain this strong anomeric preference: 1) the anomeric effect, 2) the axial OH at C-2 that enhances the stereoelectronic effect, 3) the strong preference of the methylene phosphonate group for an equatorial position, to avoid a repulsion with the axial protons H-3 and H-5.



Scheme 3 – Synthesis of 1-phosphono analogues of HBP and H1P: a) PCC, 4 Å MS, DCM, overnight, 81%; b) $CH_3OH/H_2O/Et_3N$, r.t., 12h, 98%; c) dibenzyl methylphosphonate **20**, *n*-BuLi, THF, -78°C-r.t., 77%; d) (i) TFAA, Pyridine, THF, 0°C-r.t., 2h; (ii) Et_3N, CH_3OH, 1h, 83%; e) dibenzylphosphate, P(*p*-ClPh)$_3$, DIAD, Et_3N, r.t., overnight, 85%; f) H_2, $Pd(OH)_2/C$, 1,4-dioxane/H_2O, then Dowex 50-WX8 (Na^+-form); for **8**, 83%, for **5**, 82% g) BCl_3, DCM, -78°C -r.t., then Et_3N; for **9**, 85%, for **6**, 86%.

In 2006, our group disclosed the first synthesis of fluorinated phosphono-*exo*-glycals *via* Selectfluor-mediated fluorination/elimination on phosphono-*exo*-glycals.[23] We applied this Selectfluor-mediated fluorination on intermediates **22** and **23**, and obtained fluorinated phosphonate **24** and **25**, respectively (Scheme 4). The absolute configuration of the newly generated asymmetric center (C*1'-F) of **25** was assessed by vibrational circular dichroism (VCD) along with computations of the VCD and IR spectra (See ESI). The (S) configuration indicates that Selectfluor reacted with *exo*-glycal **23** from the less hindered α face.[24] Hydrogenolysis of **24** and **25** furnished phosphonates **10** and **7** (triethylammonium salts) in 82% and 85% yield, respectively.



Scheme 4 – Synthesis of fluorinated 1-phosphono analogues of HBP **1** and H1P **2**: a) Selectfluor, 4 Å MS, CH_3CN/CH_3NO_2, 12h, 30°C, for **24**, 52%, for **25**, 78%; b) H_2, $Pd(OH)_2/C$, 1,4-dioxane/H_2O, then Et_3N; for **10**, 82%, for **7**, 85%.

Biological evaluations – The current state of knowledge in this field does not provide information on whether close structural analogues of HBP or H1P can also trigger the TIFA-NF-*k*B signalling. Moreover, such molecules may act as inhibitors of HBP/H1P-triggered inflammatory responses, potentially by blocking the penetration of HBP/H1P into the host cell, or by binding the intracellular proteins that interact with or modify HBP/H1P.

To assess the ability of the non-hydrolyzable phosphonate analogues of H1P and HBP **4**-**10** to modulate the TIFA-NF-*k*B signalling, we initiated two distinct experiments following established protocols [6a, 7] 1) An experiment in which all molecules are tested *in vivo* in parallel and compared to HBP (the level of phosphor NF-*k*B p65 and TIFA are measured by Western blotting and compared with cells that are not induced) 2) In case the phosphonate analogues of HBP and H1P did not activate TIFA-NF-*k*B signalling, they are assayed in competition with an equimolar amount of HBP with the same experimental setting. By assessing the activated TIFA-NF-*κ*B axis as a surrogate of the inflammatory responses and in comparison with HBP alone, the inflammation modulatory ability of these molecules can be estimated accordingly.

The first experiment (Figure 3A) examined HEK293T cells treated with or without 6 nM of phosphonate analogues **4**-**10**, HBP (**1**), and H1P (**2**) by Western blotting for the levels of TIFA and phospho-NF-*κ*B p65, as indicative of the induced inflammatory

responses.[6a, 7] To ascertain these results, the NF-κB activation was also estimated by the luciferase-based reporter assay (Figure 3B).[7] The results showed that glycomimetics **5**, **8**, **10** can significantly increase TIFA expression and activate NF-*κ*B, although to a lesser extent than HBP and inflammatory cytokine TNFα (tumor necrosis factor alpha), in contrast to the other molecules (**2, 3, 6, 7, 9**) and mock treatment. The H1P analogue **8** gave a slightly better pro-inflammatory response, a result that could not be predicted from the observations published to date with natural bacterial heptosides.[8] Even though the proinflammatory effect of these compounds are lower than that of HBP, the non-hydrolyzable nature of these analogues should make them useful for further applications.

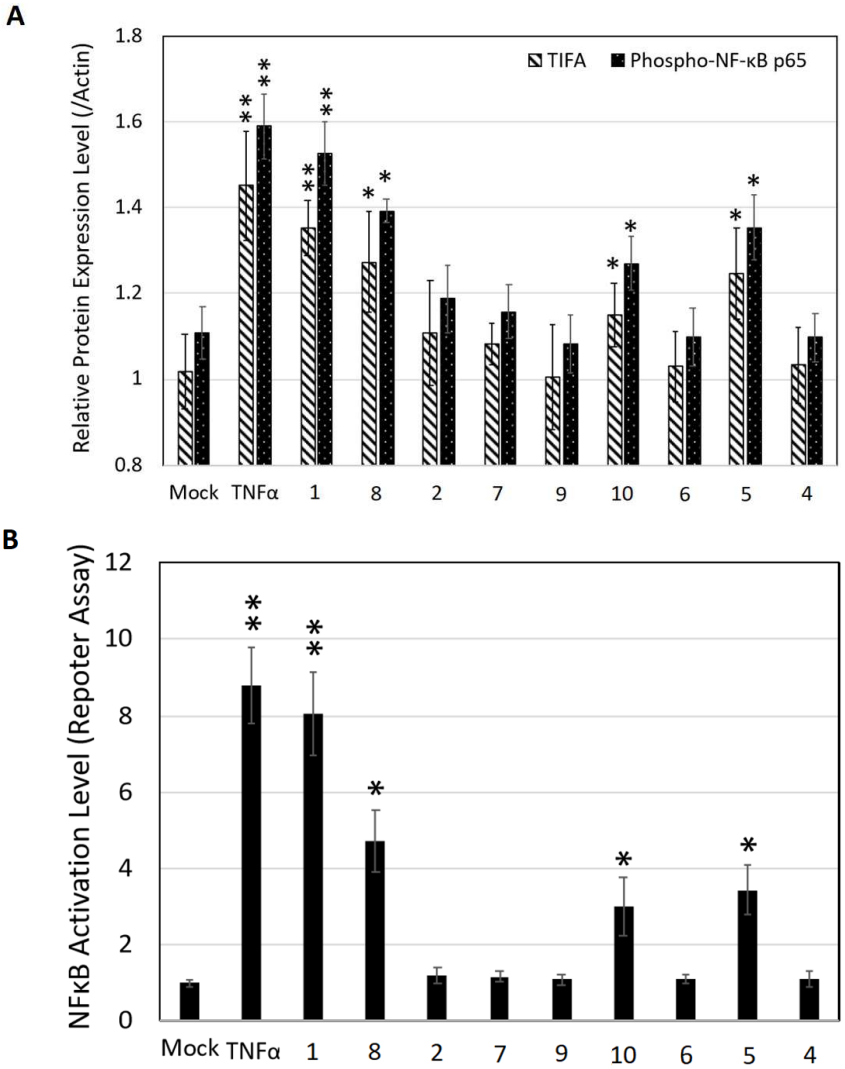


Figure 3. (**A**) Western blot analysis of TIFA and phospho-NF-*κ*B p65 expression in HEK293T cells treated with 6 nM of compounds **1**, **2**, **4**-**10**. (**B**) Luciferase-based reporter assay of NF-*κ*B activity in HEK293T cells with the same treatments as in A. Details are as described previously.[6a, 7]

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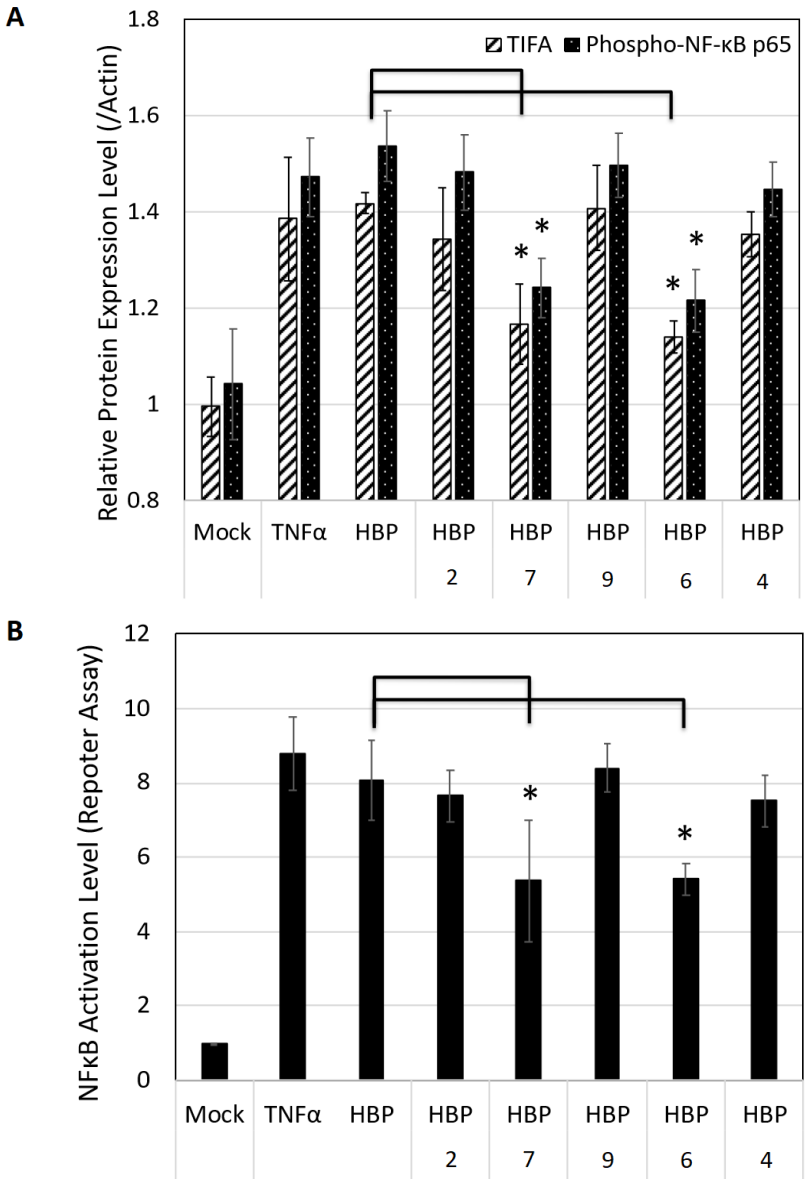

Figure 4 (**A**) Western blot analysis of the expression of TIFA and phospho-p65 induced by HBP alone or co-treatments with phosphonate **2**, **7**, **9**, **6**, or **4** in HEK293T cells. (**B**) The same as in Figure 3B, except that cells were treated the same way than in A.

To assess whether the five compounds that do not activate TIFA-NF-*k*B signalling alone (compounds **2**, **4**, **6**, **7**, **9**) antagonize the stimulatory activity of HBP, the same cells were simultaneously treated with an equimolar amount of HBP and each of these selected phosphonates. The activation of NF-*κ*B was monitored by Western blotting (Figure 4 A), as well as by the luciferase-based reporter assay (Figure 4 B), under the same conditions as Figure 3. These experiments showed that **6** and **7** can antagonize HBP in activating the TIFA-NF-*k*B signalling, with equal

10.1002/cbic.202000319

potency (evaluated at 33% inhibition). Both antagonists are HBP analogues. Their only structural difference is the presence of a F atom. Here, the steric and electronic changes due to the presence of this F atom in **7**, compared to **6**, does not affect the inhibiting activity of these HBP analogues. The luciferase-based reporter assay further showed that the proinflammatory effect of H1P analogue **8** and the antagonistic activity of HBP analogues **7** and **6** against HBP were in dose- and time-dependent manners (Figure 5).

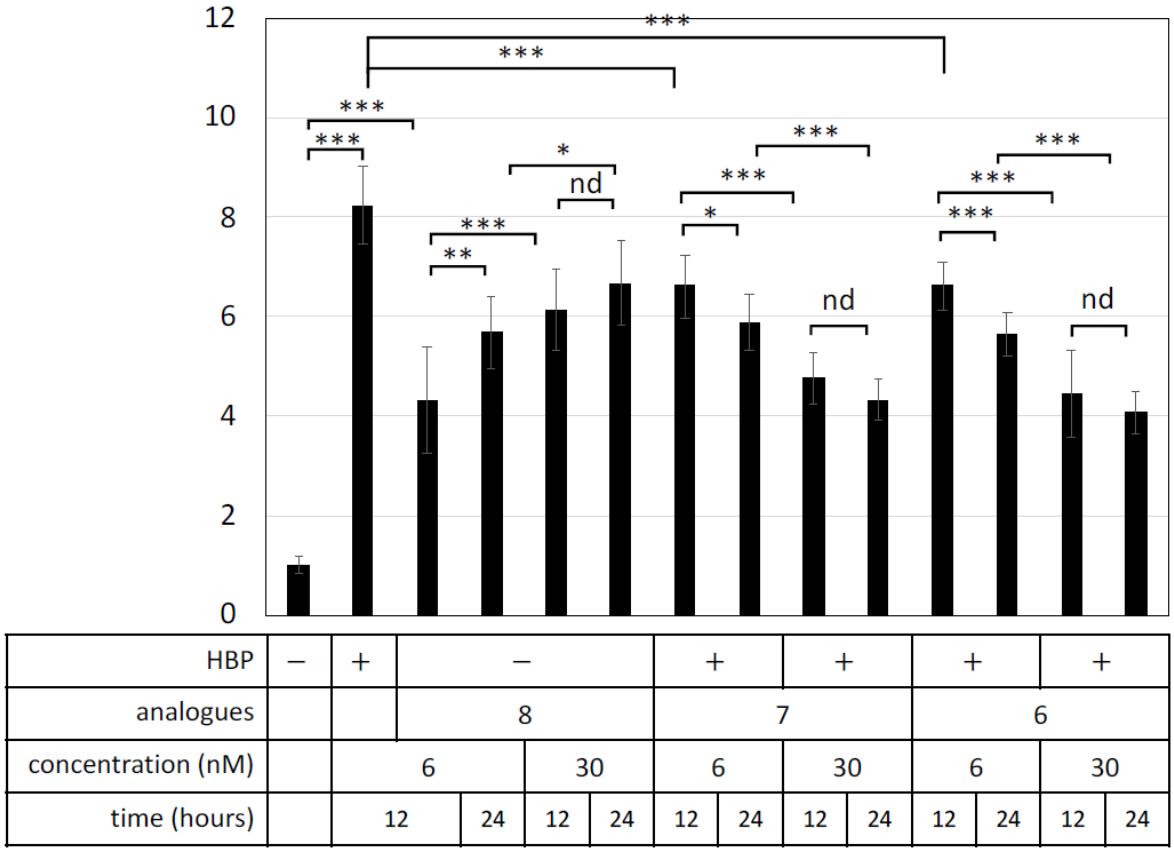

Figure 5 Luciferase-based reporter assay of NF-*κ*B activity induced by HBP or high (30 nM) and low (6 nM) dosage of phosphonate **8** alone or co-treatments with phosphonate **7**, **6** at the indicated time points in HEK293T cells.

Our results demonstrate that modest structural modifications at the vicinity of the anomeric phosphonate can impact the pro-inflammatory activity of the molecules (Figure 6). In particular, presence of the anomeric OH group in molecules **6** and **9** is sufficient to abolish the pro-inflammatory activity as compared to **5** and **8**, respectively. Presence of the additional F atom in **10** partially restores the activity of **9**. On the one hand, the result obtained with molecule **4** also shows that transforming

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the 7-phosphate of HBP into a phosphonate is detrimental for either activating the TIFA pro-inflammatory pathway or to antagonized it. On the other hand, the results obtained with molecules **5**-**8** and **10** clearly demonstrate that it is possible to replace the anomeric phosphate of HBP by a phosphonate and observe either an activation or an antagonization of the pro-inflammatory signalling. Comparison of molecules **6** and **7** shows that the presence of the fluorine atom has no significant effect in the competition against HBP, despite the lowering of the pKa of the vicinal phosphonate of **7** by the fluorine atom. Comparison of molecules **7** and **10** shows that the phosphorylation at position 7 can turn an activator (**10**, H1P analogue) into an antagonist (**7**, HBP analogue). In addition, replacing the exocyclic anomeric oxygen atom of HBP by a monofluoromethylene group CHF also transformed the natural activator HBP into an antagonist **7**.

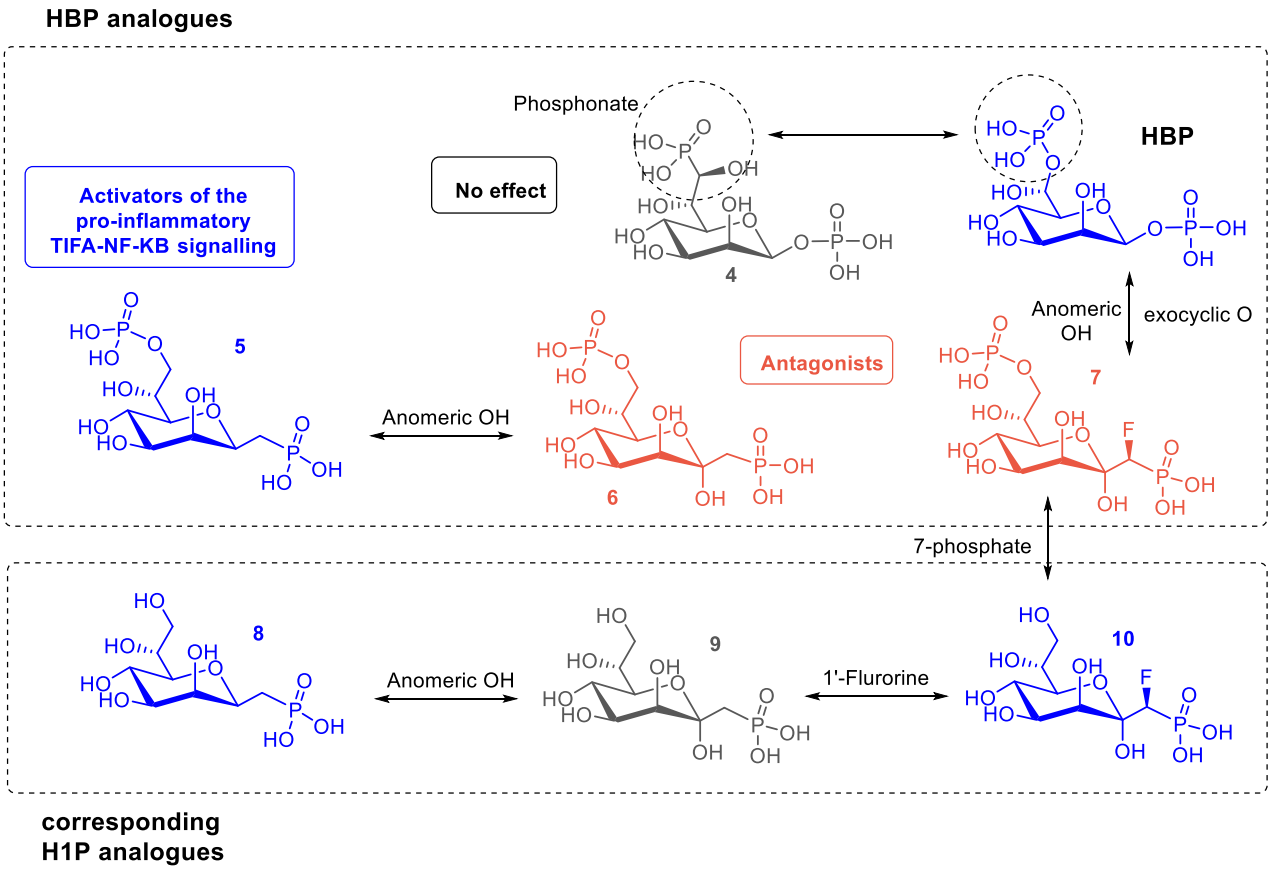

Figure 6 – Structures of the molecules assayed in this study highlighting their ability to modulate (activators in blue, antagonists in red) the pro-inflammatory TIFA-NF-*κ*B signalling. Arrows highlight the structural changes that significantly affect the activity of the molecules.

Given the complexity of this inflammatory cascade, a precise interpretation of these results at the molecular level is challenging. Indeed, these experiments are whole cell assays and involve many potential biochemical partners, intracellular (for instance AMP-transferase, or any protein of the TIFA pathway) or at the cell surface (receptors, transporters etc.). Indeed, the different pathogens investigated to date use distinct secretion systems. It remains unclear, from the recent literature, whether the natural heptose metabolites activate a common pattern recognition receptor ALPK1,[13b, c] and the way H1P, HBP and ADP-heptose are internalized in the host will require further investigation.[13c, d] In addition, in some of the above-mentioned studies, the cell assays were performed with lipofection or permeabilizing reagents, to facilitate the metabolite's uptake.[5, 10] Given the polarity of these molecules, an active transport of these heptose glycomimetics is likely. Such a transporter assisted uptake can by itself be a source of differentiation of these phosphonates.

While the mechanism of action of these molecules requires further investigations, we believe that these results constitute a very important step towards generation of novel molecules acting either as inducer or inhibitor of the HBP-induced inflammatory response.

Conclusions –

In this study, a series of parent analogues of bacterial heptose-1-phosphate H1P and heptose-1,7-bisphosphate HBP have been prepared. Our synthetic strategy was based on the construction of common intermediates that could be derivatized to generate molecules whose structures could be directly compared. The central D,D-heptose scaffold was constructed through a sequence of Swern oxidation, Wittig olefination and dihydroxylation from D-mannose, followed by a β-stereoselectivity Mitsunobu phosphorylation. Beta-configured anomeric phosphonates were constructed thanks to the diastereoselective hydrogenation of phosphonylated *exo*-glycals.

The assessment of the activation of the TIFA- NF-*κ*B signalling by these heptose mimetics revealed that phosphonates **5**, **8**, **10** resemble the pro-inflammatory activity of natural HBP and TNFα. The competition experiment also suggested that two HBP analogues **6** and **7** that did not display pro-inflammatory activity were potent to

supress the HBP-triggered activation of NF-κB. Globally this study evidenced that the best pro-inflammatory inducers are H1P analogues whereas the best antagonists are phosphonate analogues of HBP.

Interestingly, apparently small structural variations in the heptoside skeleton eventually lead to significantly different biological responses. In fact, this observation is certainly not surprising if one considers that already, at the protein/small molecule level, small structural variations can lead to dramatic changes in the affinity of a ligand towards a biomacromolecule. These results also revealed that, despite their highly polar nature, glycomimetics can trigger a specific biological response. The uptake of these glyco-phosphonates into the epithelial cell is still an open question, but it is likely that there are internalized to activate the TIFA-dependent inflammation pathway. This transport step is essential in the whole process: neither the level of specificity nor the biomolecules involved in this process are known. Thus, it will be imperative, as a next objective, to identity the protein(s) that interact with HBP.

The recent discoveries of the role of natural heptose metabolites as PAMPs has raised new questions related to the mechanisms of innate immunity. It has also opened new perspectives in the design of molecules that could be exploited as vaccine adjuvants or for the treatment of allergies.[13] This study provides novel structural insights towards the design of such molecules.

Acknowledgements –

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