DOI: 10.1002/chem.201100396

Systematic Synthesis of Inhibitors of the Two First Enzymes of the Bacterial Heptose Biosynthetic Pathway: Towards Antivirulence Molecules Targeting Lipopolysaccharide Biosynthesis

Maxime Durka,[a] Abdellatif Tikad,[a] Régis Périon,[a] Michael Bosco,[a] Mounir Andaloussi,[a] Stéphanie Floquet,[b] Elodie Malacain,[b] François Moreau,[b] Mayalen Oxoby,[b] Vincent Gerusz,[b] and Stéphane P. Vincent*[a]

Abstract: L-Heptoses (L-*glycero*-D-*manno*-heptopyranoses) are constituents of the inner core of lipolysaccharide (LPS), a molecule playing key roles in the mortality of many infectious diseases as well as in the virulence of many human pathogens. The inhibition of the first enzymes of the bacterial heptose biosynthetic pathway is an almost unexplored field to date although it appears to be a very novel way for the development of antivirulence drugs. We report the synthesis of a series of D-*glycero*-D-*manno*-heptopyranose 7-phosphate (H7P) analogues and their inhibition properties against the isomerase GmhA and the the kinase HldE, the two first enzymes of the bacterial heptose biosynthetic pathway. The heptose structures have been modified at the 1-, 2-, 6- and 7-positions to probe the importance of the key structural features of H7P that allow a tight binding to the target enzymes; H7P being the product of GmhA and the substrate of HldE, the second objective was to find structures that could simultaneously inhibit both enzymes. We found that GmhA and HldE were extremely sensitive to structural modifications at the 6- and 7- positions of the heptose scaffold. To our surprise, the epimeric analogue of H7P displaying a D-glucopyranose configuration was found to be the best inhibitor of both enzymes but also the only molecule of this series that could inhibit GmhA ($IC_{50}=34\ \mu M$) and HldE ($IC_{50}=9.4\ \mu M$) in the low micromolar range. Noteworthy, this study describes the first inhibitors of GmhA ever reported, and paves the way to the design of a second generation of molecules targeting the bacterial virulence.

Keywords: bactericides · cell wall · heptose · inhibition · virulence

Introduction

The main polysaccharide present at the surface of gram-negative bacteria is lipopolysaccharide (LPS). This complex molecule plays key roles in the mortality of many infectious diseases as well as in the virulence of numerous human pathogens.[1] As cell wall glycolipids, LPS insures a protection against hydrophobic molecules and participate to the bacterial cell integrity.[2]

LPS is an amphipathic molecule that can be decomposed into three main substructures: lipid A, the oligosaccharide core and the O-antigen.[3] The oligosaccharide core can be divided into two parts: the inner core is formed at least of one molecule of 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) and two molecules of L-*glycero*-α-D-*manno*-heptose (heptose), and the outer core is composed of hexoses.[4] Lipid A and one Kdo is the minimal structure for maintaining cell viability.[2] Gram-negative bacteria that lack heptose display the deep-rough phenotype[1] and show a dramatically increased sensitivity towards detergents or hydrophobic antibiotics and are much more susceptible to phagocytosis by macrophages,[5] as well as to the bactericidal effect of the host.[6] Therefore, the inhibition of the heptose biosynthetic pathway can be seen as a novel approach to develop new weapons to combat bacterial pathogens: instead of targeting the central metabolism or the cell wall construction, this approach consists in attenuating or even abolishing the virulence of the microorganism without the need to kill it.

Indeed, antivirulence has emerged as an alternative chemotherapeutic strategy to the discovery of new bactericides or bacteriostatic molecules.[7–9] With this concept in mind, we have recently reported on the inhibition of heptosyltransferase WaaC as a novel approach to inhibit the bacterial cell wall resistance to innate immune response.[10,11] Moreover, we just disclosed the synthesis and the inhibition properties of glycosylated fullerenes designed to inhibit the adhesion of uropathogenic *E. coli* strains to bladder cells.[12] Thus, the inhibition of the heptose biosynthesis appears to be a very novel field for the development of antivirulence

[a] Dr. M. Durka, Dr. A. Tikad, Dr. R. Périon, Dr. M. Bosco, Dr. M. Andaloussi, Prof. S. P. Vincent
Chemistry Department, University of Namur
rue de Bruxelles 61, 5000 Namur (Belgium)
Fax: (+32) 81-72-45-17
E-mail: stephane.vincent@fundp.ac.be

[b] S. Floquet, E. Malacain, Dr. F. Moreau, Dr. M. Oxoby, Dr. V. Gerusz
Mutabilis S.A., 102, route de Noisy
93230 Romainville (France)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201100396.

drugs. Interestingly, the inhibition of this pathway has been relatively unexplored compared, for instance, to the peptidoglycan biosynthesis. This can be explained by the fact that i) the enzymes involved in the heptose biosynthesis have been cloned and characterized only recently, and ii) their substrates are not commercially available and their synthesis is not straightforward.

The bacterial heptoses are constructed from sedoheptulose-7-phosphate **1**, which originates from the central metabolism (Scheme 1).[13] A keto–aldose isomerase GmhA transforms **1** into D-*glycero*-D-*manno*-heptose 7-phosphate **2** (coined H7P or Heptose-7-Phosphate for simplification in this paper) which is then phosphorylated by the kinase HldE. After hydrolysis of the terminal phosphate of **3**, intermediate **4** is transformed into nucleotide-sugar **5**. Interestingly, bacterial strains such as *E. coli* use the same HldE enzyme for two non-consecutive steps. A regioselective D to L epimerization is then catalyzed by HldD, yielding ADP-L-heptose **6**, the donor substrate of heptosyltransferases (WaaC, WaaF and WaaQ).

To date, the most studied enzymes of this pathway are the epimerase HldD and heptosyltransferase WaaC. In 2000, Kosma et al. described the first synthesis of the two anomers of ADP-L-heptose **6**,[14] thus demonstrating the anomeric configuration of **6**. Later, the same team disclosed the synthesis of C-glycosidic analogues[15] of **6** for which no inhibition data are available to date. In 2008, our group has described the synthesis and the inhibition properties of the 2-fluoro analogue of **6**.[10] Fortunately, we were able to obtain a 3D structure of WaaC in complex with this inhibitor.[11] Based to a virtual screening and a structure–activity relationship studies, Moreau et al. have been able to develop low-micromolar inhibitors of heptosyltransferase WaaC.[16]

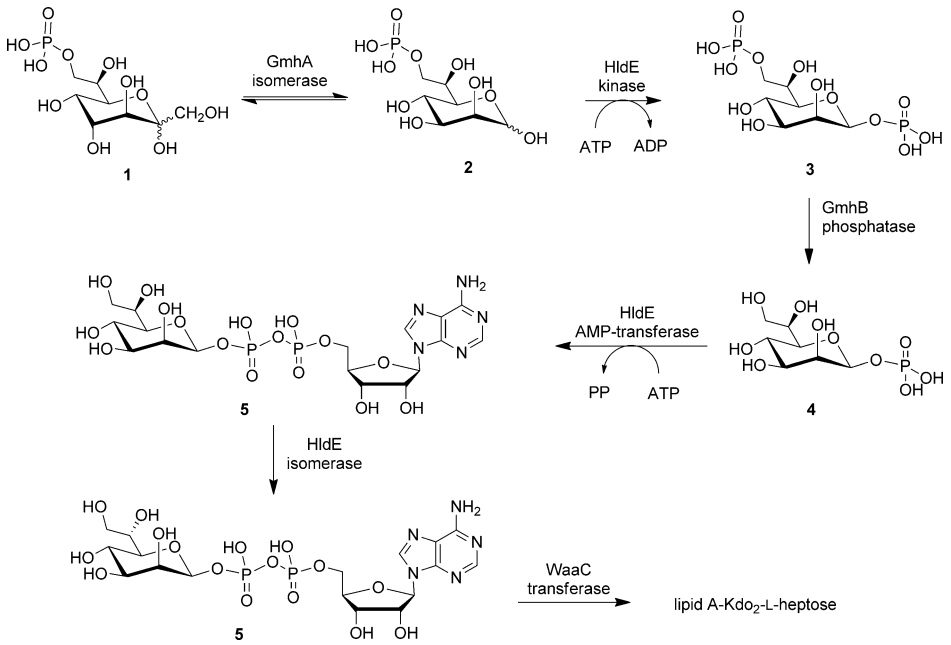
Recently, Tanner et al. have led a detailed mechanistic study on the HldD-catalyzed epimerization step.[17–22] Crystal structures of HldD have been obtained and provided important information about its catalytic pocket.[18,23] Despite this mechanistic and structural knowledge, no potent inhibitor of HldD has been described to date.

Surprisingly, the inhibition of the first enzymes of the heptose biosynthetic pathway is an almost unexplored field, although its importance had already been acknowledged by Paulsen et al. in 1994.[24] They indeed described the synthesis of a series of D-*glycero*-D-*manno*-heptosides modified at the anomeric position but the biological evaluation has, unfortunately, never been published. In 2006, Wright et al. developed an in vitro screen of bacterial lipopolysaccharide biosynthetic enzymes that allowed the identification of an inhibitor of HldE.[25] Later on, some of us identified inhibitors of HldE from a high-throughput screening that allowed a structure activity relationship studies on heterocyclic structures.[26] Noteworthy, several crystal structures of GmhB[27] and GmhA have been obtained, some of them in complex with its substrate that allowed a mapping of the interactions between the sugar-phosphate and the enzyme.[28–30] However, to the best of our knowledge, there is no GmhA inhibitor reported to date.

Thus, there is no literature report on the relative affinities (i.e., the hierarchy of interactions) of all the functional groups present in the heptose skeleton with the first enzymes of this therapeutically relevant biosynthetic pathway. Therefore, we defined, as a first objective, to determine the tolerance of structural modifications of the heptose phosphates as ligands (substrates or inhibitors) of these enzymes, especially the two first ones. Moreover, H7P **2** being the product of GmhA and the substrate of HldE, our second objective was to find structures that could simultaneously inhibit both enzymes.

To answer to these important questions, we report here the synthesis and the inhibition evaluation of a series of heptose-7-phosphate analogues. The heptose structures have been modified at the 1-, 2-, 6- and 7-positions and assayed against GmhA and HldE.

Strategically, we decided that our first synthetic targets should be analogues of the key structure heptose-7-phosphate **2** for two important reasons. First, the initial step of the heptose biosynthesis being in fact an equilibrium between **1** and **2**,[28] H7P is thus *substrate* of the two first enzymes of this pathway. As we will show below, molecules mimicking **2** can thus



Scheme 1. Bacterial heptose biosynthetic pathway.

be inhibitors of both enzymes. Second, sedoheptulose-7-phosphate (**1**) being also a product of the central metabolism of eukaryotes, glycomimetics of this biosynthetic intermediate may lead to non-selective inhibition profiles. In contrast, mannoheptosides such as compounds **2–6** are only found in prokaryotes.[4]

To probe the importance of the key structural features of H7P, we designed and synthesized a series of analogues of **2** depicted in Figure 1. For the carbohydrate substructure, the two main stereochemical characterics of heptoside **2** are its configurations at C2 (D-*manno*) and C6 (D-*glycero*) that confers to this biosynthetic intermediate its sugar identity. Compounds **7** (D-*glycero*-D-*manno*), **8** (L-*glycero*-D-*manno*) and **9** (D-*glycero*-D-*gluco*) were thus designed to address the question of defining the extent of which modifications at these positions hamper the binding process or at least decrease the affinity of the heptosidic analogues towards the enzymes. To prevent these molecules to be substrates of HldE, an anomeric α-methyl group was installed on each of these molecules.

To probe the importance of the 7-phosphate group in terms of interactions and binding strength with the enzymes, a small series of phosphate mimics was also synthesized: carboxylates **10** and **11**, phosphonates **12** and **13**, phosphoramide **14**, sulfonamide **15** and triazole **16**.

To evaluate the impact of the anomeric α-methyl group on the binding process, lactols **11** and **13** were designed to be directly compared to **10** and **12**, respectively.

Overall, this series comprises five methyl glycosides (compounds **7**–**10**, **12**) and five free anomeric sugars (compounds **11**, **13**–**16**) that will enable us to scan the possibilities of structural variations on the D-*glycero*-D-*manno*-pyranose 7-phosphate **2** central structure.

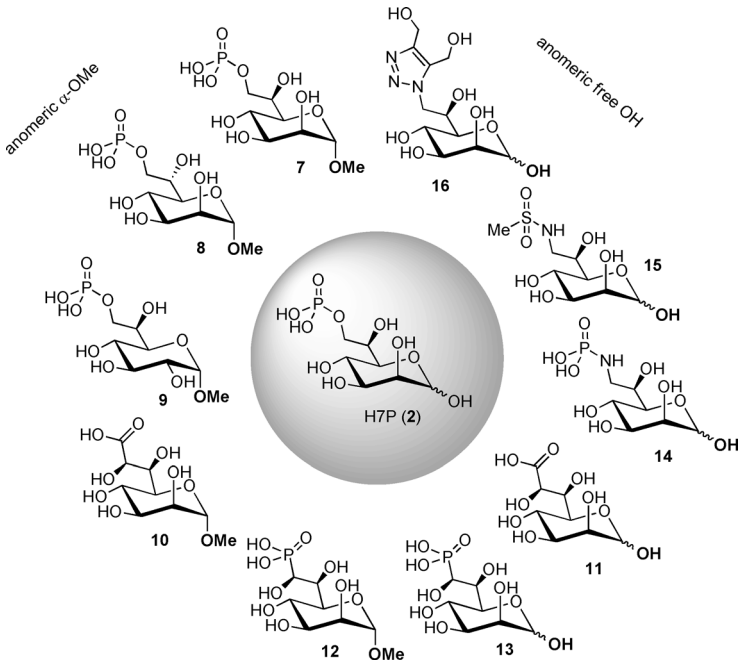
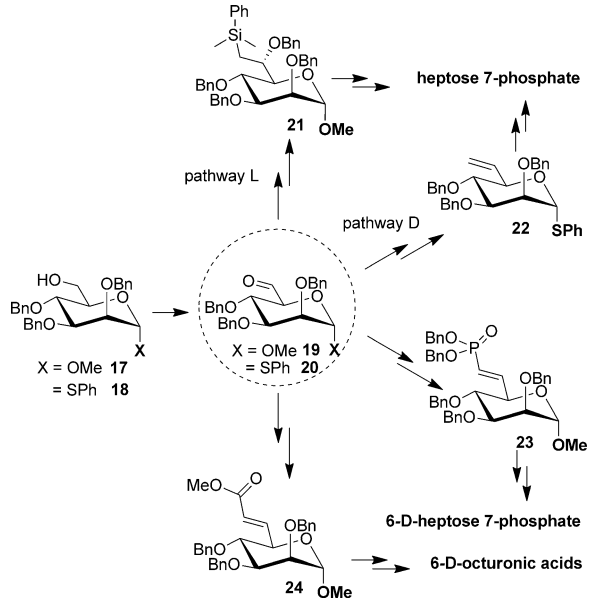


Figure 1. Targeted H7P analogues.

Results and Discussion

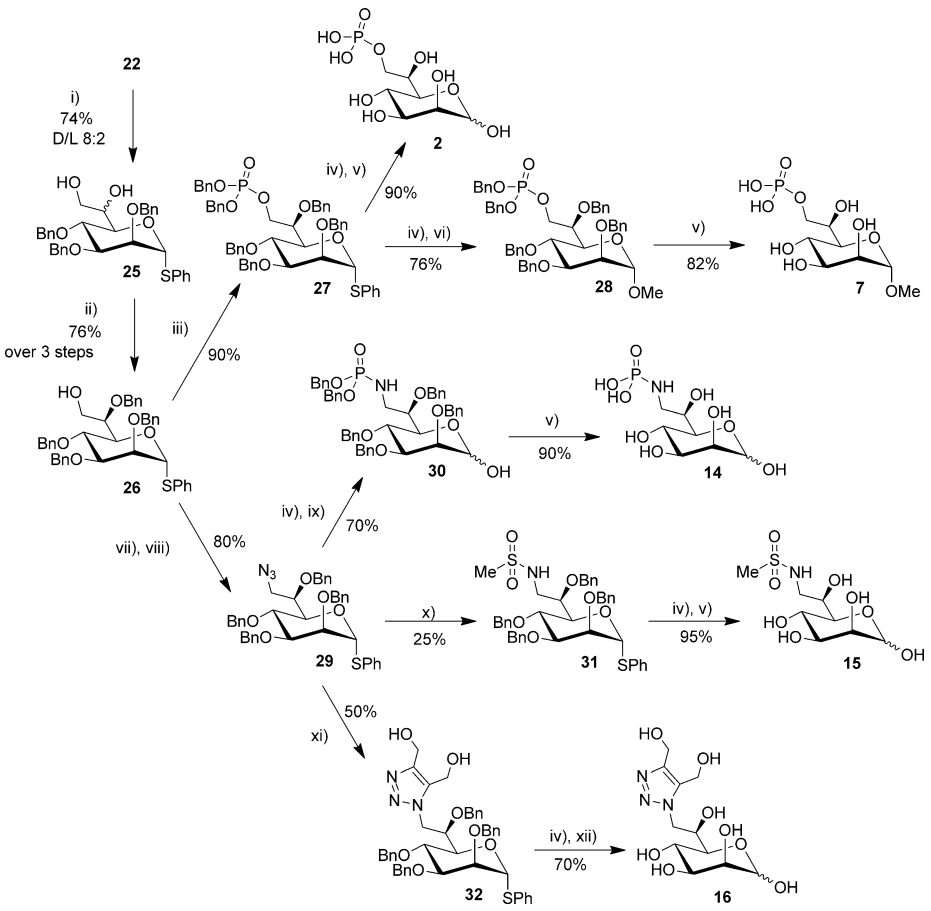
Several synthetic procedures have been developed for the construction of L-heptoses, either through a homologation of a mannoside,[31,32] or by reconstitution of the heptose skeleton through a central asymmetric aldol reaction.[33] For the synthesis of fluoro-heptosides, we also explored the stereoselective epoxide formation followed by a cesium acetate opening.[10] The two strategies that are the most commonly used to date are a D-selective dihydroxylation of alkene **22** (Scheme 2, pathway D)[34] followed, if L-heptosides are required, by a Mitsunobu inversion,[35–37] and the L-selective addition of Grignard reagents (pathway L).[38–46]



Scheme 2. General synthetic scheme.

General synthesis of D-*glycero*-D-*manno*heptosides: For the synthesis of all analogues of **2** with the D-*glycero*-D-*manno*-heptose scaffold, we followed a general synthetic pathway depicted in Scheme 3. This strategy relies on the known pathway described in the literature for the construction of D-heptosides (see Scheme 2).[47,48] The anomeric thiophenyl functionality was selected for its robustness and because it allows both the introduction of α-methyl or a chemoselective hydrolysis to provide lactols. Starting alkene **22** was obtained by a Swern oxidation of alcohol **18** directly followed by a Wittig olefination (Scheme 2).

The latter was dihydroxylated in 74% yield with a 4:1 D/L ratio. The pure D-*glycero* diol **25** could be separated at this stage by careful silica gel chromatography and was transformed into the desired 6-benzylated heptoside **26** through a three-step sequence of protective group manipulations that required a single purification final step. Intermediate **26**, the central knot of our divergent synthetic approach could be produced in 11 steps in a robust multigram scale with an overall yield of 27% from D-mannose. Phosphorylation of **26** under standard Mitsunobu conditions yielded 7-phosphate **27** in 90% yield. This intermediate could easily be de-



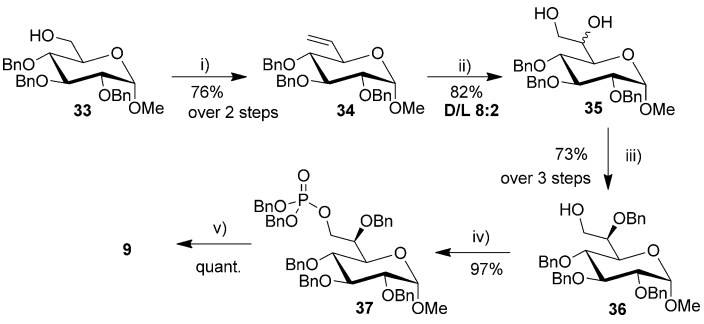
Scheme 3. Synthesis of H7P analogues with a D-*glycero*-D-*manno*-heptoside core structure: i) 10% $K_2OsO_4(H_2O)_2$, $K_3Fe(CN)_6$, K_2CO_3, *t*BuOH/H_2O 1:1, 0°C→RT; ii) a) TIPSCl, imidazole, THF, 0°C→RT, then ii) b) BnBr, NaH, DMF, 0°C→RT, then ii) c) TBAF, THF, 0°C→RT; iii) PPh_3, $(BnO)_2PO_2H$, NEt_3, DEAD 40% in toluene, THF, RT; iv) NBS, acetone/H_2O, −15°C; v) Pd/C 10%, H_2, AcOEt/EtOH/H_2O 3:5:2, RT; vi) MeI, Ag_2O, DMF, RT; vii) MsCl, DMAP, Py; viii) NaN_3, TBABr, DMF, 90°C; ix) $(BnO)_3P$, toluene, 90°C; x) a) PPh_3, toluene, then x) b) MsCl, then x) c) $NaHCO_{3\,aq}$; xi) 2-butyn-1,4-diol, 110°C; xii) H_2, 20% $Pd(OH)_2$, MeOH.

protected to give the natural occuring compound **2**,[22,48] that was used as substrate of HldE for the inhibition assays. In parallel, the thioacetal **27** was easily transformed into methyl heptoside **28** under standard glycosidation conditions and hydrogenolyzed to furnish **7**, the first key analogue of H7P **2**.

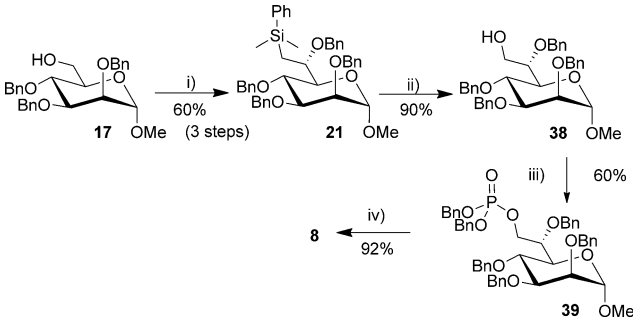
A two-step azidation protocol furnished 7-azide **29** in 80% yield from **26**. A Huisgen cycloaddition involving this second key intermediate **29** and a symmetrical butynediol yielded triazole **16** after the two usual deprotection steps. On the other hand, the azide functionality of **29** could be chemoselectively reduced into an intermediate primary amine that was transformed either into sulfonamide **31** with mesyl chloride or phosphoramide **30** using dibenzyl chlorophosphate. The optimized deprotection protocol thus provided two additional heptosides **14** and **15**.

Synthesis of the D-*glycero*-D-*gluco* epimeric analogue of inhibitor 7: As mentioned above, we considered that it was important to probe the tolerance of the targeted enzymes regarding modifications at the 2-position of the heptose skeleton. In fact, for the synthesis of ADP-2-fluoroheptose that we synthesized as a potent inhibitor of heptosyltransferase WaaC, we had already studied the homologation of methyl α-glucosides and demonstrated the D and L configurations of the heptosides obtained either by dihydroxylation of **34** (see Scheme 4) or by nucleophilic epoxide opening.[10] The sequence silylation/benzylation/desilylation produced alcohol **36** in 60% yield over three steps. A Mitsunobu phosphorylation followed by hydrogenolysis furnished the desired glucoheptoside **9** in 97% yield for the two last steps.

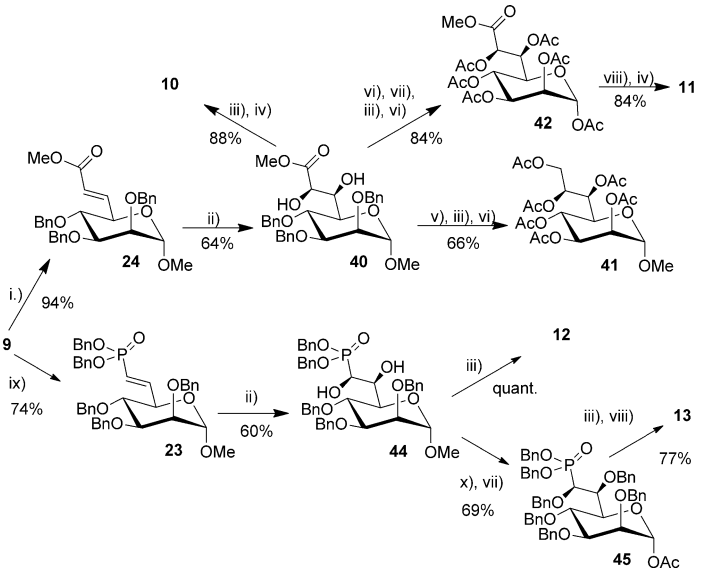
Synthesis of 8, the L-*glycero*-D-*manno* epimeric analogue of inhibitor 7: With the same rationale than for the preceding molecule, we developed the synthesis of the epimer at the 6-position of the mannoheptose core structure. Due to its extremely high L-diastereoselectivity (Scheme 1, pathway L), the most appealing strategy for us was the sequence developed by van Boom and co-workers[39,40,49] which consists in the addition of the silylated Grignard reagent $PhMe_2SiCH_2MgCl$ followed by a Fleming–Tamao oxidation (Scheme 5).[50–52]



Scheme 4. Synthesis of the D-*glycero*-D-*gluco* analogue **9**: i) a) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2, −78°C → 0°C, then i) b) Ph_3PCH_3Br, *n*BuLi (2.5 M), THF, −78°C→RT; ii) $K_2OsO_4(H_2O)_2$ 10%, NMO, H_2O/acetone 1:1, 0°C; iii) a) TIPSCl, Im, THF, 0°C → RT, then iii) b) BnBr, NaH, DMF, 0°C → RT, then iii) c) TBAF, THF, RT; iv) PPh_3, $(BnO)_2PO_2H$, NEt_3, DEAD 40% in toluene, THF, RT; v) 10% Pd/C, H_2, AcOEt/EtOH/H_2O 3:5:2, RT.



Scheme 5. Synthesis of the L-*glycero*-D-*manno* analogue **8**: i) a) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2, −78 °C → 0 °C, i) b) $PhSi(Me)_2$-CH_2-MgCl, THF, 0 °C, then i) c) BnBr, NaH, DMF, 0 °C → RT; ii) $Hg(OTFA)_2$, AcOH/Ac_2O 1:1, AcOK, 40% AcOOH in AcOH, 10 °C → RT; iii) PPh_3, $(BnO)_2PO_2H$, NEt_3, 40% DEAD in toluene, THF, RT; iv) 10% Pd/C, H_2, AcOEt/EtOH/H_2O 3:5:2, RT.



Scheme 6. Synthesis of octoses and phosphonates analogues of H7P **2**: i) $P(Ph_3)$=CH-CO_2Me, CH_2Cl_2, 0 °C → RT; ii) 10% $K_2OsO_4(H_2O)_2$, NMO, H_2O/acetone 1:2, 0 °C → RT; iii) 10% Pd/C, H_2, AcOEt/EtOH/H_2O 3:5:2, RT; iv) LiOH, H_2O, RT; v) LAH, THF, 0 °C → RT; vi) Ac_2O, Py, DMAP, RT; vii) Ac_2O, AcOH, H_2SO_4, 0 °C; viii) MeONa, MeOH,RT; ix) $CH_2(P(OBn)_2O)_2$, NaH, CH_2Cl_2, 0 °C → RT; x) BnBr, NaH, DMF, 0 °C → RT.

Indeed, this reaction gave the L-adduct in 75% yield with a D/L diastereoselectivity superior to 14:1 as reported in the literature. In our strategy, this sequence was also attractive because we could achieve the direct benzylation of the intermediate alcohol and obtain L-heptoside **21** ready for regioselective functionalizations at the 7-position without the need to manipulate protective groups to differentiate the 6- and the 7-position of heptosides. For the Fleming–Tamao step, we could reproduce the yields reported in the literature on a 50 mg scale (>60%) but we faced, however, major difficulties when we scaled-up this oxidation. Side reactions such as partial debenzylation very significantly decreased the yields (<30%). After inspection of the recent literature data, we found that Ley and co-workers had performed Fleming–Tamao oxidations on a very complex and sensitive structure exploiting a milder procedure based on the use of mercury salts.[53] Inspired by this seminal work, we developed a novel procedure for the Fleming–Tamao oxidation of intermediate **21** under almost neutral conditions. The yields were always in the range of 80–90% on a multigram scale. The resulting L-heptoside **38** could thus be engaged in a Mitsunobu phosphorylation. The subsequent hydrogenolysis yielded the desired final L-stereoisomer **8**. The same molecule had been already prepared from another Grignard reagent and a different sequence.[54,55]

Synthesis of octosides and phosphonates analogues of 2: The synthesis of the targeted octosides (Scheme 6) began with the Horner–Wadsworth–Emmons olefination of aldehyde **19** giving the expected (*E*) unsaturated ester **24** in 94% yield. A similar sequence has been followed in the literature for generating analogues of mannose-6-phosphates.[56] The subsequent *syn*-dihydroxylation yielded the (6*S*,7*R*)-isomer as expected from the Kishi's rule.[57] The two configurations were ascertained by X-ray crystallography of peracetate **41** obtained after reduction of the ester.[58] A careful acetolysis of **40** allowed the deprotection of the anomeric acetal and was directly followed by a debenzylation to give peracetate **42** in 84%. A sequence of Zemplén deacetylation followed by LiOH mediated methyl ester hydrolysis gave the best yield of target molecule **11** and was preferred as the direct total saponification of intermediate **42**.

A similar sequence could be applied from dibenzyl phosphonate **23**. The same *syn*-dihydroxylation gave diol **44** in 60% yield that could be deprotected either directly to furnish **12** or after the acetolysis sequence to provide lactol **13** in quantitative (one step) and 53% yield (three steps) from **44**, respectively.

Enzymatic assays: The IC_{50} values of compounds **7** to **16** were measured following the procedure we already described.[26] Among the two catalytic activities of the enzyme HldE, we naturally assayed the heptosides against the kinase activity.

An analysis of the IC_{50} values reported in Table 1 could lead us to draw clear conclusions about the effect of modifications of the heptose-7-phosphate structure and thus allowed a "mapping" of the interactions with these two bacterial enzymes. Compound **7**, the closest analogue of H7P **2** displayed a good inhibition level only against the kinase HldE. The inhibition profile of **7** against GmhA strongly suggests that mimicking the *product* of the first step of the heptose biosynthetic pathway is not the proper strategy to inhibit it. Then, we could demonstrate that the inversion of the configuration at C6 dramatically affects the affinity of the heptoside towards both enzymes. The L-heptose-phosphate **8** is a poor inhibitor of GmhA and does not display any inhibition on the kinase activity of HldE.

Table 1. Inhibition data.[a]

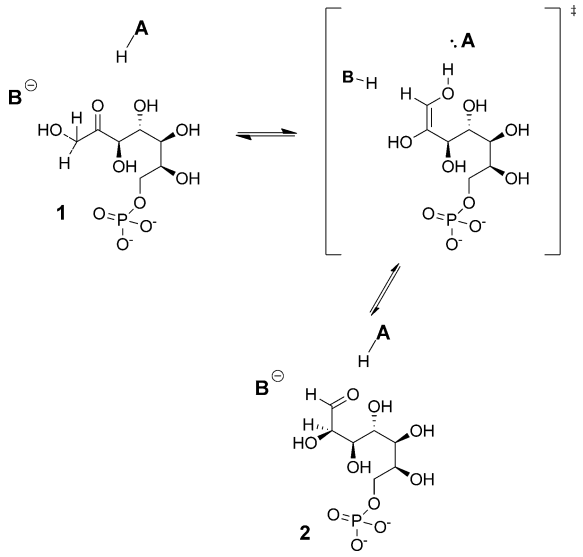
Compounds	Isomerase GmhA IC_{50} [μM]	Kinase HldE IC_{50} [μM]
7	>100	*16*
8	679	>1000
9	*34*	*9.4*
10	>1000	>1000
11	239	>1000
12	438	>1000
13	*47*	>1000[b]
14	1096	>1000[c]
15	>1000	>1000
16	756	>1000

[a] All measurements were repeated 3 to 6 times, then averaged. [b] 35% Inhibition at a concentration of 1000 μM. [c] 31% Inhibition at 1000 μM.

For the inhibition of isomerase GmhA, the presence of the anomeric α-methyl group affects the binding strength of the inhibitors. The relative IC_{50} values of carboxylates **10** and **11**, as well as phosphonates **12** and **13**, clearly show that the removal of the anomeric methyl group significantly enhances the binding affinities (almost 10 times for **13**). Such an effect cannot be discussed for the kinase activity of HldE, since the methyl glycoside **9** displayed a low micromolar inhibition profile while the other analogues were not inhibitors (with or without anomeric methyl groups).

It is tempting to rationalize our results in light with the crystallographic structure of GmhA. To date eight structures of the isomerase orthologues of 5 bacterial species are reported in the literature. The structures of GmhA are described in *apo*, substrate and product-bound forms. It has been suggested that GmhA can adopt two distinct conformations during isomerization through reorganization of the quaternary structure (open and closed). It was suggested[29] that the closed conformation of *B. pseudomallei*'s GmhA is catalytically relevant for the development of therapeutics. Unfortunately, for *E. coli*'s GmhA, only the open form was crystallized in *apo* or substrate-bound forms. Since our inhibition study has been performed on *E. coli*'s GmhA, we will only refer here to the *E. coli* structure.[28] The latter has been cocrystallized only with sedoheptulose-7-phosphate **1** (see Scheme 1 for the pyranose form). Very interestingly, this sugar is present in an acyclic ketone form (represented in Scheme 7) suggesting that the enzyme stabilizes the heptose in a linear tautomeric form. This stabilization is consistent with the two possible isomerization mechanisms, especially the one involving an enediol intermediate depicted in Scheme 7. This stabilization might also explain why lactols **11** and **13**, that can adopt an acyclic form within GmhA catalytic pocket, display significantly lower IC_{50} values than their corresponding methyl pyranosides **10** and **12** that are forced to bind the enzyme as a 6-membered ring.

Very interestingly, the two enzymes were found much more permissive to modifications at the 2-position of the carbohydrate scaffold. Thus, low-micromolar inhibition levels were measured for the *gluco* analogue **9** of *manno*-heptose H7P **2**. To our surprise, this epimeric analogue of H7P was the best inhibitor of both enzymes but also the



Scheme 7. Putative enzymatic isomerization of **2**.

only molecule of this series that could inhibit GmhA (IC_{50} = 34 μM) and HldE (IC_{50} = 9.4 μM) in the low micromolar range. This result is in deep contrast with the inhibition data discussed above for *manno*-heptoside **8**, another epimeric analogue of H7P. Regarding isomerase GmhA, this surprising result might be rationalized by the fact that this enzyme indeed transforms the 2-position by catalyzing a 1,2-ketol-to-aldol isomerization. Although a mechanism involving a hydride shift has not been totally ruled out, a double tautomerization involving an intermediate 1,2-enediol seems likely, as proposed by Junop[28] and Harmer[29] (see Scheme 7).

Thus, the conformation(s) adopted by the carbohydrate scaffold at the transition state(s) may place the 2-hydroxyl in an intermediate position between a pure "*manno*" (axial) or "*gluco*" (equatorial) positions. The slightly better inhibition level observed against HldE for the D-*gluco* derivative **9** compared to the D-*manno* analogue **7** is more difficult to rationalize. To study the binding mode of **9**, we performed a Lineweaver–Burk experiment (Figure 2) that led us to confirm that this *gluco*-heptose was indeed a competitor of the *manno*-heptose substrate **2**. This experiment also gave us a K_i of 5.2 μM for **9**, a value in agreement with its IC_{50}.

As for the epimer at C-6, the two enzymes were extremely sensitive to modifications of their substrate at C-7 despite the fact that in both cases, the catalytic transformation occurs at the anomeric position. This lack of tolerance to modification of the phosphate moiety was especially strong with the kinase HldE for which IC_{50}'s were always above 1 mM. Phosphate mimics **14**, **15** and **16** displayed weak inhibition profiles of GmhA, close to 1 mM. Phosphoramides such as **14** might be labile in aqueous solution and degrade under the assay conditions, thus causing a lack of inhibition. Surprisingly, carboxylate **11** displayed a significantly lower IC_{50} than phosphoramide **14**. This result could not be really anticipated from the simple examination of the structures of

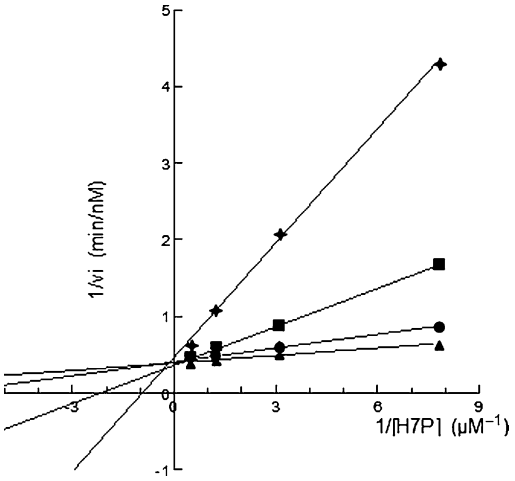


Figure 2. Competition between HldE substrate H7P ($K_M = 0.095\,\mu M$) and inhibitor **9** ($K_i = 5.2\,\mu M$; *: 33 μM, ■: 11 μM, ●: 3.7 μM, ▲: 0) for HldE (Lineweaver–Burk plot).

these molecules compared to **2**. To our delight, phosphonate **13** displayed a low micromolar IC_{50} against GmhA, a result that was rather unexpected given the poor inhibition of all other phosphate mimics.

An examination of *E. coli* GmhA structures shows that a collection of four serine and one threonine residues constitute the phosphate binding pockets.[28,29] Moreover, site-directed mutagenesis of the threonine residue led to an inactive enzyme.[28,29] These residues are structurally conserved and form tight contacts with the substrate **1**. Thus, even slight structural modifications of the phosphate moiety of substrate analogues might give repulsive interactions and dramatically decrease the binding affinity of the inhibitors.

Conclusion

In summary, we have reported a systematic study in which we synthesized and evaluated the binding affinities of a series of Heptose-7-phosphate analogues (H7P, D-*glycero*-D-*manno*-pyranose), an important intermediate in the LPS biosynthesis. Derivatizations of a central scaffold allowed the preparation of heptosides with structural modifications at the 1-, 2-, 6- and 7-positions. The inhibition profile of the whole inhibitor family towards the two first enzymes of the heptose biosynthetic pathway clearly indicated that the two enzymes are extremely sensitive to structural modifications of the heptose scaffold at the 6- and 7-positions. Interestingly, we could also show that both enzymes tolerated an epimerization at the 2-position and maintain low-micromolar inhibition levels with *gluco*-heptose analogues.

Overall, this study also showed that the two first enzymes of the heptose biosynthetic pathway do not show the same level of tolerance regarding the structural modifications of the H7P scaffold. Indeed, the isomerase GmhA was more permissive than the kinase HldE which only allowed structural changes at the 2-position of the heptose moiety. Noteworthy, this study describes the first inhibitors of GmhA ever reported.

From this systematic study and the recently published 3D-structures of GmhA, we are confident that the structural bases of the development of powerful inhibitors of this important biosynthetic pathway will be found and will ultimately lead to the discovery of novel therapeutic strategies to fight infectious diseases. Inhibitors of bacterial heptose synthesis are expected to prevent full LPS development in Gram negative bacteria, inducing a high sensitivity to the host complement and preventing or inhibiting bacterial infection. Small molecules inhibitors of heptose synthesis may therefore provide a novel way to treat bloodstream infections caused by pathogenic Gram-negative bacteria, without affecting the commensal flora and with less selective pressure than conventional antibacterial agents.

Experimental Section

General techniques: All reactions were carried out under an argon atmosphere. Yields refer to chromatographically and spectroscopically homogeneous materials. Reagents and chemicals were purchased from Sigma–Aldrich and Acros at ACS grade and were used without purification. All reactions were performed using purified and dried solvents: tetrahydrofuran (THF) was refluxed over sodium/benzophenone, dichloromethane (CH_2Cl_2), triethylamine (NEt_3), and pyridine were refluxed over calcium hydride (CaH_2). All reactions were monitored by thin-layer chromatography (TLC) carried out on Merck aluminum roll silica gel 60-F254 using UV light and a molybdate/sufuric acid solution as staining reagent. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a JEOL (JNM EX-400) or a Bruker (AMX-400) spectrometer at 20 °C. All compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR as well as by $^1H,^1H$ and $^1H,^{13}C$ correlation experiment when necessary. The following abbreviations were used to describe the multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, brs = broad singlet. The numbering of the protons and carbons is analogous to the proton numbers resulting from the name of the compound. Aromatic, benzyl, acetyl and methyl (carbons and protons) are respectively labeled with "arom", "Bn", "Ac" and "Me" subscript, quaternary carbons are indicated with a "q" subscript. Chemical shifts (δ) are reported in ppm and referenced indirectly to TMS via the solvent (or residual solvent) signals. Merck silica gel (60, particle size 0.040–0.063 mm) was employed for flash column chromatography and preparative thin layer chromatography using technically solvent distilled prior to use as eluting solvents. LC-MS measurement were performed on an Agilent 6200 series TOF mass spectrometer using an Agilent 1200 series LC system. Purifications of final molecules were realized by semi-preparative HPLC using a Waters Delta prep 4000 chromatography system equipped with a RP-C18 column (Agilent). Compounds **17**,[59] **18**,[60] **21**,[39] were prepared from known procedures.

Inhibition of the enzymatic activity of GmhA (luminescent assay): The assay buffer "AB" contained 50 mM Hepes pH7.5, 1 mM $MnCl_2$, 25 mM KCl, 0.012% Triton-X100 and 1 mM dithiothreitol (DTT) and 0.1 μM Myelin basic protein (MBP). The following components were added in a white polystyrene Costar plate up to a final volume of 30 μL: 10 μL inhibitor dissolved in DMSO/water 50/50, and 20 μL GmhA of *E. coli* in AB. After 30 min of pre-incubation at room temperature, 30 μL of Substrates mix in AB were added in each well to a final volume of 60 μL. This reaction mixture was then composed of 2 nM GmhA, 3 μM sedoheptulose-7-phosphate (Sigma), 3 μM ATP (Sigma) and 50 nM HldE of *E. coli* in assay buffer. After 30 min of incubation at room temperature, 100 μL of the revelation mix were added to a final volume of 160 μL, including the following constituents at the respective final concentrations: 10000 light units mL^{-1} luciferase (Sigma), 20 μM D-luciferin (Sigma),

100 μM N-acetylcysteamine (Aldrich). Luminescence intensity was immediately measured on Luminoskan (Thermofischer) and converted into inhibition percentages. For IC_{50} determinations, the inhibitor was tested at 6 to 10 different concentrations, and the related inhibitions were fitted to a classical langmuir equilibrium model using XLFIT (IDBS).

Inhibition of the enzymatic activity of HldE (luminescent assay on kinase activity): The assay buffer "AB" contained 50 mM Hepes pH7.5, 1 mM $MnCl_2$, 25 mM KCl, 0.012% Triton-X100 and 1 mM dithiothreitol (DTT) and 0.1 μM Myelin basic protein (MBP). The following components were added in a white polystyrene Costar plate up to a final volume of 30 μL: 10 μL inhibitor dissolved in DMSO/water 50:50, and 20 μL HldE of *E. coli* in AB. After 30 min of pre-incubation at room temperature, 30 μL of substrate mix in AB were added in each well to a final volume of 60 μL. This reaction mixture was then composed of 3 nM HldE, 0.2 μM of heptose-7-phosphate H7P **2** and 0.2 μM ATP (Sigma) in assay buffer. After 30 min of incubation at room temperature, 200 μL of the revelation mix were added to a final volume of 260 μL, including the following constituents at the respective final concentrations: 5000 light units mL^{-1} luciferase (Sigma), 30 μM D-luciferin (Sigma), 100 μM N-acetylcysteamine (Aldrich). Luminescence intensity was immediately measured on Luminoskan (Thermofischer) and converted into inhibition percentages. For IC_{50} determinations, the inhibitor was tested at 6 to 10 different concentrations, and the related inhibitions were fitted to a classical Langmuir equilibrium model using XLFIT (IDBS).

Please see the Supporting Information for synthetic experimental details and analytical data of all compounds.

Acknowledgements

This work was funded by FNRS (FRFC grant 2.4.625.08.F and "Crédit au chercheur" 1.5.167.10) and a Marie Curie RTN grant (2005-019561 "Dynamic"). We are also grateful to Mutabilis S.A. for their financial and technical supports.

[1] E. T. Rietschel, T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, *FASEB J.* **1994**, *8*, 217–225.
[2] S. Gronow, H. Brade, *J. Endotoxin Res.* **2001**, *7*, 3–23.
[3] A. Silipo, A. Molinaro, T. Ierano, A. De Soyza, L. Sturiale, D. Garozzo, C. Aldridge, P. A. Corris, C. M. A. Khan, R. Lanzetta, M. Parrilli, *Chem. Eur. J.* **2007**, *13*, 3501–3511.
[4] P. Kosma, *Curr. Org. Chem.* **2008**, *12*, 1021–1039.
[5] W. G. Coleman, L. Leive, *J. Bacteriol.* **1979**, *139*, 899–910.
[6] C. R. H. Raetz, C. Whitfield, *Annu. Rev. Biochem.* **2002**, *71*, 635–700.
[7] S. Escaich, *Curr. Opin. Chem. Biol.* **2008**, *12*, 400–408.
[8] M. A. Valvano, P. Messner, P. Kosma, *Microbiology* **2002**, *148*, 1979–1989.
[9] L. Cegelski, G. R. Marshall, G. R. Eldridge, S. J. Hultgren, *Nat. Rev. Microbiol.* **2008**, *6*, 17–27.
[10] H. Dohi, R. Périon, M. Durka, M. Bosco, Y. Roué, F. Moreau, S. Grizot, A. Ducruix, S. Escaich, S. P. Vincent, *Chem. Eur. J.* **2008**, *14*, 9530–9539.
[11] S. Grizot, M. Salem, V. Vongsouthi, L. Durand, F. Moreau, H. Dohi, S. Vincent, S. Escaich, A. Ducruix, *J. Mol. Biol.* **2006**, *363*, 383–394.
[12] M. Durka, K. Buffet, J. Iehl, M. Holler, J.-F. Nierengarten, J. Taganna, J. Bouckaert, S. P. Vincent, *Chem. Commun.* **2011**, *47*, 1321–1323.
[13] B. Kneidinger, C. Marolda, M. Graninger, A. Zamyatina, F. McArthur, P. Kosma, M. A. Valvano, P. Messner, *J. Bacteriol.* **2002**, *184*, 363–369.
[14] A. Zamyatina, S. Gronow, C. Oertelt, M. Puchberger, H. Brade, P. Kosma, *Angew. Chem.* **2000**, *112*, 4322–4325; *Angew. Chem. Int. Ed.* **2000**, *39*, 4150–4153.
[15] A. Graziani, H. Amer, A. Zamyatina, A. Hofinger, P. Kosma, *Tetrahedron: Asymmetry* **2007**, *18*, 115–122.
[16] F. Moreau, N. Desroy, J. M. Genevard, V. Vongsouthi, V. Gerusz, G. Le Fralliec, C. Oliveira, S. Floquet, A. Denis, S. Escaich, K. Wolf, M. Busemann, A. Aschenbrenner, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4022–4026.
[17] A. Mayer, M. E. Tanner, *Biochemistry* **2007**, *46*, 6149–6155.
[18] T. Kowatz, J. P. Morrison, M. E. Tanner, J. H. Naismith, *Protein Sci.* **2010**, *19*, 1337–1343.
[19] J. P. Morrison, J. A. Read, W. G. Coleman, M. E. Tanner, *Biochemistry* **2005**, *44*, 5907–5915.
[20] J. P. Morrison, M. E. Tanner, *Biochemistry* **2007**, *46*, 3916–3924.
[21] J. A. Read, R. A. Ahmed, J. P. Morrison, W. G. Coleman, M. E. Tanner, *J. Am. Chem. Soc.* **2004**, *126*, 8878–8879.
[22] J. A. Read, R. A. Ahmed, M. E. Tanner, *Org. Lett.* **2005**, *7*, 2457–2460.
[23] A. M. Deacon, Y. S. Ni, W. G. Coleman, S. E. Ealick, *Structure* **2000**, *8*, 453–462.
[24] H. Paulsen, M. Pries, J. P. Lorentzen, *Liebigs Ann. Chem.* **1994**, 389–397.
[25] G. P. De Leon, N. H. Elowe, K. P. Koteva, M. A. Valvano, G. D. Wright, *Chem. Biol.* **2006**, *13*, 437–441.
[26] N. Desroy, F. Moreau, S. Briet, G. Le Fralliec, S. Floquet, L. Durant, V. Vongsouthi, V. Gerusz, A. Denis, S. Escaich, *Bioorg. Med. Chem.* **2009**, *17*, 1276–1289.
[27] H. H. Nguyen, L. B. Wang, H. Huang, E. Peisach, D. Dunaway-Mariano, K. N. Allen, *Biochemistry* **2010**, *49*, 1082–1092.
[28] P. L. Taylor, K. M. Blakely, G. P. De Leon, J. R. Walker, F. McArthur, E. Evdokimova, K. Zhang, M. A. Valvano, G. D. Wright, M. S. Junop, *J. Biol. Chem.* **2007**, *283*, 2835–2845.
[29] N. J. Harmer, *J. Mol. Biol.* **2010**, *400*, 379–392.
[30] J. Seetharaman, K. R. Rajashankar, V. Solorzano, R. Kniewel, C. D. Lima, J. B. Bonanno, S. K. Burley, S. Swaminathan, *Proteins Struct. Funct. Bioinf.* **2006**, *63*, 1092–1096.
[31] J. Hannson, S. Oscarson, *Curr.Org. Chem.* **2000**, *4*, 535–564.
[32] S. Oscarson, *Carbohydr. Polym.* **2001**, *44*, 305–311.
[33] T. Ohara, A. Adibekian, D. Esposito, P. Stallforth, P. H. Seeberger, *Chem. Commun.* **2010**, *46*, 4106–4108.
[34] N. C. R. van Straten, N. M. A. J. Kriek, C. M. Timmers, S. C. M. Wigchert, G. A. van der Marel, J. H. van Boom, *J. Carbohydr. Chem.* **1997**, *16*, 947–966.
[35] D. Crich, A. Banerjee, *Org. Lett.* **2005**, *7*, 1395–1398.
[36] D. Crich, A. Banerjee, *J. Am. Chem. Soc.* **2006**, *128*, 8078–8086.
[37] L. Dong, J. M. Roosenberg, M. J. Miller, *J. Am. Chem. Soc.* **2002**, *124*, 15001–15005.
[38] H. Kubo, K. Ishii, H. Koshino, K. Toubetto, K. Naruchi, R. Yamasaki, *Eur. J. Org. Chem.* **2004**, 1202–1213.
[39] G. J. P. H. Boons, M. Overhand, G. A. van der Marel, J. H. van Boom, *Carbohydr. Res.* **1989**, *192*, c1–c4.
[40] G. J. P. H. Boons, G. A. van der Marel, J. T. Poolman, J. H. van Boom, *Recl. Trav. Chim. Pays-Bas* **1989**, *108*, 339–343.
[41] M. Dasser, F. Chretien, Y. Chapleur, *J. Chem. Soc. Perkin Trans. 1* **1990**, 3091–3094.
[42] K. Dziewiszek, A. Banaszek, A. Zamojski, *Tetrahedron Lett.* **1987**, *28*, 1569–1572.
[43] M. Kim, B. Grzeszczyk, A. Zamojski, *Tetrahedron Lett.* **2000**, *43*, 9313–9337.
[44] C. Bernlind, S. Bennett, S. Oscarson, *Tetrahedron: Asymmetry* **2000**, *11*, 481–492.
[45] C. Bernlind, S. Oscarson, *J. Org. Chem.* **1998**, *63*, 7780–7788.
[46] M. Jørgensen, E. H. Iversen, R. Madsen, *J. Org. Chem.* **2001**, *66*, 4625–4629.
[47] J. S. Brimacombe, A. K. M. S. Kabir, *Carbohydr. Res.* **1988**, *179*, 21–30.
[48] H. Güzlek, A. Graziani, P. Kosma, *Carbohydr. Res.* **2005**, *340*, 2808–2811.
[49] G. J. P. H. Boons, M. Overhand, G. A. van der Marel, J. H. van Boom, *Angew. Chem.* **1989**, *101*, 1538–1539; *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 1504–1506.
[50] I. Fleming, P. E. J. Sanderson, *Tetrahedron Lett.* **1987**, *28*, 4229–4232.

[51] G. J. P. H. Boons, C. J. J. Elie, G. A. van der Marel, J. H. van Boom, *Tetrahedron Lett.* **1990**, *31*, 2197–2200.
[52] G. J. P. H. Boons, G. A. Vandermarel, J. H. van Boom, *Tetrahedron Lett.* **1989**, *30*, 229–232.
[53] S. V. Ley, A. Abad-Somovill, J. C. Anderson, C. Ayats, R. Bänteli, E. Beckmann, A. Boyer, M. G. Brasca, A. Brice, H. B. Broughton, B. J. Burke, E. Clenator, D. Craig, A. A. Denholm, R. M. Denton, T. Durand-Reville, L. B. Gobbi, M. Göbel, B. L. Gray, R. B. Grossmann, C. E. Gutteridge, N. Hahn, S. L. Harding, D. C. Jennens, L. Jennens, P. J. Lovell, H. J. Lovell, M. L. de la Puente, H. C. Kolb, W.-J. Koot, S. L. Maslen, C. F. McCusker, A. Mattes, A. R. Pape, A. Pinto, D. Santafianos, J. S. Scott, S. C. Smith, A. Q. Somers, C. D. Spilling, F. Stelzer, P. L. Toogood, R. M. Turner, G. E. Veitch, A. Wood, C. Zumbrunn, *Chem. Eur. J.* **2008**, *14*, 10683–10704.
[54] A. Stewart, C. Bernlind, A. Martin, S. Oscarson, J. C. Richards, E. K. H. Schweda, *Carbohydr. Res.* **1998**, *313*, 193–202.
[55] B. Grzeszczyk, O. Holst, A. Zamojski, *Carbohydr. Res.* **1996**, *290*, 1–15.
[56] S. Vidal, C. Vidil, A. Morere, M. Garcia, J. L. Montero, *Eur. J. Org. Chem.* **2000**, 3433–3437.
[57] J. K. Cha, W. J. Christ, Y. Kishi, *Tetrahedron* **1984**, *40*, 2247–2255.
[58] M. Durka, J. Wouters, S. P. Vincent, unpublished results.
[59] H. B. Borén, K. Eklind, P. J. Garegg, B. Lindberg, A. Pilotti, *Acta Chem. Scand.* **1972**, *26*, 4143.
[60] Z. Szurmai, L. Baaltoni, A. Liptak, *Carbohydr. Res.* **1994**, *254*, 301–309.
[61] P. C. B. Page, M. J. McKenzie, J. A. Gallagher, *J. Org. Chem.* **2001**, *66*, 3704–3708.
[62] B. Grzeszczyk, O. Holst, S. Müller-Loennies, A. Zamojski, *Carbohydr. Res.* **1998**, *307*, 55–67.

Received: February 4, 2011
Revised: May 26, 2011
Published online: August 26, 2011

