Carbohydrate Research 344 (2009) 869–880

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Competitive inhibitors of type B ribose 5-phosphate isomerases: design, synthesis and kinetic evaluation of new D-allose and D-allulose 6-phosphate derivatives

Sandrine Mariano [a], Annette K. Roos [b,†], Sherry L. Mowbray [c], Laurent Salmon [a,*]

[a] Laboratoire de Chimie Bioorganique et Bioinorganique, CNRS-UMR 8182, Institut de Chimie Moléculaire et des Matériaux d'Orsay, Bâtiment 420, Université Paris-Sud XI, F-91405 Orsay, France
[b] Department of Cell and Molecular Biology, Uppsala University, Biomedical Center, Box 596, SE-751 24 Uppsala, Sweden
[c] Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Center, Box 590, SE-751 24 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 26 November 2008
Received in revised form 20 February 2009
Accepted 23 February 2009
Available online 3 March 2009

Keywords:
Sugar phosphates
Enzyme inhibitor
Hydroxamic acids
Ribose 5-phosphate isomerase
Allose 6-phosphate isomerase
Mycobacterium tuberculosis

ABSTRACT

This study reports syntheses of D-allose 6-phosphate (All6P), D-allulose (or D-psicose) 6-phosphate (Allu6P), and seven D-ribose 5-phosphate isomerase (Rpi) inhibitors. The inhibitors were designed as analogues of the 6-carbon high-energy intermediate postulated for the All6P to Allu6P isomerization reaction (Allpi activity) catalyzed by type B Rpi from *Escherichia coli* (EcRpiB). 5-Phospho-D-ribonate, easily obtained through oxidative cleavage of either All6P or Allu6P, led to the original synthon 5-dihydrogenophospho-D-ribono-1,4-lactone from which the other inhibitors could be synthesized through nucleophilic addition in one step. Kinetic evaluation on Allpi activity of EcRpiB shows that two of these compounds, 5-phospho-D-ribonohydroxamic acid and *N*-(5-phospho-D-ribonoyl)-methylamine, indeed behave as new efficient inhibitors of EcRpiB; further, 5-phospho-D-ribonohydroxamic acid was demonstrated to have competitive inhibition. Kinetic evaluation on Rpi activity of both EcRpiB and RpiB from *Mycobacterium tuberculosis* (MtRpiB) shows that several of the designed 6-carbon high-energy intermediate analogues are new competitive inhibitors of both RpiBs. One of them, 5-phospho-D-ribonate, not only appears as the strongest competitive inhibitor of a Rpi ever reported in the literature, with a K_i value of 9 μM for MtRpiB, but also displays specific inhibition of MtRpiB versus EcRpiB.

1. Introduction

Ribose 5-phosphate isomerase (Rpi, E.C. 5.3.1.6), a key enzyme of the pentose phosphate pathway and of the Calvin cycle of plants, is the metal-independent aldose–ketose isomerase that catalyzes the reversible conversion of D-ribose 5-phosphate (R5P) into D-ribulose 5-phosphate (Ru5P). As observed in the case of triosephosphate isomerase (TIM),[1] phosphoglucose isomerase (PGI),[2,3] and phosphomannose isomerase (PMI),[3,4] the isomerization reaction catalyzed is thought to proceed through a proton transfer mechanism between the two carbon atoms C-1 and C-2 of the substrates, concomitant with a proton transfer between the two oxygen atoms O-1 and O-2, and thus involves a 1,2-*cis*-enediol(ate) high-energy intermediate (HEI) as depicted in Chart 1.

Two unrelated forms of the enzyme, with no amino acid sequence similarity and different structures, are known to catalyze the isomerization. RpiA is the most common form and is found in almost all organisms including humans, *Spinacia oleracea*, and *Escherichia coli* (Ec). The second type, RpiB, is found mainly in bacteria, including *Mycobacterium tuberculosis* (Mt) and *E. coli*. Due to important differences in the two active sites including a change in the sequence position and nature of the catalytic base, *M. tuberculosis* and *E. coli* RpiBs were proposed to be representative of two distinct sub-families.[5] Indeed, we recently demonstrated, through kinetic analyses and structural studies, that EcRpiB, but not MtRpiB is also a functional allose 6-phosphate isomerase (Allpi), catalyzing the reversible isomerization of the 6-carbon sugars D-allose 6-phosphate (All6P) and D-allulose (also called D-psicose) 6-phosphate (Allu6P).[6,7] A proton transfer mechanism similar to that proposed for the Rpi activity would involve the 6-carbon HEI depicted in Chart 1.

While a number of strong competitive inhibitors designed as mimics of the HEI proposed to be involved in the isomerization of the 5-carbon sugars R5P and Ru5P have been reported in the literature, such as 4-phospho-D-erythronate,[8,9] 4-deoxy-4-phosphonomethyl-D-erythronate,[10] and 4-phospho-D-erythronohydroxamic acid,[9] to date no corresponding inhibitors based on the All6P to Allu6P isomerization reaction have been kinetically evaluated. Competitive inhibitors of Allpi activity would be

* Corresponding author. Tel.: +33 1 69 15 63 11; fax: +33 1 69 15 72 81.
E-mail address: laurent.salmon@u-psud.fr (L. Salmon).
† Present address: Structural Genomics Consortium, Oxford University, Old Road Campus Research Building, Roosevelt Drive, Oxford, OX3 7DQ, UK.

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.02.024

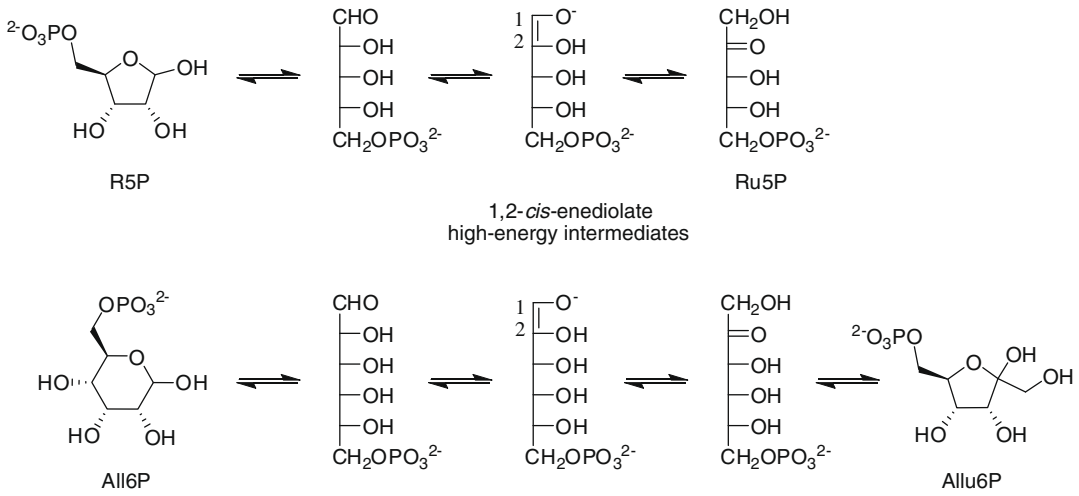


Chart 1. Isomerization reactions of D-ribose 5-phosphate (R5P) to D-ribulose 5-phosphate (Ru5P) and D-allose 6-phosphate (All6P) to D-allulose 6-phosphate (Allu6P).

important tools for future structural and mechanistic studies, as well as promising compounds for the design of species-specific therapeutic agents. Indeed, Allpi activity was suggested to be a characteristic of certain type B Rpis,[11,12] which are mainly found in bacteria, but not of type A Rpis (like human Rpi), which display only Rpi activity. Thus, specific inhibition of enzymes possessing Allpi activity while leaving unaffected those that have only Rpi activity could lead to a new type of highly selective antibiotics. For these reasons, we have actively sought inhibitors that are able to distinguish between enzymes catalyzing the two types of reactions.

A knockout study in *M. tuberculosis* has not yet been performed, but some evaluation of *rpiB* essentiallity is still possible.[6] Using transposon site hybridization experiments, Sassetti et al. identified *rpiB* as being essential for optimal growth of *M. tuberculosis* under some, but not all, circumstances.[13] Further, an Rpi of either type A or B (or both) is present in every complete genome, indicating that the activity is likely to play an important role in an organism's ability to maintain the correct balance of different nutrients in the cell. This is supported by a recent medical study that revealed that a human deficiency in RpiA causes destruction of myelin sheaths (leukoencephalopathy), which in turn leads to extensive brain abnormalities.[14] In the pathogens *Trypanosoma cruzi* and *M. tuberculosis*, the fact that an RpiB is the only Rpi present, and that such enzymes have no homologues in upper eukaryotic organisms, opens up the possibility of considering them as potential targets for the treatment of Chagas disease and tuberculosis.[15]

In this study, we report the synthesis of five new potential Rpi inhibitors, and an improved preparation of two previously described (one potential and one known) Rpi inhibitors, designed as analogues of the HEI postulated for the mechanism of the All6P to Allu6P isomerization (Chart 2). We also report a kinetic evaluation of these compounds on the Allpi activity of EcRpiB, as well as on the Rpi activities of both EcRpiB and MtRpiB. One of them appears as the strongest Rpi inhibitor reported to date.

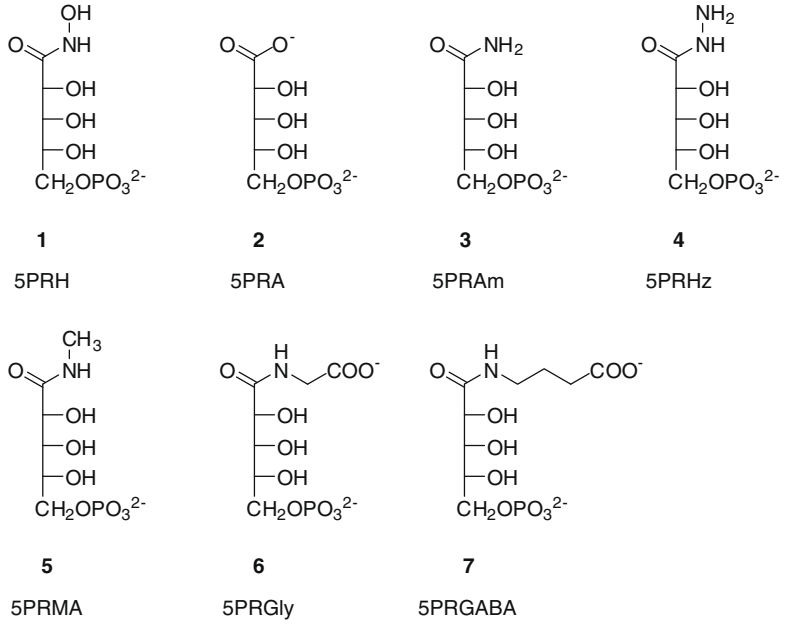
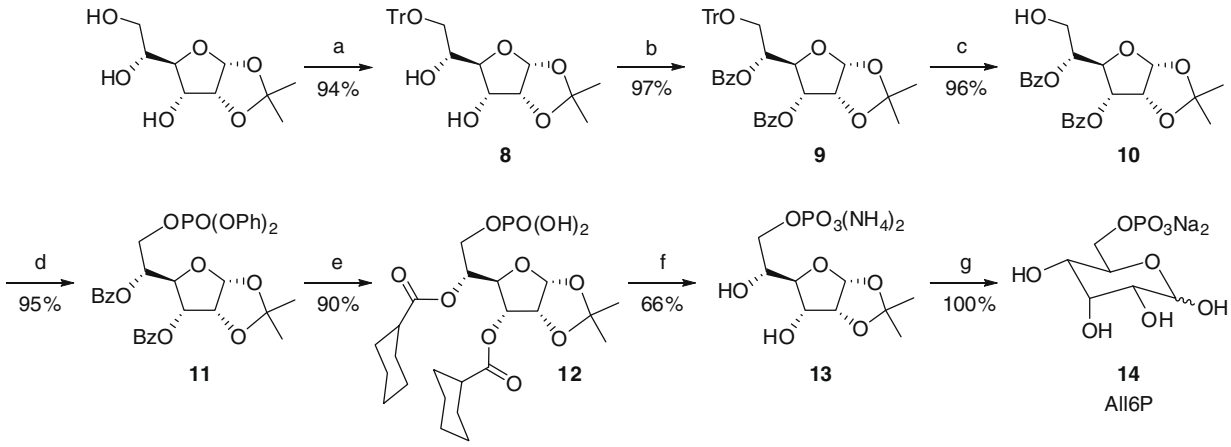
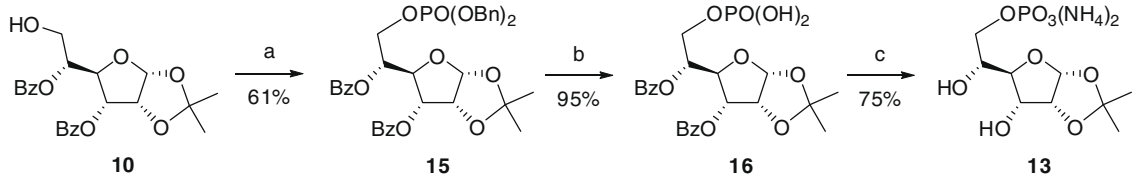


Chart 2. Structure of the inhibitors evaluated in this study: 5-phospho-D-ribonohydroxamic acid (5PRH, **1**), 5-phospho-D-ribonate (5PRA, **2**), 5-phospho-D-ribonamide (5PRAm, **3**), *N*-(5-phospho-D-ribonoyl)-hydrazine (5PRHz, **4**), *N*-(5-phospho-D-ribonoyl)-methylamine (5PRMA, **5**), *N*-(5-phospho-D-ribonoyl)-glycine (5PRGly, **6**), *N*-(5-phospho-D-ribonoyl)-γ-aminobutanoate (5PRGABA, **7**).



Scheme 1. Synthesis of D-allose 6-phosphate, disodium salt (All6P, **14**) using diphenylphosphochloridate as the phosphorylating agent. Reagents and conditions: (a) TrCl, DMAP, pyridine, 60 °C, 12 h; (b) BzCl, pyridine, rt, 12 h; (c) H_2 (30 bar)/Pd–C (10%), CH_2Cl_2/MeOH (4/1), 5 days; (d) $(PhO)_2POCl$, pyridine, rt, 5 h; (e) H_2 (4 bar)/PtO_2, MeOH, rt, 4 days; (f) NH_3/H_2O, MeOH, rt, 12 h; (g) 1-TFA, H_2O, rt, 2 h, 2-Dowex®-50WX8 (H^+), 3-Dowex®-50WX8 (Na^+).



Scheme 2. Synthesis of 1,2-*O*-isopropylidene-α-D-allofuranose 6-phosphate, diammonium salt (**13**) using dibenzyl *N*,*N*-diisopropylphosphoramidite as the phosphorylating agent. Reagents and conditions: (a) 1-$(BnO)_2P$–$N[CH(CH_3)_2]_2$, 1,2,4-triazole, CH_3CN, rt, 12 h; 2-^{t}BuOOH, CH_2Cl_2, rt, 2 h; (b) H_2 (10 bar)/Pd–C (10%), MeOH, rt, 4 h; (c) NH_3/H_2O, MeOH, rt, 12 h.

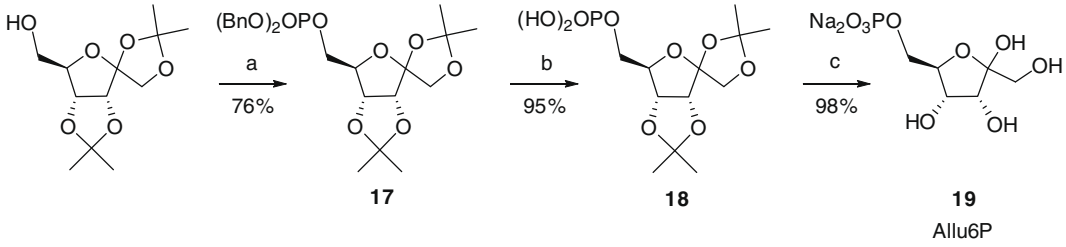
2. Results and discussion

As depicted in Chart 2, 5-phospho-D-ribonohydroxamic acid (5PRH, **1**),[16] which can be considered as a weak acid with a generally accepted pK_a in the range of 9–9.5 for hydroxamic acids, and 5-phospho-D-ribonate (5PRA, **2**)[8,17] were designed as mimics of the anionic 1,2-*cis*-enediolate HEI. 5-Phospho-D-ribonamide (5PRAm, **3**) and *N*-(5-phospho-D-ribonoyl)-hydrazine (5PRHz, **4**), which can be considered as the respective neutral analogues of **2** and **1**, as well as *N*-(5-phospho-D-ribonoyl)-methylamine (5PRMA, **5**), were designed as mimics of the neutral 1,2-*cis*-enediol HEI. Finally, *N*-(5-phospho-D-ribonoyl)-glycine (5PRGly, **6**) and *N*-(5-phospho-D-ribonoyl)-4-aminobutanoate (5PRGABA, **7**) were designed as new types of amino acid derivatives of phosphorylated monosaccharides valuable for studying the influence of the size of the upper moiety of Allpi inhibitors. With the exception of **2**, the inhibitors depicted in Chart 2 were all synthesized from a common synthon, itself obtained from All6P or Allu6P (**2** was directly obtained from All6P or Allu6P).

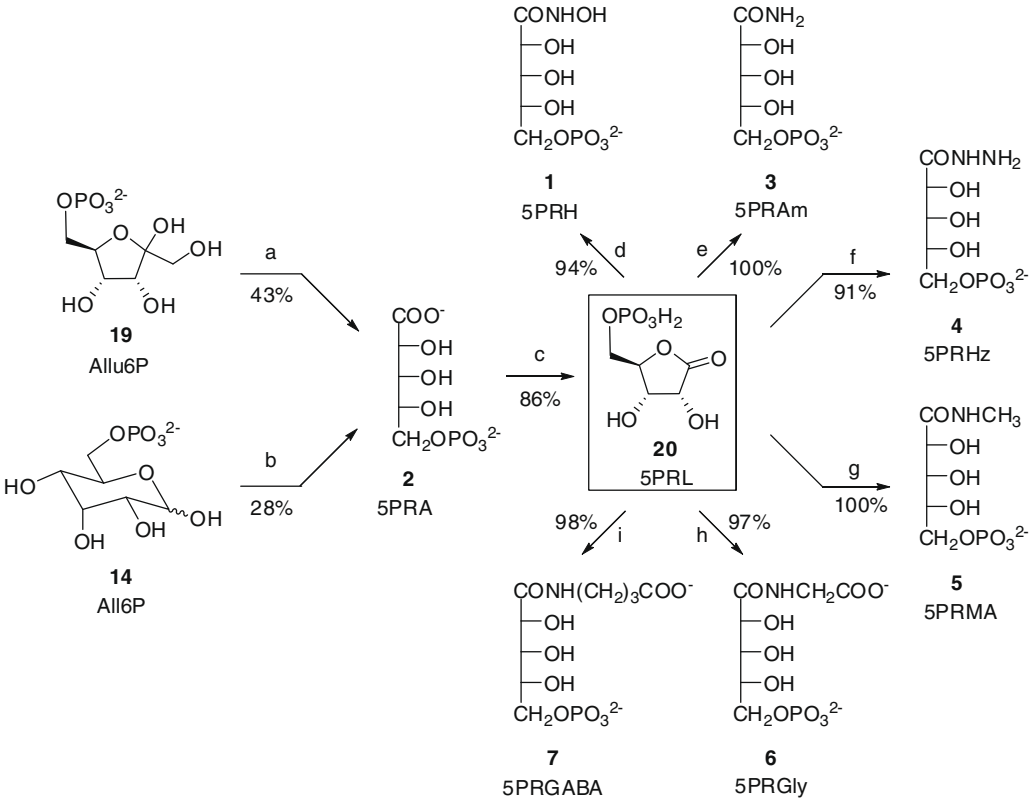
The synthesis of All6P is depicted in Schemes 1 and 2. Allu6P, the synthesis of which is depicted in Scheme 3, was used for the calibration of the thiobarbituric acid (TBA) solution (see Section 4), as well as for the kinetic studies. Synthesis of All6P (or Allu6P) provided the starting compounds needed for a straightforward synthesis of the new potential Allpi inhibitor, 5-phospho-D-ribonate (5PRA, **2**), which, upon cyclization in acidic medium, led to the original synthon 5-dihydrogenophospho-D-ribono-1,4-lactone. Indeed, we had previously reported the successful synthesis of several new and strong PGI inhibitors from the synthon 5-dihydrogenophospho-D-arabinono-1,4-lactone,[18] easily obtained from its corresponding aldonate 5-phospho-D-arabinonate.[19] Similarly, the reaction of defined nucleophilic amines with the synthon here allowed us to achieve the one-step and almost quantitative syntheses of new potential Allpi inhibitors **1** and **3**–**7,** as depicted in Scheme 4.

2.1. Synthesis of enzyme substrates

D-Allose 6-phosphate (All6P) was obtained in seven steps from 1,2-*O*-isopropylidene-α-D-allofuranose[20,21] with an overall yield of 50% (Scheme 1). Alternative chemical and enzymatic syntheses of All6P have been reported earlier starting from much less afford-



Scheme 3. Synthesis of D-allulose (or D-psicose) 6-phosphate, disodium salt (Allu6P, **19**). Reagents and conditions: (a) 1-$(BnO)_2P$–$N[CH(CH_3)_2]_2$, 1,2,4-triazole, CH_3CN, rt, 12 h; 2-^{t}BuOOH, CH_2Cl_2, rt, 1 h; (b) H_2 (10 bar)/Pd–C (10%), MeOH, rt, 4 h; (c) 1-TFA, H_2O, rt, 2 h, 2-Dowex®-50WX8 (H^+), 3-Dowex®-50WX8 (Na^+).



Scheme 4. Synthesis of 5-phospho-D-ribonohydroxamic acid (5PRH, **1**), 5-phospho-D-ribonate (5PRA, **2**), 5-phospho-D-ribonamide (5PRAm, **3**), *N*-(5-phospho-D-ribonoyl)-hydrazine (5PRHz, **4**), *N*-(5-phospho-D-ribonoyl)-methylamine (5PRMA, **5**), *N*-(5-phospho-D-ribonoyl)-glycine (5PRGly, **6**), *N*-(5-phospho-D-ribonoyl)-4-aminobutanoate (5PRGABA, **7**), and 5-dihydrogenophospho-D-ribono-1,4-lactone (5PRL, **27**) from D-allulose (or D-psicose) 6-phosphate (Allu6P, **19**) or D-allose 6-phosphate (All6P, **14**): (a) O_2, NaOH 0.5 M, rt, 48 h; (b) O_2, NaOH 0.5 M, rt, 72 h; (c) 1-Dowex®-50WX8 (H^+), 2-lyophilization; (d) NH_2OH/H_2O, rt, 15 min; (e) NH_3/H_2O, rt, 2 h; (f) H_2NNH_2/H_2O, rt, 3 h; (g) CH_3NH_2, rt, 12 h; (h) glycine, MeONa/MeOH, reflux, 10 min, then rt, 2 h; (i) γ-aminobutyric acid, reflux, 10 min, then rt, 2 h.

able D-ribose 5-phosphate[22] and D-allose,[23] respectively. The partially protected 1,2-*O*-isopropylidene-α-D-allofuranose, synthesized in three steps from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose according to literature procedures,[20,21,24–26] allowed selective tritylation of the primary hydroxyl group to obtain **8** in 94% yield. The fully orthogonally protected D-allose intermediate **9** was obtained almost quantitatively through benzoylation of the secondary hydroxyl groups. The primary alcohol **10** was then easily formed by hydrogenolysis which allowed its phosphorylation by diphenylphosphochloridate in pyridine to furnish the fully protected D-allose 6-phosphate derivative **11** in 91% yield (two steps). Compound **12** was obtained in 90% yield by hydrogenation over PtO_2, which not only deprotected the phosphate group, but also reduced benzoyl to cyclohexanoyl groups, as we previously reported for 2,3-di-*O*-benzoyl-5-diphenyphospho-D-ribono-1,4-lactone.[16] Removal of the cyclohexanoyl groups by aqueous ammonia gave the side product cyclohexylamide and the ammonium salt of 1,2-*O*-isopropylidene-6-phospho-D-allofuranose **13** (66% yield); the latter was quite hygroscopic. Acid hydrolysis of the remaining isopropylidene group followed by ion-exchange chromatography gave D-allose 6-phosphate **14** as the disodium salt in quantitative yield. As an alternative to the use of the platinium catalyst described above, we also achieved phosphorylation of compound **10** by dibenzyl *N*,*N*-diisopropylphosphoramidite and subsequent oxidation by *tert*-butylhydroperoxide[27] to obtain the phosphorylated D-allofuranose intermediate **15** in 61% yield (Scheme 2). Deprotection of the phosphate group through hydrogenation over Pd/C yielded the dihydrogenophosphate intermediate **16** (95%), which reacted with aqueous ammonia as above to give **13** in 75% yield and the side product benzylamide.

D-Allulose 6-phosphate (Allu6P), also called D-psicose 6-phosphate, was prepared in three steps from 1,2:3,4-di-*O*-isopropylidene-α-D-psicofuranose[28–30] with an overall yield of 71% (Scheme 3). The synthesis was based on the strategy initially developed by Herve du Penhoat and Perlin,[31] with the exception of the phosphorylation step. The starting compound was obtained in four steps from D-fructose according to literature procedures.[28–37] Phosphinylation of the primary hydroxyl group by dibenzyl *N*,*N*-diisopropylphosphoramidite and oxidation by *tert*-butylhydroperoxide[27] yielded the new phosphorylated psicofuranose **17** (76%). Although direct introduction of the phosphate group using diphenylphosphochloridate gave us the expected product as described,[31] the subsequent hydrogenolysis step over PtO_2 that would have led to **18** repeatedly did not work. The dihydrogenophosphate **18** was instead obtained quantitatively through hydrogenolysis of **17** over Pd/C 10%. Final hydrolysis of the isopropylidene groups with trifluoroacetic acid and ion-exchange chromatography gave D-allulofuranose (or D-psicofuranose) 6-phosphate **19** as the disodium salt[31] in almost quantitative yield (98%).

2.2. Synthesis of substrate-derived enzyme inhibitors

All6P and Allu6P, which have the same absolute configuration at carbon atoms C-3, C-4, and C-5 as compounds **1**–**7** have at carbon atoms C-2, C-3, and C-4, respectively, were considered as two possible starting compounds (Scheme 4). The synthesis of 5-phospho-D-ribonate **2** was achieved by oxidative cleavage of All6P **14** in 28% yield or from Allu6P **19** in 43% yield with molecular dioxygen and NaOH (Spengler–Pfannenstiel conditions), following the

strategy reported for the synthesis of 5-phospho-D-arabinonate from D-glucose 6-phosphate (G6P)[38] or from D-fructose 6-phosphate (F6P).[19] For reasons which remain unclear to us, the reaction appears less efficient on All6P or Allu6P than on G6P or F6P (where 78% yields were obtained). 5-Dihydrogenophospho-D-ribono-1,4-lactone (5PRL, **20**) was then obtained in 86% yield by freeze-drying of an aqueous solution of the acidic form of **2**. Although formation of **20** was mentioned in the kinetic study of the oxidation of R5P by chromium(VI)[39] or vanadium(V)[40] in perchloric acid media, we report here the first synthesis of this important phosphorylated sugar. Indeed, treatment of synthon **20**, which can be considered as an activated ester, with the nucleophilic amine derivatives hydroxylamine, ammonia, hydrazine, methylamine, glycine, and 4-aminobutyric acid easily and efficiently gave 5-phospho-D-ribonohydroxamic acid (5PRH, **1**), 5-phospho-D-ribonamide (5PRAm, **3**), *N*-(5-phospho-D-ribonoyl)-hydrazine (5PRHz, **4**), *N*-(5-phospho-D-ribonoyl)-methylamine (5PRMA, **5**), *N*-(5-phospho-D-ribonoyl)-glycine (5PRGly, **6**), and *N*-(5-phospho-D-ribonoyl)-4-aminobutanoate (5PRGABA, **7**), respectively, with yields ranging from 91% to quantitative (Scheme 4). With the exception of the hydroxamic acid **1,** for which we had previously reported a different synthesis,[16] all the products obtained are original compounds and were fully characterized.

2.3. Kinetic evaluation of substrate-derived enzyme inhibitors

Substrate-derived compounds **1**–**7** were tested for inhibition of the All6P to Allu6P isomerization (Allpi activity) catalyzed by EcRpiB, and of the R5P to Ru5P isomerization (Rpi activity) catalyzed by both EcRpiB and MtRpiB. Inhibition constants (K_i) and/or IC_{50} values obtained are reported in Table 1.

Kinetic evaluation on Allpi activity of EcRpiB shows that two of these compounds, 5-phospho-D-ribonohydroxamic acid (5PRH, **1**) and *N*-(5-phospho-D-ribonoyl)-methylamine (5PRMA, **5**), are new efficient inhibitors of the enzyme, with IC_{50} values of 0.62 and 0.35 mM, respectively. Because the TBA assay is not optimized for K_i determination, only 5PRH (**1**) was tested further; this compound displayed competitive inhibition, as shown by the Lineweaver–Burk plots depicted in Figure 1A, which gave a K_i value of 0.43 mM (Fig. 1B). In comparison to the K_m value of 2.9 mM we determined in this study for All6P isomerization, 5PRH (**1**) appears as a relatively good HEI analogue inhibitor of EcRpiB Allpi activity with a K_m/K_i ratio of about 7. As expected, shorter (**2** and **3**) and longer (**6** and **7**) HEI analogues gave higher IC_{50} values and thus behave as weak inhibitors (except **6** which does not inhibit EcRpiB Allpi activity). Although closely related to 5PRH (**1**) in structure, 5PRHz (**4**) is also a weak inhibitor of EcRpiB Allpi activity. Indeed, as discussed in the case of other aldose–ketose isomerases such as PGI,[18] this observation would be in accordance with the rather anionic nature of the HEI thought to be involved in the rate-determining step of the isomerization reaction of All6P catalyzed by EcRpiB.

Considering inhibition of EcRpiB Rpi activity by compounds **1**–**7**, Table 1 shows that, most often, close if not identical IC_{50} and/or K_i values were obtained (slight differences observed in some cases appear to be due to instability of EcRpiB when diluted). As K_i is the simple dissociation constant for the enzyme–inhibitor complex, and the same active site of EcRpiB acts on both 5- and 6-carbon linear substrates,[7] we indeed expect the K_i values to be the same for both of the activities. The K_i values we report for 5PRH (**1**), 5PRAm (**3**), and 5PRMA (**5**), 0.09, 0.07, and 0.18 mM, respectively, are among the best values ever reported against EcRpiB (Hamada et al. reported a value of about 0.07 mM using D-glucose 6-phosphate[41]). In comparison to the K_m value of 1.5 mM we measured for R5P, the three new EcRpiB inhibitors have 10–20 times more affinity for EcRpiB than the substrate. Although not as low as we had initially expected, these K_i values show that the design of inhibitors as mimics of the 6-carbon HEI postulated for Allpi activity is appropriate for inhibition of EcRpiB.

Table 1
Inhibition parameters (IC_{50} and K_i values) of compounds **1**–**7** for the All6P to Allu6P isomerization (Allpi activity) catalyzed by EcRpiB, and the R5P to Ru5P isomerization (Rpi activity) catalyzed by EcRpiB and MtRpiB

Inhibitor	Enzyme inhibition	EcRpiB Allpi[a]	EcRpiB Rpi[b]	MtRpiB Rpi[c,d]
5PRH (**1**)	IC_{50}	*0.62 ± 0.05*	*0.17 ± 0.02*	*0.44 ± 0.02*
	K_i	0.43 ± 0.04	0.09 ± 0.02	0.26 ± 0.01[e]
5PRA (**2**)	IC_{50}	*1.33 ± 0.09*	*1.31 ± 0.06*	*0.031 ± 0.002*
	K_i	n.d.[f]	n.d.	*0.009 ± 0.001*
5PRAm (**3**)	IC_{50}	*1.8 ± 0.3*[g]	*0.20 ± 0.02*	*0.22 ± 0.02*
	K_i	n.d.	0.07 ± 0.05	0.04 ± 0.01
5PRHz (**4**)	IC_{50}	*1.9 ± 0.3*	n.d.	*1.0 ± 0.1*
	K_i	n.d.	n.d.	n.d.
5PRMA (**5**)	IC_{50}	*0.35 ± 0.01*	*0.36 ± 0.02*	*0.19 ± 0.01*
	K_i	n.d.	0.18 ± 0.03	0.11 ± 0.02
5PRGly (**6**)	IC_{50}	n.i.[i]	*9 ± 1*	*0.57 ± 0.04*
	K_i	n.d.	n.d.	0.34 ± 0.01
5PRGABA (**7**)	IC_{50}	*1.6 ± 0.3*	*2.0 ± 0.2*	*1.42 ± 0.03*
	K_i	n.d.	n.d.	n.d.

IC_{50} values (in italic) and K_i values are given in mM.
[a] K_m (All6P) = 2.9 ± 0.8 mM (lit.[7]: K_m (All6P) = 0.5 ± 0.2 mM).
[b] K_m (R5P) = 1.5 ± 0.4 mM (lit.[7]: K_m (R5P) = 1.1 ± 0.2 mM; lit.[47]: K_m (R5P) = 1.23 mM).
[c] K_m (R5P) = 3.1 ± 0.7 mM (lit.[5]: K_m (R5P) = 3.7 mM; lit.[7]: K_m (R5P) = 1.0 ± 0.4 mM).
[d] IC_{50} (All6P) = 2.0 ± 0.4 mM; IC_{50} (Allu6P) = 6.3 ± 0.5 mM.
[e] Lit.[16]: K_i = 0.40 ± 0.04 mM.
[f] n.d.: not determined.
[g] Estimated value.
[i] n.i.: no inhibition.

In addition to EcRpiB Allpi and Rpi activities, inhibition properties of compounds **1**–**7** were evaluated on the Rpi activity of MtRpiB (Table 1), a more interesting enzyme from a therapeutic point of view, and for which we previously demonstrated that no Allpi activity exists.[7] Indeed, while most of compounds **1**–**7** were originally designed as mimics of the 6-carbon HEI postulated for Allpi activity, not for Rpi activity, several of them behave as new good competitive inhibitors of MtRpiB, such as 5PRH (**1**), 5PRMA (**5**), and even the rather long 5PRGly (**6**), with respective K_i values of 0.26, 0.11, and 0.34 mM (to be compared to the K_m value of 3.1 mM under the conditions used in this study). More interestingly, the two shortest of the evaluated compounds, namely 5-phospho-D-ribonate (5PRA, **2**) and 5-phospho-D-ribonamide (5PRAm, **3**), behave as new strong competitive inhibitors of MtRpiB with K_i values of 0.009 (Fig. 1C and D) and 0.04 mM, respectively, which gives corresponding K_m/K_i ratios of 344 and 77. To our knowledge, 5PRA (**2**) is the strongest competitive inhibitor of an Rpi ever reported in the literature. It should be noted that, from a theoretical point of view, the two inhibitors 5PRA (**2**) and 5PRAm (**3**) are slightly too long to act as mimics of the 5-carbon HEI postulated for the R5P isomerization reaction, having an additional oxygen atom and NH_2 group, respectively. Nevertheless, they prove to have favorable interactions in the MtRpiB active site that explain the observed strong stabilization of these 'imperfect' HEI analogues. We recently reported the high-resolution 3D crystal structures of a MtRpiB-5PRA complex [PDB (The Protein Data Bank:[42] www.rcsb.com) ID code: 2VVQ], which was obtained by fortuitious hydrolysis of 5PRH (**1**) under the crystallization conditions. This structure clearly shows the unexpected conformation of the upper moiety of the inhibitor in the enzyme active site (Fig. 2A) when compared to the structures of the enzyme complexed to linear R5P/Ru5P (PDB ID code: 2VVP) or 4-phospho-D-erythronohydroxamic acid (PDB ID code: 2BES).[7]

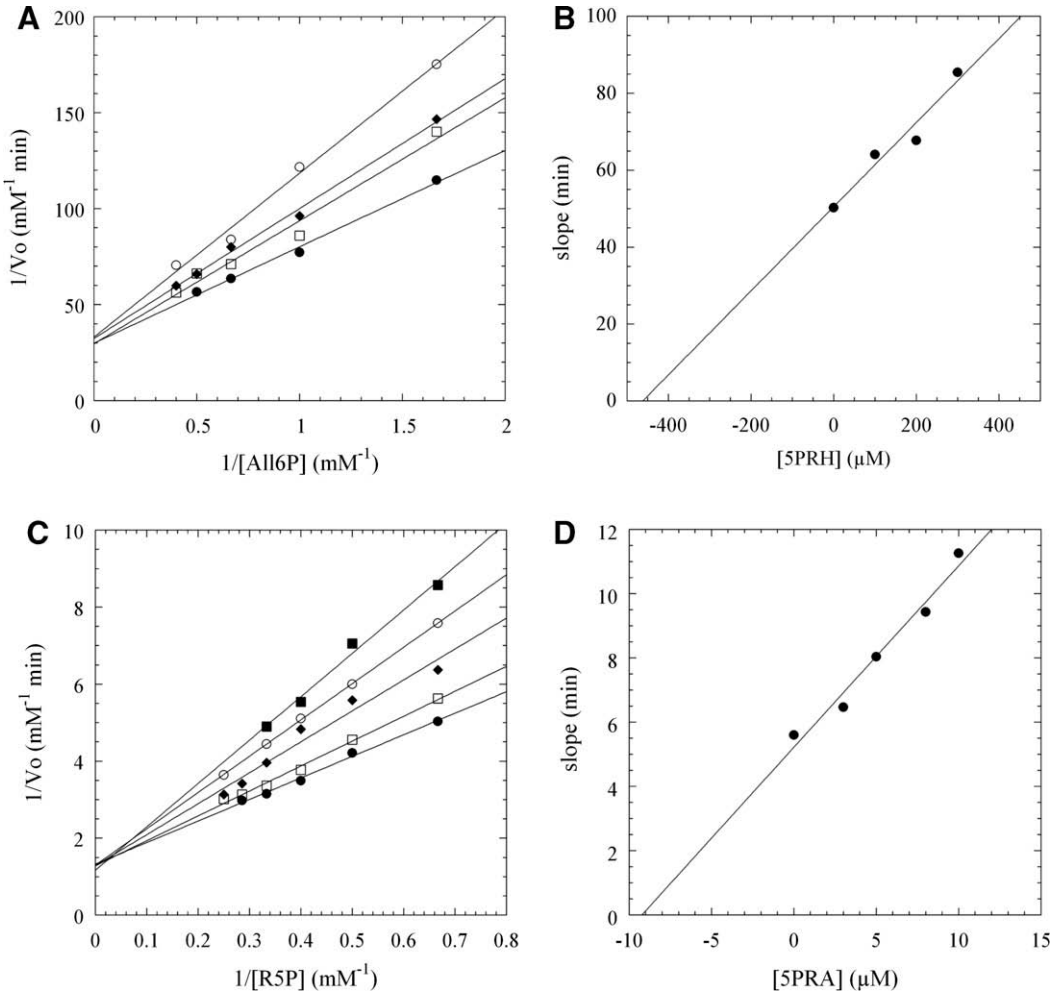


Figure 1. (A) Inhibition of EcRpiB (50 mM Tris·HCl buffer, 37 °C, pH 7.5) by 5-phospho-D-ribonohydroxamic acid (5PRH, **1**); double reciprocal plot of initial reaction velocity versus All6P concentration obtained at various concentrations of inhibitor: ●, no inhibitor; □, [*I*] = 100 μM; ♦, [*I*] = 200 μM; ○, [*I*] = 300 μM. (B) Secondary plot of apparent K_m/V_{max} values (slopes of the straight lines in the primary graph A) versus inhibitor concentration. (C) Inhibition of MtRpiB (50 mM Tris·HCl buffer, 37 °C, pH 7.5) by 5-phospho-D-ribonate (5PRA, **2**); double reciprocal plot of initial reaction velocity versus R5P concentration obtained at various concentrations of inhibitor: ●, no inhibitor; □, [*I*] = 3 μM; ♦, [*I*] = 5 μM; ○, [*I*] = 8 μM; ■, [*I*] = 10 μM. (D) Secondary plot of apparent K_m/V_{max} values (slopes of the straight lines in the primary graph C) versus inhibitor concentration. In graphs A and C, lines drawn were obtained from a linear regression fit of the observed data using Michaelis–Menten equation for competitive inhibition. In graphs B and D, the *x* intercept, which is equal to $-K_i$, was determined by linear regression.

Interestingly, while 5PRAm (**3**) is also a rather strong inhibitor of EcRpiB Rpi activity, with a K_i value of 0.07 mM, 5PRA (**2**) and 5PRGly (**6**) are quite weak inhibitors of the Rpi activity, with IC_{50} values of 1.31 and 9 mM, respectively. The observed specific inhibition of MtRpiB versus EcRpiB by 5PRA (**2**) and 5PRGly (**6**) clearly points out the important differences between the two RpiBs active sites in their stabilization of the HEI, confirming the initially proposed existence of two sub-families of type B Rpis, based on their sequences alone.[5]

The most striking difference between the active sites of *M. tuberculosis* and *E. coli* enzymes is the nature of the catalytic base, Glu75 and Cys66, respectively. Consideration of the active sites helps to explain some of the kinetic results we obtained. Figure 2 shows the active site of MtRpiB complexed to 5PRA (Fig. 2A, actual structure from 2VVQ) and to 5PRH (Fig. 2B, manually docked, using the position of the phosphate group and carbon atoms 2–5 of the inhibitor). The analysis clearly predicts steric hindrance for 5PRH with an improbable H-bond of 1.5 Å between the *N*-bonded hydroxyl group and the backbone NH group of Ser71, to be compared to 5PRA which gives a much more satisfactory H-bond of 2.7 Å. This structural comparison is in accordance with the respective K_i values of 0.26 and 0.009 mM we obtained. Figure 2 also shows the active site of EcRpiB complexed to 5PRA (Fig. 2C) and 5PRH (Fig. 2D) based on the partial ligand electron density seen in the EcRpiB-5PRH structure (Roos et al., unpublished). The figure clearly shows loss of two H-bonds when 5PRH is replaced by 5PRA. Again, this structural comparison is in accordance with the IC_{50} values we determined for the two compounds, 0.17 and 1.31 mM, respectively. Other results were more difficult to link to particular aspects of the protein structures. More structural work will be required to determine how the differences arise among the compounds, and between the two enzymes, particularly in the case of the *E. coli* enzyme, for which no well-defined inhibitor complexes are at present available.

3. Conclusion

This study reports syntheses of All6P, Allu6P and subsequently, Rpi inhibitors **1**–**7,** designed as analogues of the 6-carbon HEI postulated for EcRpiB Allpi activity. Several analogues, notably 5PRH (**1**), 5PRAm (**3**), and 5PRMA (**5**), appear as new efficient competitive inhibitors of Allpi/Rpi activity of EcRpiB. In addition, compounds **1**–**7** not only inhibit Allpi/Rpi activity of EcRpiB, but also Rpi activity of MtRpiB. Therefore, it appears that specific inhibition of Allpi/Rpi activity of EcRpiB versus Rpi activity of MtRpiB has not been

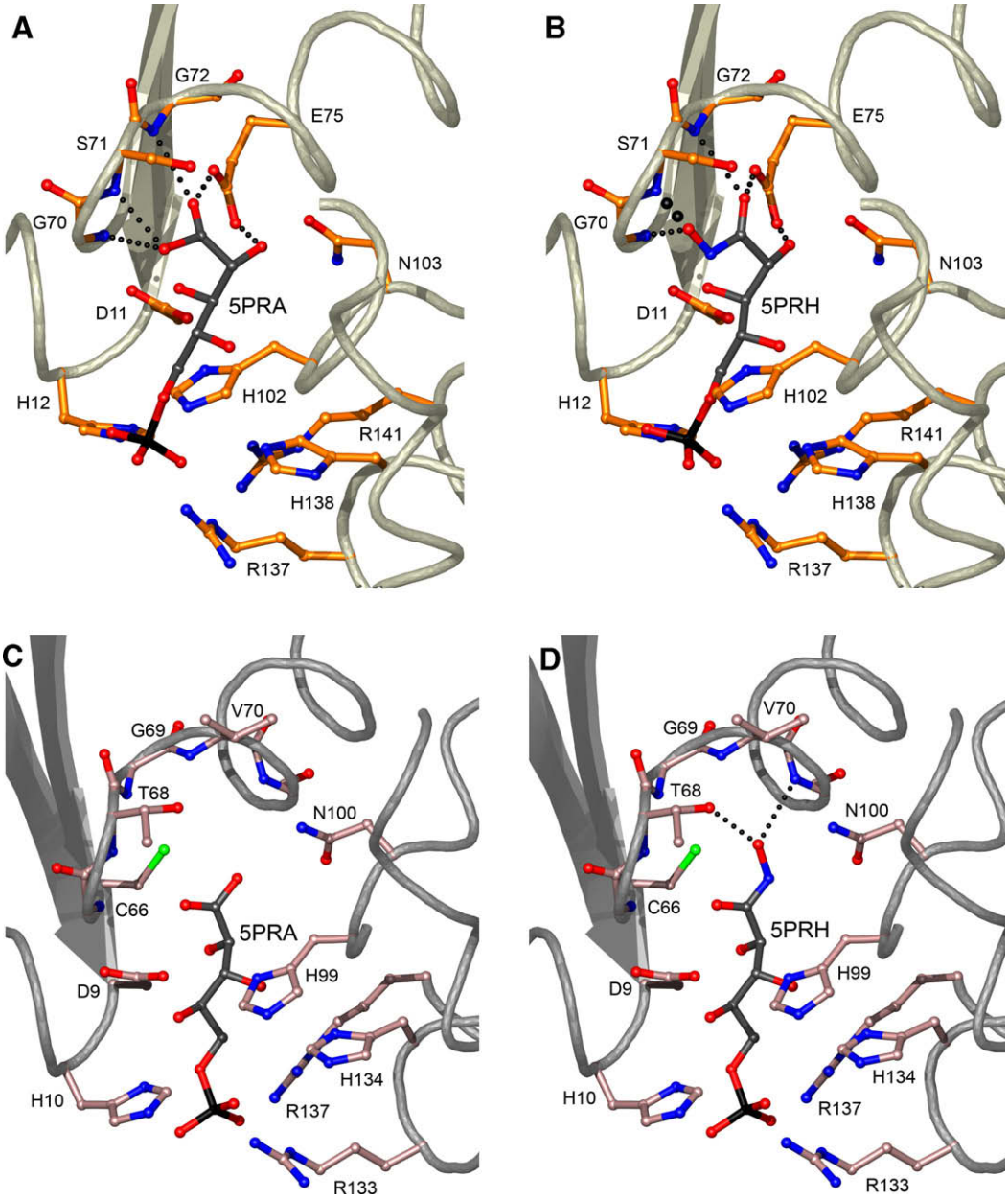


Figure 2. (A) Representation of the active site of MtRpiB complexed to 5PRA (**2**) from the parent X-ray 3D crystal structure (PDB ID code 2VVQ). (B) Model of the active site of MtRpiB complexed to 5PRH (**1**) obtained by manual docking based on the same structure (steric hindrance of the hydroxamic acid function is depicted as large circles). (C) Representation of the active site of EcRpiB complexed to 5PRA (**2**) obtained by manual docking using an EcRpiB–5PRH structure (undeposited, in which the electron density allows the placement of the phosphate of the ligand with some confidence, and the hydroxamic acid moiety with somewhat less precision; the OH groups of carbons 2 and 3 are not visible in the density). (D) Representation of the active site of EcRpiB complexed to 5PRH (**1**) from the same parent X-ray structure. Selected H-bonds are depicted as small circles. Some active site residues, water molecules, and H-bonds were omitted for clarity of the figure.

reached. However, species-specific inhibition of MtRpiB versus EcRpiB, which could be demonstrated for 5PRA (**2**) and 5PRGly (**6**), highlights the importance of testing the inhibition properties of 6- and 5-carbon HEI-analogue inhibitors on both Allpi and/or Rpi activities of type A Rpis (notably human RpiA) and type B Rpis of therapeutical interest, a subject that is now under investigation.

4. Experimental

4.1. General materials and methods

Unless otherwise stated, all chemical reagents were of analytical grade, obtained from Acros or Aldrich, and used without further purification. Solvents were obtained from VWR-Prolabo. Pyridine, MeOH, CH_2Cl_2, MeCN, and acetone were dried by refluxing with, respectively, KOH, Mg/I_2, CaH_2, P_2O_5, and $CaSO_4$, then distilled. Unless used immediately, dried MeOH and pyridine were stored over molecular sieves (respectively 0.3 and 0.4 nm) under argon. Column chromatography was performed using silica gel (70–200 μm, E. Merck). Flash chromatography was performed using silica gel (35–70 μm, E. Merck) under N_2 pressure. Ion-exchange chromatography was performed on Dowex®-50WX8 cation-exchange resin (H^+ or Na^+ form, 50–100 or 100–200 mesh, Acros), eluting with purified water (Millipore, 18.2 MΩ). Unless otherwise stated, all organic extracts were dried over $MgSO_4$ and filtered. Concentration of solutions was performed under diminished pressure at temperature <30 °C using a rotary evaporator. All air- and moisture-sensitive reactions were performed under an atmosphere

of argon. Analytical TLC was performed using Silica Gel 60 F_{254} precoated aluminum plates (E. Merck). Spots were visualized by treatment with 5% ethanolic H_2SO_4 followed by heating and/or by absorbance of UV light at 254 nm. Melting points were measured in open capillaries on a Büchi apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-370 digital polarimeter at 589 nm. NMR spectra were recorded at 297 K in $CDCl_3$, CD_3OD, and D_2O with a Bruker DPX 250 (^1H at 250.13 MHz, ^{13}C at 62.90 MHz, and ^{31}P at 101.26 MHz), DRX 300 (^1H at 300.13 MHz and ^{13}C at 75.47 MHz), or Avance 360 (^1H at 360.13 MHz and ^{13}C at 90.56 MHz) spectrometer using NMR Notebook 2.0 software. Chemical shifts are reported in ppm (δ) and coupling constants in hertz (J_{ij}). ^1H NMR spectra were referenced to internal residual $CHCl_3$ (δ 7.26), CD_2HOD (δ 3.31), and HOD (δ 4.80) for solns in $CDCl_3$, CD_3OD, and D_2O, respectively. For CH_2 groups, H-1′, H-5′, and H-6′ resonances appear arbitrarily at higher field than H-1, H-5, and H-6 resonances, respectively. ^{13}C NMR spectra were referenced to solvent for solns in $CDCl_3$ (δ 77.0) and CD_3OD (δ 49.1), and to dioxane (δ 67.4) for solns in D_2O. ^{31}P NMR spectra were referenced externally to 85% aq H_3PO_4 (δ 0.0). In most cases, COSY, HSQC, J-mod, and/or DEPT135 NMR spectra were recorded for assigning resonances. Infrared spectra were recorded with a FT-IR Bruker IFS-66 spectrometer. Low-resolution mass spectrometry (MS) and high-resolution mass spectrometry (HRMS) analyses were performed by the 'Service de Spectrométrie de Masse' (ICMMO, Orsay) using electrospray with positive ionization mode (ESI^+) for non-ionic compounds or negative ionization mode (ESI^-) for anionic compounds. Elemental analyses were performed by the 'Service de Microanalyse' (ICSN-CNRS, Gif-sur-Yvette).

4.2. Syntheses

4.2.1. Preparation of 5-phospho-D-ribonohydroxamic acid, monohydroxylammonium salt (1)[16]

A soln of compound **20** (98 mg, 0.43 mmol) in water (1.5 mL) was cooled to 4 °C. Aq NH_2OH (50% w/w, 260 μL, 4.4 mmol) was then added and the reaction mixture was stirred for 15 min. Following removal of excess hydroxylamine and water under high vacuum, lyophilization gave **1** (119 mg, 94%) as a very hygroscopic white powder: $[\alpha]_D^{28} + 3.3$ (*c* 1, water); ^1H NMR (250 MHz, D_2O): δ 4.10 (d, 1H, $J_{2,3}$ 3.7 Hz, H-2), 3.71–3.60 (m, 4H, H-3, H-4, H-5, H-5′); ^{13}C NMR (62.9 MHz, D_2O): δ 170.2 (C-1), 71.7 (C-3), 71.2 (C-2), 69.7 (d, $J_{C,P}$ 6.5 Hz, C-4), 65.0 (d, $J_{C,P}$ 2.9 Hz, C-5); ^{31}P NMR (101.2 MHz, D_2O): δ 3.7. All NMR data are in agreement with our previously reported values for the bis(hydroxylammonium) salt;[16] ESI^-MS: *m*/*z* 260.0 (100) $[M-NH_3OH]^-$, lit.[16] 260.1; $HRESI^-$MS: calcd for $C_5H_{11}NO_9P$: 260.0177 $[M-NH_3OH]^-$, found *m*/*z* 260.0173.

4.2.2. Preparation of 5-phospho-D-ribonate, trisodium salt (2)[8,17]

(A) D-Allulofuranose 6-phosphate, disodium salt **19** (0.5 g, 1.65 mmol) was dissolved in 0.5 M aq NaOH (7.5 mL) and vigorously stirred under oxygen (1 bar) for 72 h, during which time oxygen consumption was 25 mL (1.12 mmol, 68%). Stirring for an additional 24 h period did not increase oxygen consumption. The reaction mixture was adjusted to pH 1.5 with concentrated HCl (0.5 mL) and bubbled with N_2. Adjustment to pH 5 was then achieved with a satd aq $Ba(OH)_2$ soln (5.5 mL) and the barium salt of 5-phospho-D-ribonic acid precipitated by the addition of abs EtOH (2 vols, 27 mL). The filtered barium salt was suspended in water (3 mL) and the soln was adjusted again to pH 1.5 with concd HCl, bubbled with N_2, adjusted to pH 5 with satd aq $Ba(OH)_2$ (3.5 mL), and precipated as before (13 mL abs EtOH). The filtered barium salt was suspended again in water (3.5 mL) and a third precipitation was performed in the same manner, but with only a single volume (7 mL) of abs EtOH. The filtered and dried barium salt was dissolved in a minimum vol of water with 1 g of Dowex®-50WX8 (H^+ form) resin. The product was then successively eluted with water on two Dowex®-50WX8 ion-exchange columns (1-H^+ form, 2-Na^+ form) and lyophilized to afford **2** (220 mg, 43%) as a white powder. (B) The corresponding procedure was applied starting from D-allopyranose 6-phosphate **14** (1 g, 3.3 mmol) to afford **2** (270 mg, 26%): $[\alpha]_D^{28} - 2.8$ (*c* 1, water); ^1H NMR (250 MHz, D_2O): δ 4.16 (d, 1H, $J_{2,3}$ 2.3 Hz, H-2), 4.10–3.80 (m, 4H, H-3, H-4, H-5, H-5′); ^{13}C NMR (62.9 MHz, D_2O): δ 178.1 (C-1), 73.2 (C-3), 72.5 (C-2), 70.0 (d, $J_{C,P}$ 7.1 Hz, C-4), 66.4 (d, $J_{C,P}$ 4.3 Hz, C-5), in relatively good agreement with the data reported for the dilithium salt;[17] ^{31}P NMR (101.2 MHz, D_2O): δ 1.30; ESI^-MS: *m*/*z* 288.9 (19) $[M-Na]^-$, 266.9 (100) $[M-2Na+H]^-$.

4.2.3. 5-Phospho-D-ribonamide, disodium salt (3)

Compound **20** (105 mg, 0.46 mmol) was dissolved in 30% aq ammonia (3 mL, 2.3 mmol) and stirred at rt for 14 h. Following evaporation under diminished pressure, the bis(ammonium) salt of the title compound was eluted with water through a Dowex®-50WX8 (Na^+ form) ion-exchange column. Lyophilization afforded the disodium salt **3** (140 mg, 100%) as a white solid: ^1H NMR (250 MHz, D_2O): δ 4.39 (d, ^1H, $J_{2,3}$ 3.3 Hz, H-2), 4.03–3.88 (m, 6H, H-3, H-4, H-5, H-5′, NH_2); ^{13}C NMR (62.9 MHz, D_2O): δ 177.2 (C-1), 72.2 (C-2, C-3), 70.1 (d, $J_{C,P}$ 5.8 Hz, C-4), 65.1 (d, $J_{C,P}$ 4.5 Hz, C-5); ^{31}P NMR (101.2 MHz, D_2O): δ 4.3; ESI^-MS: *m*/*z* 244.0 (100) $[M-2Na+H]^-$; $HRESI^-$MS: calcd for $C_5H_{11}NO_8P$: 244.0228 $[M-2Na+H]^-$. Found *m*/*z* 244.0227.

4.2.4. *N*-(5-Phospho-D-ribonoyl)-hydrazine, bis(hydrazinium) salt (4)

Compound **20** (96 mg, 0.42 mmol) was dissolved in water (1.5 mL) and the soln was cooled to 4 °C and stirred. Aq hydrazine (64%, 205 μL, 4.2 mmol) was added and the soln stirred for 15 min. Water and excess hydrazine were removed under high vacuum. The residue was lyophilized to afford the bis(hydrazinium) salt **4** (125 mg, 91%) as a very hygroscopic white powder: ^1H NMR (250 MHz, D_2O): δ 4.24 (d, ^1H, J_{23} 3.2 Hz, H-2), 3.83–3.70 (m, 5H, H-3, H-4, H-5, H-5′, NH); ^{13}C NMR (62.9 MHz, D_2O): δ 172.4 (C-1), 72.1 (C-3), 71.7 (C-2), 69.9 (d, $J_{C,P}$ 5.7 Hz, C-4), 64.8 (d, $J_{C,P}$ 3.7 Hz, C-5); ^{31}P NMR (101.2 MHz, D_2O): δ 4.5; ESI^-MS: *m*/*z* 259.0 (100) $[M-2NH_3NH_2+H]^-$; $HRESI^-$MS calcd for $C_5H_{12}N_2O_8P$: 259.0337 $[M-2NH_3NH_2+H]^-$, found *m*/*z* 259.0348.

4.2.5. *N*-(5-Phospho-D-ribonoyl)-methylamine, disodium salt (5)

Compound **20** (94 mg, 0.41 mmol) was dissolved in 40% aq methylamine (2 mL, 2.3 mmol) and the soln was stirred at rt for 14 h. Evaporation, under diminished pressure, of water and excess methylamine gave the bis(methylammonium) salt of the title compound, which was thereafter eluted with water through a Dowex®-50WX8 (Na^+ form) ion-exchange column. Lyophilization afforded the disodium salt **5** (160 mg, 100%) as a white solid: ^1H NMR (250 MHz, D_2O): δ 4.19 (d, 1H, $J_{2,3}$ 3.8 Hz, H-2), 3.75 (m, 5H, H-3, H-4, H-5, H-5′, NH), 2.59 (s, 3H, CH_3); ^{13}C NMR (62.9 MHz, D_2O): δ 174.9 (C-1), 72.6 (C-3), 72.2 (C-2), 70.1 (d, $J_{C,P}$ 5.2 Hz, C-4), 64.9 (d, $J_{C,P}$ 3.7 Hz, C-5), 25.4 (C-6, CH_3); ^{31}P NMR (101.2 MHz, D_2O): δ 4.8; ESI^-MS: *m*/*z* 258.0 (100) $[M-2Na+H]^-$; $HRESI^-$MS: calcd for $C_6H_{13}NO_8P$: 258.0384 $[M-2Na+H]^-$. Found: *m*/*z* 258.0382.

4.2.6. *N*-(5-Phospho-D-ribonoyl)-glycine, trisodium salt (6)

Compound **20** (100 mg, 0.43 mmol) was dissolved under argon in anhyd MeOH (10 mL) and the soln was stirred. Glycine (330 mg, 4.4 mmol) and MeONa (140 mg, 2.6 mmol) were added and the soln was stirred at reflux for 10 min, then at rt for 2 h. The solvent was removed under diminished pressure and the residue was eluted with water on a Dowex®-50WX8 (Na^+ form) ion-exchange

column to furnish **6** (156 mg, 97%) as a white solid after lyophilization: ^{1}H NMR (360 MHz, D_2O): δ 4.33 (d, 1H, $J_{2,3}$ 2.4 Hz, H-2), 3.96–3.85 (m, 5H, H-3, H-4, H-5, H-5′, NH), 3.80 (d, 1H, $J_{6,6'}$ 17.5 Hz, H-6), 3.68 (d, 1H, H-6′); ^{13}C NMR (75.0 MHz, D_2O): δ 178.6 (COO^-), 173.8 (C-1), 72.4 (C-3), 72.3 (C-2), 69.9 (d, $J_{C,P}$ 5.8 Hz, C-4), 65.4 (d, $J_{C,P}$ 4.2 Hz, C-5), 42.9 (CH_2COO^-); ^{31}P NMR (101.2 MHz, D_2O): δ 2.41.

4.2.7. *N*-(5-Phospho-D-ribonoyl)-γ-aminobutanoate, trisodium salt (7)

Compound **20** (98 mg, 0.43 mmol) was dissolved under argon in anhyd MeOH (10 mL) and the soln was stirred. 4-Aminobutanoic acid (450 mg, 4.3 mmol) and MeONa (145 mg, 2.6 mmol) were added and the soln was stirred at reflux for 10 min, then at rt for 2 h. The solvent was removed under diminished pressure and the residue was eluted with water on a Dowex®-50WX8 (Na^+ form) ion-exchange column, then lyophilized to give **7** (170 mg, 98%) as a white solid: ^{1}H NMR (300 MHz, D_2O): δ 4.33 (d, 1H, $J_{2,3}$ 3.2 Hz, H-2), 3.98–3.90 (m, 5H, H-3, H-4, H-5, H-5′, NH), 3.19 (m ,2H, $NHCH_2$), 2.19 (m, 2H, CH_2COO^-), 1.74 (m, 2H, $NHCH_2CH_2$). ^{13}C NMR (75.0 MHz, D_2O): δ 182.7 (COO^-), 173.9 (C-1), 72.5 (C-3), 72.1 (C-2), 69.9 (d, $J_{C,P}$ 6.1 Hz, C-4), 65.5 (d, $J_{C,P}$ 3.8 Hz, C-5), 38.8 ($NHCH_2$), 34.7 (CH_2COO^-), 25.3 ($NHCH_2CH_2$); ESI$^-$MS: *m*/*z* 330.1 (100) [M−3Na+2H]; HRESI$^-$MS: calcd for $C_9H_{17}NO_{10}P$: 330.0596 [M−3Na+2H], found: *m*/*z* 330.0598.

4.2.8. 1,2-*O*-Isopropylidene-6-*O*-trityl-α-D-allofuranose (8)

1,2-*O*-Isopropylidene-α-D-allofuranose[21] (3.03 g, 13.7 mmol), trityl chloride (4.21 g, 15.1 mmol), and DMAP (0.42 g, 3.43 mmol) were dissolved under argon in pyridine (6 mL) at rt. The reaction mixture was stirred at 60 °C for 12 h and concentrated. The crude product was purified by column chromatography (1:1 pentane–EtOAc) to give **8** (5.97 g, 94%) as a white powder: mp 64 °C; $[\alpha]_D^{28}$ + 12.9 (*c* 1, CH_2Cl_2); R_f 0.48 (1:1 pentane–EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.51–7.27 (m, 15H, 3Ph), 5.81(d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 4.65 (dd, 1H, $J_{2,3}$ 5.1 Hz, H-2), 4.15 (m, 1H, $J_{5,6}$ 3.8, $J_{5,6'}$ 5.6, $J_{5,OH}$ 7.0, $J_{5,4}$ 8.8 Hz, H-5), 4.06 (m, 1H, $J_{3,OH}$ 3.2, $J_{3,4}$ 4.4 Hz, H-3), 3.92 (dd, 1H, H-4), 3.45 (dd, 1H, $J_{6,6'}$ 10.1 Hz, H-6), 3.32 (dd, 1H, H-6′), 2.84 (d, 1H, OH-5), 2.48 (d, 1H, OH-3), 1.61, 1.40 (2*s*, 6H, $2CH_3$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 143.5 (3*C*ipso Ph), 128.6 (6*C*m Ph), 127.8 (6*C*o Ph), 127.1 (3*C*p Ph), 112.8 (CMe_2), 103.8 (C-1), 87.1 (CPh_3), 79.8 (C-4), 79.3 (C-2), 71.9 (C-5), 71.0 (C-3), 64.3 (C-6), 26.6 and 26.4 ($2CH_3$); ESI$^+$MS: *m*/*z* 485.3 (100) [M+Na]$^+$; HRESI$^+$MS: calcd for $C_{28}H_{30}O_6Na$: 485.1935 [M+Na]$^+$. Found *m*/*z* 485.1950.

4.2.9. 3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-6-*O*-trityl-α-D-allofuranose (9)

Compound **8** (1.9 g, 4.1 mmol) was dissolved under argon in dry pyridine (6.5 mL) before addition of benzoyl chloride (1 mL, 8.62 mmol). The reaction mixture was stirred at rt for 15 h, then concentrated under diminished pressure. Following addition of water and extraction with CH_2Cl_2, evaporation under diminished pressure of the solvent yielded pure **9** (2.67 g, 97%) as a white powder: mp 72 °C; $[\alpha]_D^{26}$ + 71.5 (*c* 1, CH_2Cl_2); R_f 0.84 (1:1 pentane–EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.03 (dd, 2H, C*H* PhCO), 7.92 (dd, 2H, C*H* PhCO), 7.46–7.33 (m, 11H, C*H* arom), 7.25–7.21 (m, 10H, C*H* arom), 5.85 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 5.74 (ddd, 1H, $J_{5,6'}$ 4.1, $J_{5,4}$ 5.0, $J_{5,6}$ 6.4 Hz, H-5), 5.16 (dd, 1H, $J_{3,2}$ 5.0, $J_{3,4}$ 8.8 Hz, H-3), 4.99 (dd, 1H, H-2), 4.72 (dd, 1H, H-4), 3.52 (dd, 1H, $J_{6,6'}$ 10.1 Hz, H-6), 3.48 (dd, 1H, H-6′), 1.60, 1.35 (2*s*, 6H, $2CH_3$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 165.4 (2PhC=O), 143.6 (3*C*ipso Ph trityl), 133.1, 133.0 (2*C*p PhC=O), 129.7 (4*C*m PhC=O), 129.0 (2*C*ipso PhC=O), 128.6 (6*C*m Ph trityl), 128.2 (4*C*o PhC=O), 127.7 (6*C*o Ph trityl), 127.0 (3*C*p Ph trityl), 113.1 (CMe_2), 104.3 (C-1), 86.7 (CPh_3), 77.5 (C-2), 76.2 (C-4), 73.7 (C-3), 72.3 (C-5), 62.5 (C-6), 26.7 and 26.6 ($2CH_3$); ESI$^+$MS: *m*/*z* 693.4 (100) [M+Na]$^+$; HRESI$^+$MS: calcd for $C_{42}H_{38}O_8Na$: 693.2459 [M+Na]$^+$. Found *m*/*z* 693.2469.

4.2.10. 3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-allofuranose (10)

Compound **9** (2.04 g, 3.0 mmol) was dissolved in a mixture of anhyd CH_2Cl_2 (22 mL) and MeOH (6 mL). Following addition of Pd/C 10% (350 mg), the reaction mixture was stirred under H_2 (30 bar) for 4 d. The soln was then filtered and concentrated under diminished pressure. The residue was purified by column chromatography using initially CH_2Cl_2 as eluent to remove triphenylmethane, then 97:3 CH_2Cl_2–MeOH to furnish **10** (1.25 g, 96%) as a white crystalline powder (very hygroscopic): $[\alpha]_D^{27}$ + 105.3 (*c* 1, CH_2Cl_2); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.95–7.91 (m, 4H, C*H*m Ph), 7.48–7.42 (m, 2H, C*H*p Ph), 7.34–7.22 (m, 4H, C*H*o Ph), 5.88 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 5.43 (ddd, 1H, $J_{5,6}$ 4.0, $J_{5,6'}$ 5.4, $J_{5,4}$ 6.0 Hz, H-5), 5.17 (dd, 1H, $J_{3,2}$ 5.0, $J_{3,4}$ 8.9 Hz, H-3), 4.99 (dd, 1H, H-2), 4.65 (dd, 1H, H-4), 4.02 (dd, 1H, $J_{6,6'}$ 12.2 Hz, H-6), 3.96 (dd, 1H, H-6′), 1.57, 1.32 (2s, 6H, $2CH_3$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 166.0 and 165.5 (2PhC=O), 133.1 and 133.0 (2*C*p Ph), 129.6 (4*C*m Ph), 129.3 and 128.8 (2*C*ipso Ph), 128.2 and 128.1 (4*C*o Ph), 113.3 (CMe_2), 104.2 (C-1), 77.6 (C-2), 76.0 (C-4), 74.4 (C-3), 74.2 (C-5), 61.8 (C-6), 26.6 and 26.5 ($2CH_3$); ESI$^+$MS: *m*/*z* 451.1 (100) [M+Na]$^+$; HRESI$^+$MS: calcd for $C_{23}H_{24}O_8Na$: 451.1363 [M+Na]$^+$. Found *m*/*z* 451.1373. Anal. Calcd for $C_{23}H_{24}O_8$: C, 64.48; H, 5.65; O, 29.88. Found: C, 63.84; H, 5.78; O, 28.95.

4.2.11. 3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-allofuranose 6-diphenylphosphate (11)

Compound **10** (1.15 g, 2.68 mmol) was dissolved in anhyd pyridine under argon. Diphenylphosphate chloride was added and the reaction mixture was stirred at rt for 5 h. The soln was concentrated, water (50 mL) was added to the residue, and the product was extracted with CH_2Cl_2 (3 × 50 mL). The organic phases were dried on Na_2SO_4, filtered, and concentrated to afford pure **11** (1.68 g, 95%) as a white powder: mp 115 °C; $[\alpha]_D^{27}$ + 82.4 (*c* 1, CH_2Cl_2); R_f 0.51 (2:1 pentane–EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.86 (d, 4H, C*H*m PhC=O), 7.49–7.40 (m, 2H, C*H*p PhC=O), 7.30–7.15 (m, 14H, C*H*o PhC=O, C*H* PhO), 5.91 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 5.55 (m, 1H, H-5), 5.14 (dd, 1H, $J_{3,2}$ 4.9, $J_{3,4}$ 8.8 Hz, H-3), 5.02 (dd, 1H, H-2), 4.66 (m, 3H, H-4, H-6, H-6′), 1.57, 1.35 (2s, 6H, $2CH_3$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 164.9 and 164.8 (2PhC=O), 150.0 (d, $J_{C,P}$ 6.7 Hz, *C*ipso PhO), 149.9 (d, $J_{C,P}$ 6.7 Hz, *C*ipso PhO), 132.8 (2*C*p PhC=O), 129.4 and 129.3 (4*C*m PhC=O), 129.3 (2*C*p PhO), 128.5 and 128.3 (2*C*ipso PhC=O), 127.8 and 127.7 (4*C*o PhC=O), 125.1 and 125.0 (4*C*m PhO), 119.6 (d, $J_{C,P}$ 5.3 Hz, 2*C*o PhO), 119.5 (d, $J_{C,P}$ 5.3 Hz, 2*C*o PhO), 113.0 (CMe_2), 104.0 (C-1), 77.5 (C-2), 74.5 and 74.4 (C-3, C-4), 71.7 (d, $J_{C,P}$ 7.1 Hz, C-5), 66.8 (d, $J_{C,P}$ 4.6 Hz, C-6), 26.4 and 26.3 ($2CH_3$); ^{31}P NMR (101.2 MHz, $CDCl_3$): δ −12.1; ESI$^+$MS: *m*/*z* 683.1 (100) [M+Na]$^+$; HRESI$^+$MS: calcd for $C_{35}H_{33}O_{11}PNa$: 683.1676 [M+Na]$^+$, found *m*/*z* 683.1662. Anal. Calcd for $C_{35}H_{33}O_{11}P$: C, 63.63; H, 5.04; P, 4.69. Found: C, 63.93; H, 5.08; P, 4.86.

4.2.12. 3,5-Di-*O*-cyclohexanoyl-1,2-*O*-isopropylidene-α-D-allofuranose 6-dihydrogenophosphate (12)

Compound **11** (1.33 g, 2.01 mmol) dissolved in anhyd MeOH (20 mL) and PtO_2 (133 mg) were stirred under H_2 (4 bar) for 3 d. The soln was then filtered and concentrated under diminished pressure to give pure **12** (1.1 g, 100%) as a white solid: mp 57 °C; $[\alpha]_D^{29}$ + 82.6 (*c* 1, MeOH); ^{1}H NMR (250 MHz, $CDCl_3$): δ 9.16 (m, 2H, $PO(OH)_2$), 5.82 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.31 (m, 1H, H-5), 5.85 (dd, $J_{2,3}$ 4.8 Hz, H-2), 4.78 (dd, 1H, $J_{3,4}$ 8.5 Hz, H-3), 4.31 (dd, 1H, $J_{4,5}$ 4.5 Hz, H-4), 4.23 (m, 1H, H-6), 4.10 (m, 1H, H-6′), 2.37 (m, 2H, C*H*ipso C_6H_{11}), 1.95–1.26 (m, 20H, CH_2 C_6H_{11}), 1.53, 1.33 (2s, 6H, $2CH_3$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 176.3 and 176.2 ($2C_6H_{11}CO$), 114.1 (C-7), 105.9 (C-1), 78.7 (C-2), 77.4 (C-4), 74.4

(C-3), 72.4 (d, $J_{C,P}$ 8.4 Hz, C-5), 65.3 (d, $J_{C,P}$ 3.0 Hz, C-6), 44.2 and 43.9 (2*C*Hipso C_6H_{11}), 30.2, 30.1, 30.0 and 29.8 (4*C*H_2CHipso C_6H_{11}), 27.0 (2CH_3); 26.8 (2$CH_2CH_2CH_2$CHipso C_6H_{11}), 26.4 and 26.3 (4CH_2CH_2CHipso C_6H_{11}); ^{31}P NMR (101.2 MHz, $CDCl_3$): δ 0.95; ESI$^-$MS: *m*/*z* 519.2 (100) [M−H]$^-$; ESI$^+$MS: *m*/*z* 543.2 (100) [M+Na]$^+$; ESI$^-$HRMS: calcd for $C_{23}H_{36}O_{11}P$: 519.1990 [M−H]$^-$, found *m*/*z* 519.2005. Anal. Calcd for $C_{23}H_{37}O_{11}P$: C, 53.07; H, 7.16. Found: C, 52.64; H, 7.51.

4.2.13. 1,2-*O*-Isopropylidene-α-D-allofuranose 6-phosphate, diammonium salt (13)

Aq ammonia (60 mL) was added to compound **16** (5.1 g, 99 mmol) dissolved in MeOH (15 mL). The soln was stirred at rt for 8 h. Co-evaporation of solvents with toluene under diminished pressure yielded a white powder that was dissolved in MeOH (5 mL). Addition of diethylether allowed precipitation of the title compound with the side-product benzamide staying in the liquid phase. Upon filtration and drying, pure **13** was obtained as a white powder (2.5 g, 75%). The same procedure was used starting from compound **12** (1.33 g, 2.55 mmol) to obtain **13** (0.564 g, 66%) with the side-product cyclohexylamide: $[\alpha]_D^{26}$ + 26.8 (*c* 1, water); ^{1}H NMR (250 MHz, CD_3OD): δ 5.75 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 4.59 (dd, 1H, $J_{2,3}$ 4.6 Hz, H-2), 5.18 (dd, $J_{3,4}$ 8.8 Hz, H-3), 4.05–4.02 (m, 1H, H-5), 3.99–3.92 (m, 3H, H-4, H-6, H-6′), 1.56, 1.36 (2s, 6H, 2CH_3); ^{13}C NMR (62.9 MHz, CD_3OD): δ 113.8 (*C*Me_2), 105.3 (C-1), 81.5 (C-2), 72.2 (C-3, C-4), 67.0 (C-5), 66.6 (d, $J_{C,P}$ 4.9 Hz, C-6), 27.2 and 26.8 (2s, 2CH_3); ^{13}C NMR (62.9 MHz, D_2O): δ 114.0 (*C*Me_2), 104.2 (C-1), 80.1 (C-2), 79.7 (C-4), 70.6 (C-3), 70.3 (d, $J_{C,P}$ 7.5 Hz, C-5), 65.9 (d, $J_{C,P}$ 2.5 Hz, C-6), 26.2 and 26.0 (2CH_3); ^{31}P NMR (101.2 MHz, CD_3OD): δ 3.35; ESI$^-$MS: *m*/*z* 299.0 (100) [M−2NH_4+H]$^-$; HRESI$^-$MS: calcd for $C_9H_{16}O_9P$: 299.0526 [M−2NH_4+H]$^-$, found *m*/*z* 299.0538.

4.2.14. Preparation of D-allopyranose 6-phosphate, disodium salt (14)[22,23]

Compound **13** (431 mg, 1.29 mmol) was dissolved in water (3 mL) and trifluoroacetic acid (20 mL). The soln was stirred at rt for 2 h and concentrated under diminished pressure. The residue was eluted with water through an ion-exchange column (Dowex-50WX8, H^+ form) to remove ammonium salts. Evaporation of the solvents afforded 6-dihydrogenophospho-D-allopyranose (350 mg, 100%): ^{1}H NMR (250 MHz, D_2O): δ 4.60 (d, 1H, $J_{1,2}$ 8.2 Hz, H-1), 3.89–3.77 (m, 3H, H-3, H-6, H-6′), 3.61 (m, 1H, H-5), 3.41 (dd, 1H, $J_{4,3}$ 2.3, $J_{4,5}$ 9.9 Hz, H-4), 3.12 (dd, 1H, $J_{2,3}$ 2.7 Hz, H-2); ^{13}C NMR (62.9 MHz, D_2O): δ 93.3 (C-1β), 92.7 (C-1α), 71.9 (d, $J_{C,P}$ 7.4 Hz, C-5), 70.9 (C-2), 70.8 (C-3), 66.1 (C-4), 65.3 (d, $J_{C,P}$ 4.3 Hz, C-6); ^{31}P NMR (101.2 MHz, D_2O): δ −0.087. 6-Dihydrogenophospho-α-D-allopyranose was then eluted with water on an ion-exchange column (Dowex®-50WX8, Na^+ form) and lyophilized to afford **14** (392 mg, 100%) as a white solid: $[\alpha]_D^{27}$ + 11.5 (c 1, water); FTIR (KBr): ν_{max} 3415, 1037 cm^{-1}; ^{1}H NMR (250 MHz, D_2O): δ 5.05 (d, 1H, $J_{1\alpha,2}$ 3.5 Hz, H-1), 4.79 (d, 1H, $J_{1\beta,2}$ 8.2 Hz, H-1β), 4.08 (dd, 1H, $J_{3,2}$ 2.8, $J_{3,4}$ 9.7 Hz, H-3), 4.00 (m, 1H, $J_{6,5}$ 4.6, $J_{6,6'}$ 11.4 Hz, H-6), 3.93 (m, 1H, $J_{6',5}$ 5.5 Hz, H-6′), 3.80 (ddd, 1H, $J_{5,4}$ 3.0 Hz, H-5), 3.61 (dd, 1H, H-4), 3.33 (dd, 1H, H-2), in agreement with the literature for the monosodium salt;[23] ^{13}C NMR (62.9 MHz, D_2O): δ 93.5 (C-1β), 92.6 (C-1α), 72.6 (d, $J_{C,P}$ 7.4 Hz, C-5α, C-5β), 71.3 (C-3α), 71.1 and 71.0 (C-2β, C-3β), 66.9 (C-2α), 66.3 (C-4β), 65.7 (C-4α), 64.3 (d, $J_{C,P}$ 3.5 Hz, C-6β), 64.0 (d, $J_{C,P}$ 3.0 Hz, C-6α), in agreement with the literature[22] (nature of the salt not reported); ^{31}P NMR (101.2 MHz, D_2O): δ −0.67, lit.[23] 3.55 for the monosodium salt.

4.2.15. 3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-allofuranose 6-dibenzylphosphate (15)

A mixture of **10** (10 g, 23 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (16 g, 46 mmol) was dried under vacuum (0.1 mbar) for 30 min. A soln of 1,2,4-triazole (7 g, 93 mmol) in anhyd MeCN (100 mL) was then added, and the soln was stirred at rt under argon for 15 h. Following dilution with CH_2Cl_2 (80 mL) and addition of *tert*-butylhydroperoxide (11 mL, 93 mmol), the soln was stirred vigorously at rt for 1 h. The mixture was neutralized with 1 M aq $Na_2S_2O_3$ (100 mL) and 1 M aq $NaHCO_3$ (100 mL), and then extracted with CH_2Cl_2 (2 × 200 mL). The combined organic phases were dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue (22 g) was purified by two successive column chromatographies (4:1 pentane–EtOAc, then 1:1 toluene–EtOAc) to give **15** (7.3 g, 60%) as a white powder: R_f 0.19 (4:1 pentane–EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.90–7.86 (m, 4H, C*H* arom), 7.50–7.41 (m, 2H, C*H* arom), 7.32–7.20 (m, 14H, C*H* arom), 5.88 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 5.52 (m, 1H, H-5), 5.12 (dd, $J_{3,2}$ 4.9, $J_{3,4}$ 8.8 Hz, H-3), 5.06 (d, 2H, $J_{H,P}$ 8.0 Hz, OCH_2Ph), 4.99 (m, 3H, H-2, OCH_2Ph), 4.62 (dd, 1H, $J_{4,5}$ 6.9 Hz, H-4), 4.41 (m, 2H, H-6, H-6′), 1.56, 1.33 (2s, 6H, 2CH_3); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 165.3 (2PhCO), 135.5 (d, $J_{C,P}$ 7.0 Hz, 2*C*ipso $PhCH_2$), 133.0 (2*C*p PhCO), 129.7 and 129.6 (2*C*m PhCO), 129.0 and 128.7 (2*C*ipso PhCO), 128.4 (2*C*p $PhCH_2$), 128.3 (2*C*o $PhCH_2$), 128.1 (2*C*o PhCO), 127.8 and 127.7 (4*C*o $PhCH_2$), 113.3 (*C*Me_2), 104.2 (C-1), 77.6 (C-2), 74.9 (C-4), 74.5 (C-3), 72.0 (d, $J_{C,P}$ 7.7 Hz, C-5), 69.2 (d, $J_{C,P}$ 5.4 Hz, 2$PhCH_2$), 65.8 (d, $J_{C,P}$ 5.1 Hz, C-6), 26.7 and 26.6 (2CH_3); ^{31}P NMR (101.2 MHz, D_2O): δ −1.1.

4.2.16. 3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-allofuranose 6-dihydrogenophosphate (16)

Compound **15** (7.2 g, 104 mmol) dissolved in MeOH (60 mL) and Pd/C 10% (2.4 g) were stirred under H_2 (15 bar) for 2.5 h. The mixture was then filtered and concentrated to give **16** (5.1 g, 95%) as a white solid: ^{1}H NMR (360 MHz, CD_3OD): δ 7.90 (d, 2H, *J* 7.3 Hz, Ph), 7.82 (d, 2H, *J* 7.3 Hz, Ph), 7.52–7.44 (m, 2H, Ph), 7.33–7.23 (m, 4H, Ph), 5.94 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.51 (m, 1H, H-5), 5.18 (dd, $J_{3,2}$ 4.9, $J_{3,4}$ 8.8 Hz, H-3), 5.02 (dd, 1H, H-2), 4.62 (dd, 1H, $J_{4,5}$ 7.7 Hz, H-4), 4.45–4.33 (m, 2H, H-6, H-6′), 1.55, 1.33 (2s, 6H, 2CH_3); ^{13}C NMR (90.5 MHz, CD_3OD): δ 167.1 and 166.7 (2C═O), 134.4 (2*C*p Ph), 130.8 and 130.7 (2*C*o Ph), 130.2 (2*C*ipso Ph), 129.4 (2*C*m Ph), 114.5 (*C*Me_2), 106.0 (C-1), 79.2 (C-2), 76.3 (C-3, C-4), 74.2 (d, $J_{C,P}$ 8.0 Hz, C-5), 66.1 (d, $J_{C,P}$ 4.6 Hz, C-6), 27.1 and 27.0 (2CH_3); ESI$^+$MS: *m*/*z* 553.1 (100) [M−H+2Na]$^+$. Anal. Calcd for $C_{23}H_{25}O_{11}P$: C, 54.33; H, 4.96; O, 34.62. Found: C, 52.66; H, 5.21; O, 34.59.

4.2.17. 1,2:3,4-Di-*O*-isopropylidene-β-D-psicofuranose 6-dibenzylphosphate (17)

A mixture of 1,2:3,4-di-*O*-isopropylidene-β-D-psicofuranose[28] (8.7 g, 33 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (23 g, 67 mmol) was dried under vacuum (0.1 mbar) for 30 min. A soln of 1,2,4-triazole (9 g, 134 mmol) in anhyd MeCN (200 mL) was then added, and the soln was stirred at rt under argon for 2 h. Following dilution with CH_2Cl_2 (200 mL) and addition of *tert*-butylhydroperoxide (18.3 mL, 134 mmol), the soln was stirred vigorously at rt for 1 h. The mixture was neutralized with 1 M aq $Na_2S_2O_3$ (300 mL) and 1 M aq $NaHCO_3$ (300 mL), and then extracted with CH_2Cl_2 (2 × 400 mL). The combined organic phases were dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue (20 g) was purified by two successive column chromatographies (4:1 pentane–EtOAc, then 9:1 toluene–EtOAc), then concentrated under diminished pressure to give **17** (11.43 g, 67%) as a white powder: R_f 0.17 (4:1 pentane–EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.34 (s, 10H, 2Ph), 5.07–5.00 (m, 4H, 2PhCH_2O), 4.66 (d, 1H, $J_{4,3}$ 5.8 Hz, H-4), 4.51 (d, 1H, H-3), 4.25 (d, 1H, $J_{1,1'}$ 9.9 Hz, H-1), 4.20 (t, 1H, $J_{5,6}$ 7.3, $J_{5,6'}$ 7.3 Hz, H-5), 4.01 (d, 1H, H-1′), 3.99 (m, 2H, H-6, H-6′), 1.41, 1.38, 1.34, 1.28 (4s, 12H, 4CH_3); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 135.6 (d, $J_{C,P}$ 6.6 Hz, 2*C*ipso Ph), 127.9 and 128.5 (4*C*o Ph, 4*C*m Ph, 2*C*p Ph), 113.5 (C-2), 112.6

and 111.6 (2CMe_2), 84.8 (C-3), 83.0 (d, $J_{C,P}$ 9.0 Hz, C-5), 81.8 (C-4), 69.5 (C-1), 69.3 (d, $J_{C,P}$ 5.4 Hz, 2PhCH_2), 66.7 (d, $J_{C,P}$ 5.8 Hz, C-6), 26.3 (2CH_3), 26.2 and 24.9 (2CH_3); ^{31}P NMR (101.2 MHz, D_2O): δ −1.1; ESI$^+$MS: *m*/*z* 543.1 (100) $[M+Na]^+$; HRESI$^+$MS: calcd for $C_{26}H_{33}O_9PNa$: 543.1754 $[M+Na]^+$, found *m*/*z* 543.1764. Anal. Calcd for $C_{26}H_{33}O_9P$: C, 59.99; H, 6.39. Found: C, 59.97; H, 6.39.

4.2.18. 1,2:3,4-Di-*O*-isopropylidene-β-D-psicofuranose 6-dihydrogenophosphate (18)

Compound **17** (11.43 g, 22 mmol) dissolved in anhyd MeOH (60 mL) and Pd/C 10% (0.11 g) were stirred under H_2 (20 bar) for 3 h. The mixture was then filtered and concentrated to give **18** (7.3 g, 100%): ^{1}H NMR (360 MHz, CD_3OD): δ 5.38 (br s, 2H, 2OH), 4.85 (d, 1H, $J_{4,3}$ 5.8 Hz, H-4), 4.66 (d, 1H, H-3), 4.26 (d, 1H, $J_{1,1'}$ 9.7 Hz, H-1), 4.23 (t, 1H, $J_{5,6}$ 7.3, $J_{5,6'}$ 7.3 Hz, H-5), 3.97–3.01 (m, 3H, H-1′, H-6, H-6′), 1.44, 1.42, 1.37, 1.31 (4CH_3); ^{13}C NMR (90.6 MHz, CD_3OD): δ 114.9 (C-2), 113.7 and 112.9 (2CMe_2), 86.2 (C-3), 84.8 (d, $J_{C,P}$ 9.3 Hz, C-5), 83.3 (C-4), 70.6 (C-1), 67.1 (d, $J_{C,P}$ 5.0 Hz, C-6), 26.7, 26.7, 26.6 and 25.1 (4CH_3); ^{31}P NMR (101.2 MHz, CD_3OD): δ 0.0.

4.2.19. Preparation of D-allulofuranose (or D-psicofuranose) 6-phosphate, disodium salt (19)[31]

Compound **18** (650 mg, 1.9 mmol) was dissolved in water (0.5 mL) and trifluoroacetic acid (4.5 mL). The soln was stirred at rt for 4 h and concentrated under diminished pressure to afford 6-dihydrogenophospho-D-psicofuranose (520 mg, 100%): ^{31}P NMR (101.2 MHz, D_2O): δ −0.8. 6-Dihydrogenophospho-D-psicofuranose was then successively eluted with water on two ion-exchange columns Dowex®-50WX8 (1-H^+ form, 2-Na^+ form) and lyophilized to afford **19** (633 mg, 100%) as a white solid: $[\alpha]_D^{28}$ − 29 (*c* 1, water); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 105.8 (C-2β), 103.4 (C-2α), 81.8 (d, $J_{C,P}$ 7.8 Hz, C-5α), 81.5 (d, $J_{C,P}$ 7.8 Hz, C-5β), 74.7 and 71.0 (C-3β, C-4β), 70.5 and 70.2 (C-3α, C-4α), 66.0 (d, $J_{C,P}$ 4.7 Hz, C-6β), 64.8 (d, $J_{C,P}$ 4.7 Hz, C-6α), 63.2 (C-1α), 62.4 (C-1β), in agreement with the literature[31] for the dihydrogenophosphate form; ^{31}P NMR (101.2 MHz, D_2O): δ 0.7 (α), 0.5 (β).

4.2.20. 5-Dihydrogenophospho-D-ribono-1,4-lactone (20)

Compound **2** (617 mg, 2.0 mmol) dissolved in water (5 mL) was deposited on a Dowex®-50WX8 (H^+ form) ion-exchange column and eluted with water. Concentration under diminished pressure, lyophilization, and drying under high vacuum for 3 d in a desiccator containing P_2O_5 yielded compound **20** (386 mg, 86%) as a pale yellow viscous oil: ^{1}H NMR (250 MHz, D_2O): δ 4.49 (d, 1H, $J_{3,2}$ 5.6 Hz, H-3), 4.44 (dd, 1H, $J_{4,5(5')}$ 6.2, $J_{4,5'(5)}$ 2.5 Hz, H-4), 4.26 (d, 1H, H-2), 3.96 (m, 2H, H-5, H-5′); ^{13}C^{13}C NMR (62.9 MHz, D_2O): δ 178.0 (C-1), 84.3 (d, $J_{C,P}$ 7.9 Hz, C-4), 69.1 (C-3), 68.6 (C-2), 64.6 (d, $J_{C,P}$ 4.6 Hz, C-5); ^{31}P NMR (101.2 MHz, D_2O): δ −0.8; ESI$^-$MS: *m*/*z* 227.0 (100) $[M-H]^-$; HRESI$^-$MS: calcd for $C_5H_8O_8P$: 226.9962 $[M-H]^-$, found *m*/*z* 226.9975.

4.3. Kinetic evaluations

Both enzymes were cloned, expressed, and purified as previously described.[5,7] In a minor modification, the last purification step consisted of a size exclusion chromatography in a buffer of 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM EDTA. For storage, the salt concentration in the buffer was reduced to 25 mM; for EcRpiB the reducing agent was also replaced by 5 mM MESNA. Protein samples were stored at −20 °C at a concentration of 1–10 mg/mL.

Prior to the kinetic experiments, aliquots were thawed and diluted with 50 mM Tris–HCl (pH 7.5); final concentrations in the assays were 93 nM for the Rpi activity measurements and 52 nM for Allpi activity. Rpi activity was evaluated by the spectrophotometric assay described previously,[43] in which the production of Ru5P is monitored as a change in absorbance at 290 nm (ε = 72 M^{-1} cm^{-1}) at 37 °C in 50 mM Tris–HCl buffer (pH 7.5). Allpi activity was evaluated by the thiobarbituric acid (TBA) assay described previously,[7] in which the production of Allu6P is measured at 37 °C in 50 mM Tris–HCl buffer (pH 7.5) and 5 mM MESNA after a 2-min reaction time (a blank of identical composition but without enzyme was used as the reference). Following dehydration, dephosphorylation, and reaction with TBA, a characteristic yellow TBA adduct is formed which strongly absorbs at 438 nm (ε = 27800 M^{-1} cm^{-1}).

Kinetic constants were determined from linear regression using Lineweaver–Burk or Hanes-Woolf plots at various concentrations of inhibitors, and replots of apparent K_m/V_{max} values *vs* inhibitor concentration. The *x* intercept, which is equal to $-K_i$, was determined by linear regression. For substances showing weak inhibition, only IC_{50} values were acquired. These were measured using a constant R5P concentration equal to the K_m value for Rpi activity measurements, or a constant All6P concentration equal to 0.8 mM (except for compounds **4** and **7,** for which it was 1.0 mM) for Allpi activity measurements.

4.4. Structural comparisons and analyses

Manual docking of 5PRA (**2**) and 5PRH (**1**) was performed in the graphics program O,[44] which was used together with OPLOT[45] and Molray[46] to prepare Figure 2.

Acknowledgments

The authors are grateful to Jean-Pierre Baltaze for COSY, HSQC, and *J*-mod NMR spectra, and to Karine Leblanc, Delphine Arquier, and Yannick Charvet for MS data. This work was supported by grants from the Swedish Research Council (VR) to SM, and by Uppsala University.

References

1. Rieder, S. V.; Rose, I. A. *J. Biol. Chem.* **1959**, *234*, 1007–1010.
2. Bruns, F.; Okada, S. *Nature* **1956**, *177*, 87–88.
3. Topper, Y. J. *J. Biol. Chem.* **1957**, *225*, 419–425.
4. Gracy, R. W.; Noltman, E. A. *J. Biol. Chem.* **1968**, *243*, 5410–5419.
5. Roos, A. K.; Andersson, C. E.; Bergfors, T.; Jacobsson, M.; Karlèn, A.; Unge, T.; Alwyn Jones, T.; Mowbray, S. L. *J. Mol. Biol.* **2004**, *335*, 799–809.
6. Roos, A. K. PhD thesis, Uppsala University, 2007.
7. Roos, A. K.; Mariano, S.; Kowalinski, E.; Salmon, L.; Mowbray, S. L. *J. Mol. Biol.* **2008**, *382*, 667–679.
8. Woodruff, W. W.; Wolfenden, R. *J. Biol. Chem.* **1979**, *254*, 5866–5867.
9. Burgos, E.; Salmon, L. *Tetrahedron Lett.* **2004**, *45*, 753–756.
10. Burgos, E.; Salmon, L. *Tetrahedron Lett.* **2004**, *45*, 3465–3469.
11. Kim, C.; Song, S.; Park, C. *J. Bacteriol.* **1997**, *179*, 7631–7637.
12. Poulsen, T. S.; Chang, Y.-Y.; Hove-Jensen, B. *J. Bacteriol.* **1999**, *181*, 7126–7130.
13. Sassetti, C. M.; Boyd, D. H.; Rubin, E. J. *Mol. Microbiol.* **2003**, *48*, 77–84.
14. Huck, J. H.; Verhoeven, N. M.; Struys, E. A.; Salomons, G. S.; Jakobs, C.; van der Knaap, M. S. *Am. J. Hum. Genet.* **2004**, *74*, 745–751.
15. Stern, A. L.; Burgos, E.; Salmon, L.; Cazzulo, J. J. *Biochem. J.* **2007**, *401*, 279–285.
16. Burgos, E.; Roos, A. K.; Mowbray, S. L.; Salmon, L. *Tetrahedron Lett.* **2005**, *46*, 3691–3694.
17. Bigham, E. C.; Gragg, C. E.; Hall, W. R.; Kelsey, J. E.; Mallory, W. R.; Richardson, D. C.; Benedict, C.; Ray, P. H. *J. Med. Chem.* **1984**, *27*, 717–726.
18. Hardré, R.; Salmon, L. *Carbohydr. Res.* **1999**, *318*, 110–115.
19. Chirgwin, J. M.; Noltmann, E. A. *J. Biol. Chem.* **1975**, *250*, 7272–7276.
20. Fischer, J.-C.; Horton, D. *Carbohydr. Res.* **1977**, *59*, 477–503.
21. Estevez, J. C.; Fairbanks, A. J.; Fleet, G. W. J. *Tetrahedron* **1998**, *54*, 13591–13620.
22. Franke, F. P.; Kapuscinsky, M.; Lyndon, P. *Carbohydr. Res.* **1985**, *143*, 69–76.
23. Chenault, H. K.; Mandes, R. F.; Hornberger, K. R. *J. Org. Chem.* **1997**, *62*, 331–336.
24. Wood, W. W.; Watson, G. M. *J. Chem. Soc., Perkin Trans. I* **1987**, 2681–2687.
25. Sowa, W.; Thomas, H. S. *Can. J. Chem.* **1966**, *44*, 836–838.
26. Andersson, F.; Samuelsson, B. *Carbohydr. Res.* **1984**, *129*, C1–C3.
27. Vieira de Almeida, M.; Dubreuil, D.; Cleophax, J.; Verre-Sebrie, C.; Pipelier, M.; Prestat, G.; Vass, G.; Gero, S. D. *Tetrahedron* **1999**, *55*, 7251–7270.
28. Brady, R. F., Jr. *Carbohydr. Res.* **1971**, *20*, 170–175.
29. Prisbe, E. J.; Smejkal, J.; Verheyden, J. P. H.; Moffatt, J. G. *J. Org. Chem.* **1976**, *41*, 1836–1845.
30. Mio, S.; Kumagawa, Y.; Sugai, S. *Tetrahedron* **1991**, *47*, 2133–2144.

31. Herve du Penhoat, P. C. M.; Perlin, A. S. *Carbohydr. Res.* **1979**, *71*, 149–167.
32. Brady, R. F., Jr. *Carbohydr. Res.* **1970**, *15*, 35–40.
33. Wang, Z. X.; Tu, Y.; Frohn, M.; Zhang, J. R.; Shi, Y. *J. Am. Chem. Soc.* **1997**, *119*, 11224–11235.
34. Guo, J.; Frost, J. W. *J. Am. Chem. Soc.* **2002**, *124*, 528–529.
35. Nieto, N.; Molas, P.; Benet-Buchholz, J.; Vidal-Ferran, A. *J. Org. Chem.* **2005**, *70*, 10143–10146.
36. McDonald, E. J. *Carbohydr. Res.* **1967**, *5*, 106–108.
37. Trifonova, A.; Földesi, A.; Dinya, Z.; Chattopadhyaya, J. *Tetrahedron* **1999**, *55*, 4747–4762.
38. Hardré, R.; Bonnette, C.; Salmon, L.; Gaudemer, A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3435–3438.
39. Sen Gupta, K. K.; Sen Gupta, S.; Mandal, S. K.; Basu, S. N. *Carbohydr. Res.* **1985**, *139*, 185–190.
40. Sen Gupta, K. K.; Sen Gupta, S.; Mandal, S. K.; Basu, S. N. *Carbohydr. Res.* **1986**, *145*, 193–200.
41. Hamada, K.; Ago, H.; Sugahara, M.; Nodake, Y.; Kuramitsu, S.; Miyano, M. *J. Biol. Chem.* **2003**, *278*, 49183–49190.
42. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235–242.
43. Wood, T. *Anal. Biochem.* **1970**, *33*, 297–306.
44. Jones, T. A.; Zou, J.-Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr., Sect. A. Found. Crystallogr.* **1991**, *47*, 110–119.
45. Jones, T. A.; Kjeldgaard, M. O. *Methods Enzymol.* **1997**, *277*, 173–288.
46. Harris, M.; Jones, T. A. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2001**, *57*, 1201–1203.
47. Zhang, R.-G.; Andersson, C. E.; Skarina, T.; Evdokimova, E.; Edwards, A. M.; Joachimiak, A.; Savchenko, A.; Mowbray, S. L. *J. Mol. Biol.* **2003**, *332*, 1083–1094.