Synthesis and Inhibition Properties of Conformational Probes for the Mutase-Catalyzed UDP-Galactopyranose/Furanose Interconversion

Audrey Caravano,[a] Dominique Mengin-Lecreulx,[b] Jean-Marc Brondello,[c] Stéphane P. Vincent,*[a] and Pierre Sinaÿ*[a]

Abstract: UDP-galactose mutase is a flavoenzyme that catalyzes the isomerization of UDP-galactopyranose into UDP-galactofuranose, a key step in the biosynthesis of important bacterial oligosaccharides. Several mechanisms for this unique ring-contraction have been proposed, one of them involving a putative 1,4-anhydrogalactopyranose as an intermediate in the reaction. The purpose of this study was to probe the mutase binding site with conformationally restricted analogues of its substrate. Thus, we describe the straightforward synthesis of two C-glycosidic UDP-galactose derivatives: analogue **1**, presenting a galactose moiety locked in a bicyclic $^{1,4}B$ boat conformation, and UDP-*C*-Gal*f* **2**, where the galactose residue is locked in the conformation of the mutase substrate. The two molecules were found to be inhibitors of UDP-galactose mutase at levels depending on the redox state of the enzyme. Strong inhibition of the native enzyme, but a low one of the reduced mutase, were observed with UDP-*C*-Gal*f* **2**, whereas **1** displayed intermediate inhibition levels under both native and reducing conditions. These data provide evidence of a significant conformational difference of the mutase binding pocket in the reduced enzyme and in the native one, the enzyme switching from a low Gal*f*-affinity state (reduced enzyme) to a very strong one (native enzyme). It is remarkable that the mutase binds the boat-locked analogue **1** with similar affinities in both its conformational states. These results support a mechanism involving the formation of 1,4-anhydrogalactopyranose as a low-energy intermediate. An alternative explanation would be that the distortion of the galactose moiety during the cycle contraction transiently brings the carbohydrate into a conformation close to a $^{1,4}B$ boat.

Keywords: C-glycosides · galactofuranose · inhibitors · locked conformations · nucleotides

Introduction

Galactan biosynthesis is emerging as an important research field at the frontiers of microbiology, enzymology and bioorganic chemistry.[1] Galactans are oligosaccharides with galacto*furanose* (Gal*f*) and galacto*pyranose* (Gal*p*) residues as main components. Gal*f* moieties are expressed in the membranes of prokaryotes but are totally absent in mammalian cells, thus making the enzymes responsible for their biosynthesis potential targets for applications in antibiotherapy.[2, 3] Galactofuranosyl moieties have been found in lipopolysaccharides (LPSs) of Gram-negative bacterial outer membranes, in capsular oligosaccharides of different bacterial strains, and also in mycobacterial cell walls.[4] Furthermore, they are key components of the arabinogalactan complex characteristic of mycobacterial membranes, where they are present as an oligo-(Gal*f*) made up of over 20 Gal*f* residues with a typical β-1,5-Gal*f*-β-1,6-Gal*f* repeated unit. The enzymes responsible for the galactan biosynthesis are a mutase[5] catalyzing the isomerization of UDP-galacto*pyranose* into UDP-galacto*furanose* and transferases[6] triggering the glycosylation of a growing oligosaccharide acceptor with UDP-Gal*f* as the donor substrate. Importantly, studies on mutants of *Mycobacterium smegmatis* have demonstrated that the inhibition of the arabinogalactan biosynthesis effected through UDP-Gal mutase inactivation prevents mycobacterial proliferation.[7] Although few Gal*f*-transferases (Gal*f*Ts) have been fully characterized, it has been shown that the same Gal*f*T can catalyze two subsequent Gal*f* transfers.[8] The most studied enzyme of the galactan biosynthesis is the UDP-Gal mutase

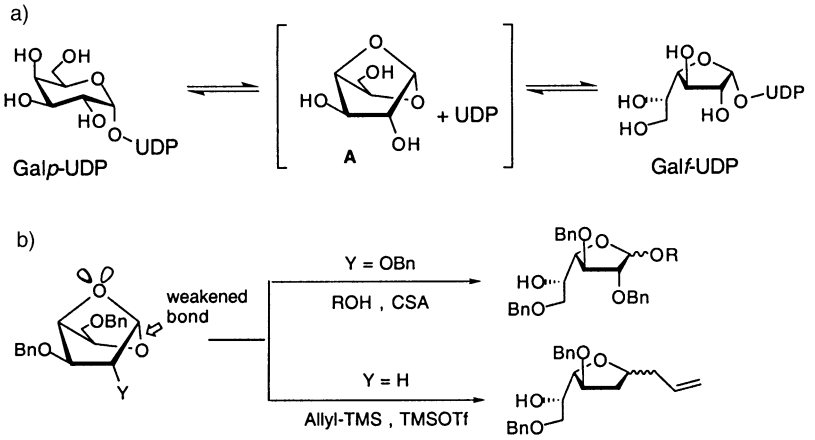
[a] Dr. S. P. Vincent, Prof. P. Sinaÿ, A. Caravano
Ecole Normale Supérieure
Département de Chimie, UMR-8642 du CNRS
24 rue Lhomond, 75231 Paris Cedex 05 (France)
Fax: (+33) 1-44-32-33-97
E-mail: sinay@ens.fr

[b] Dr. D. Mengin-Lecreulx
Enveloppes Bactériennes et Antibiotiques
IBBMC, UMR-8619 du CNRS
Université de Paris-Sud, Bât. 430
91405 Orsay Cedex (France)

[c] Dr. J.-M. Brondello
Institut de Génétique Humaine
UPR-1142 du CNRS, 141 rue de la cardonille
34396 Montpellier Cedex 05 (France)

DOI: 10.1002/chem.200305141

cloned from *Escherichia coli*, *Klebsiella pneumoniae*, and *Mycobacterium tuberculosis*, the three-dimensional structure of which is now available.[9] The mechanism by which this enzyme catalyzes this unique isomerization, formally a ring-contraction, remains very puzzling to date. First, all characterized mutases are flavoenzymes, but the exact role of the FAD cofactor is far from being well understood. From a study employing membrane extracts of *P. charlesii*, it was first hypothesized that an oxidation at the 2-position of the galactose moiety would occur during the enzymatic process.[10–12] However, several UDP-Gal analogues fluorinated at the 2-, 3-, and 4-positions have been synthesized and tested as inhibitors or substrates of the *E. coli* mutase.[13–15] Interestingly, 2F-Gal*f*-UDP and 3F-Gal*f*-UDP were found to be substrates of the enzyme when the mutase was maintained in its reduced state (thus ruling out the hypothesis of an oxidation at the 2-position), whereas the same molecules displayed inhibition properties under non-reducing conditions.[15] At about the same time, Naismith et al. concluded from analysis of the three-dimensional mutase structure that an oxidation/reduction reaction in the vicinity of the anomeric center is likely to occur.[9] In a more recent investigation, it was shown that the enzyme can stabilize the semiquinone form ($FADH^{\cdot}$) of the flavin cofactor and that the fully reduced flavin was $FADH^{-}$.[16] However, no redox turnover of the flavin cofactor has ever been found. The specific role of the FAD cofactor thus remains to be defined to allow both a clear understanding of this unique molecular rearrangement and the design of mechanism-based inhibitors of UDP-Gal mutase.



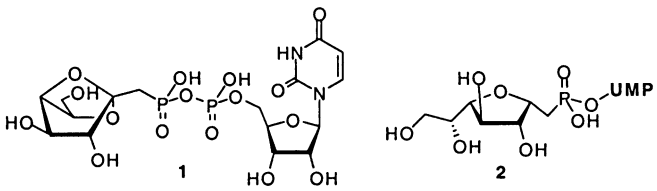
Scheme 1. Hypothetical mechanism of the mutase-catalyzed UDP-Gal*p*/UDP-Gal*f* interconversion. a) Mutase-catalyzed pyranose/furanose interconversion through a putative 1,4-anhydrogalactose intermediate. b) Chemical models for 1,4-anhydrogalactose opening reactions exclusively giving galactofuranosides.

Abstract in French: *L'UDP-galactose mutase est une flavoenzyme catalysant l'isomérisation de l'UDP-galactopyranose en UDP-galactofuranose (UDP-Galf), une étape clé de la biosynthèse d'oligosaccharides bactériens. Plusieurs mécanismes réactionnels de cette contraction de cycle ont été proposés, l'un d'entre eux faisant intervenir le 1,4-anhydrogalactopyranose comme intermédiaire hypothétique. Le but de cette étude est de sonder le site actif de cette mutase avec des analogues du substrat figés dans des conformations distinctes. Ainsi, nous avons synthétisé deux C-glycosides analogues de l'UDP-galactose: le nucléotide sucre* ***1*** *présentant un groupe galactose bloqué dans une conformation bateau* $^{1,4}B$*, et l'analogue* ***2*** *dont l'unité galactose est bloquée dans une configuration furanose. Ces deux molécules se sont avérées être des inhibiteurs de l'UDP-galactose mutase avec des niveaux d'inhibition dépendant de l'état d'oxydo-réduction de l'enzyme. L'UDP-C-Galf* ***2*** *inhibe fortement l'enzyme native, mais faiblement l'enzyme réduite, tandis que l'analogue* ***1*** *possède un pouvoir inhibiteur intermédiaire à la fois dans des conditions natives et réductrices. Ces données d'inhibition mettent clairement en évidence des différences significatives de la conformation du site actif de l'enzyme, celle-ci passant d'un état de forte affinité pour le Galf (conditions natives) à un état de faible affinité (conditions réductrices). Il est par ailleurs remarquable que ces deux états conformationnels possèdent des affinités comparables pour le groupe galactose figé dans une conformation bateau* $^{1,4}B$*. Ces résultats montrent qu'un mécanisme faisant intervenir le 1,4-anhydrogalactopyranose en tant qu'intermédiaire à basse énergie est possible. Toutefois, ces résultats peuvent également mettre en évidence le fait que l'enzyme peut stabiliser, au cours de cette isomérisation, une conformation transitoire proche d'un bateau* $^{1,4}B$*.*

Nevertheless, Blanchard et al. have provided interesting information regarding this enzymatic ring-contraction by positional isotope exchange (PIX) experiments.[17] Indeed, it was shown with *K. pneumoniae* mutase (and later reproduced with *E. coli*)[15] that a cleavage of the C1-O(UDP) bond of UDP-Gal occurs during the enzymatic process. From these results, it was hypothesized that 1,4-anhydrogalactopyranose **A** (Scheme 1) could be an intermediate in the isomerization. Although this mechanistic suggestion does not take account of any oxido-reduction process, it may, if confirmed, provide interesting knowledge about the conformation(s) of the galactose moiety at the transition(s) state(s) (if a 1,4-anhydrogalactopyranose is an intermediate in this reaction, two transition states might be invoked). Thus, to strengthen this mechanistic hypothesis, a non-enzymatic model of the mutase-catalyzed isomerization was designed by our group and showed that 2,3,6-tribenzyl-1,4-anhydrogalactopyranose, in the presence of a nucleophile and a catalytic amount of camphorsulfonic acid (CSA), yields an anomeric mixture of galactosides, exclusively in *furanose* configurations.[18] Similar selective ring-opening had previously been described in the literature and had been explained by stereoelectronic effects.[19] Given the positions of the lone-pair electrons of the oxygen O-4 and O-5 relative to the C1–O4 and C1–O5 σ bonds, the C1–O5 bond may be considered weakened by

stereoelectronic effects, due to its antiperiplanarity with one of the O-4 doublets (Scheme 1).

From the mechanistic investigations (PIX experiments) and the non-enzymatic model we designed the phosphonate **1** as a mechanistic probe with which to confirm or rule out the

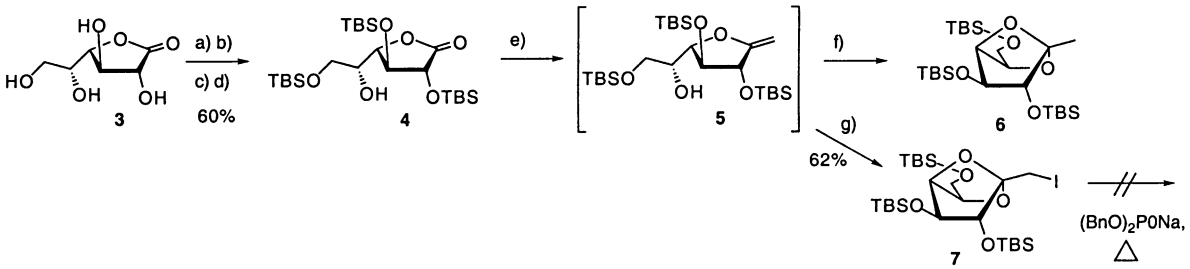


involvement of an intermediate possessing a structure close to a 1,4-anhydrogalactopyranose, or at least to measure the affinity of this enzyme for a galactose moiety locked in a $^{1,4}B$ boat conformation. This molecule is made up of a 1,4-anhydrogalactopyranose substructure, to test the mechanistic assumption, together with a UDP moiety required for tight binding to the enzyme. The two substructures are linked through a methylene group between the anomeric carbon atom of the constrained sugar and the β-phosphate of UDP, to provide both chemical and enzymatic stability to the final nucleotide sugar. Thus, **1** can also be seen as a conformational probe for this particular glycosyl processing enzyme. For comparison of the binding affinities, we also report a new synthesis of UDP-*C*-Gal*f* **2**, the phosphonate analogue of UDP-Gal*f*, in which the galactose moiety is in the ground state conformation.

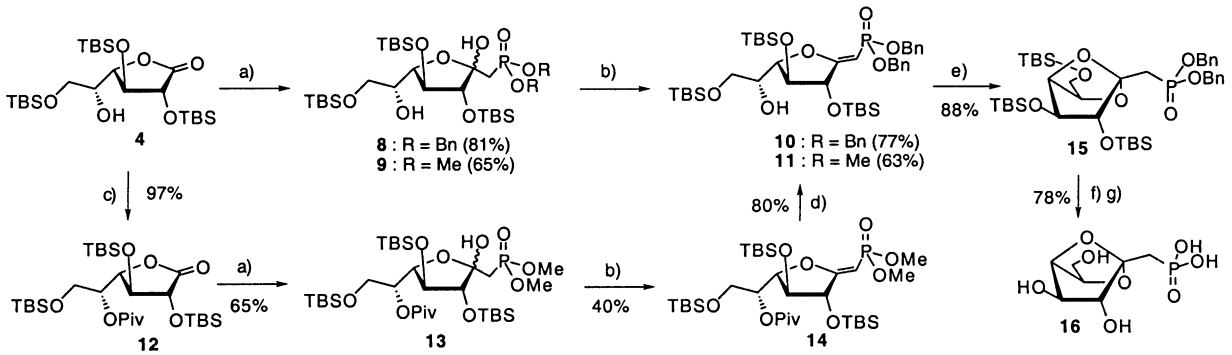
Results and Discussion

After retrosynthetic analysis, we reasoned that it should be advantageous to choose the commercially available 1,4-galactonolactone **3** as starting material. Two synthetic strategies were then explored, differing in the two key steps that had to be envisioned: a cyclization yielding the 1,4-anhydro motif and the installation of the phosphonate group. Indeed, we first had to define which step, the phosphonylation or the cyclization, should be performed first. The 2,3,6-tri-*O*-silylated lactone **4**, possessing a free hydroxy group at the 5-position, could easily be obtained on large scale, by a known procedure (Scheme 2).[20] We then found that, even in the presence of the 5-hydroxy group, a Tebbe reaction could easily be performed, giving the intermediate *exo*-glycal **5** (Scheme 2). This reactive enol ether could not be purified by standard silica gel chromatography. Interestingly, the main degradation product on silica gel was the cyclization product **6**, with a 1,4-anhydrogalactopyranose structure, thus showing the effective propensity of galactofuranosyl *exo*-glycals to cyclize under mild conditions. The intermediate **5** was therefore not isolated, but was directly treated, in one-pot fashion, with an excess of NIS in anhydrous CH_2Cl_2, to give the cyclized iodide **7** in 62% from **4**. Unfortunately, attempts to phosphonylate **7** under Arbuzoff ($(RO)_3P$, heat) or Arbuzoff–Becker ($(RO)_2PONa$, heat) conditions proved unsuccessful. This lack of reactivity is probably due to the neopentyl nature of this primary iodide.

We then turned our attention to the second strategy, based on the cyclization of a pre-phosphonylated *exo*-glycal. The desired enol ethers **10** and **11** were prepared by a straightforward two-step procedure from the galactonolactone **4** (Scheme 3). This lactone was condensed with the lithium anion of dibenzyl (or dimethyl) methylphosphonate to give the lactol **8** (or **9**).[21] The subsequent elimination was achieved through a slight modification of a procedure recently described in the literature.[22, 23] The presence of the unprotected hydroxy group at the 5-position does not significantly modify the yields of these two steps. It is worth noting that the lactols **8** and **9** were isolated, characterized, and induced to react in their furanose forms. Since the 5-OH group is free, an isomerization to a pyranose form might have been observed. However, NMR spectral data on lactols **8** and **9**, as well as on *exo*-glycals **10** and **11**, are consistent with furanosides. To demonstrate the furanosidic structure of the central intermediate **10** unambiguously, we also performed the methylenephosphonylation/elimination sequence on the 1,4-galactonolactone protected at the 5-position as a pivaloyl ester (compounds **12** to **14**, Scheme 3). Removal of the pivaloate from glycal **14** gave a 5-hydroxylated compound strictly identical to compound **11**. These experiments both confirmed the structures of compounds **10** and **11** and outlined the efficiency of this sequence: the *exo*-glycal **10** possesses a free 5-hydroxy group, ready to cyclize, without protective group manipulation. The *exo*-glycals were isolated as single diastereomers with *Z* configurations, as shown by a NOE between H-1′ (the olefinic proton) and H-2, a result consistent with literature data.[22–24] Owing to the electron-withdrawing character of the phosphoryl moiety, these *exo*-glycals are stable on silica gel.



Scheme 2. Synthesis of a non-phosphonylated 1,4-anhydrogalactopyranose substructure. Reagents and conditions: a) acetone, $CuSO_4$; b) TBDMSCl, Im, DMF; c) AcOH, H_2O; d) TBDMSCl, Im, DMF; e) Tebbe reagent, f) silica gel; g) NIS, CH_2Cl_2. TBDMS = *tert*-butyldimethylsilyl, Im = imidazole, NIS = *N*-iodosuccinimide. TBS = *tert*-butyldimethylsilyl.

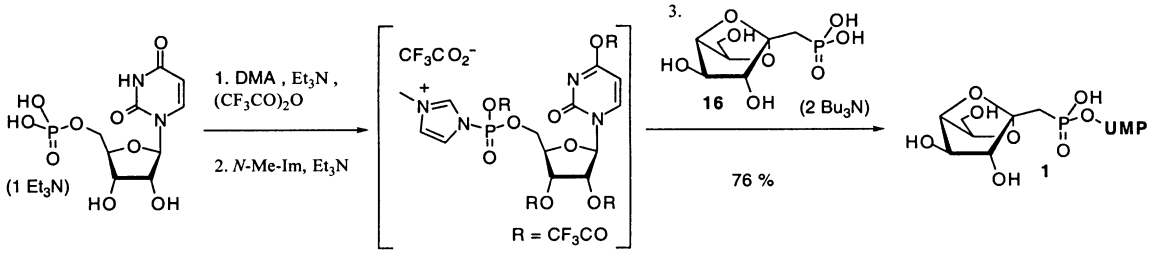


Scheme 3. Synthesis of the 1,4-anhydrogalactopyranose-phosphonate substructure. Reagents and conditions: a) $(RO)_2POCH_2Li$, THF; b) Py, $(CF_3CO)_2O$; c) PivCl; d) Dibal-H, CH_2Cl_2, O °C; e) CSA, CH_2Cl_2, reflux; f)TBAF, THF; g) H_2, Pd/C, MeOH. Piv = pivaloyl, Dibal-H = diisobutylaluminum hydride, CSA = camphorsulfonic acid.

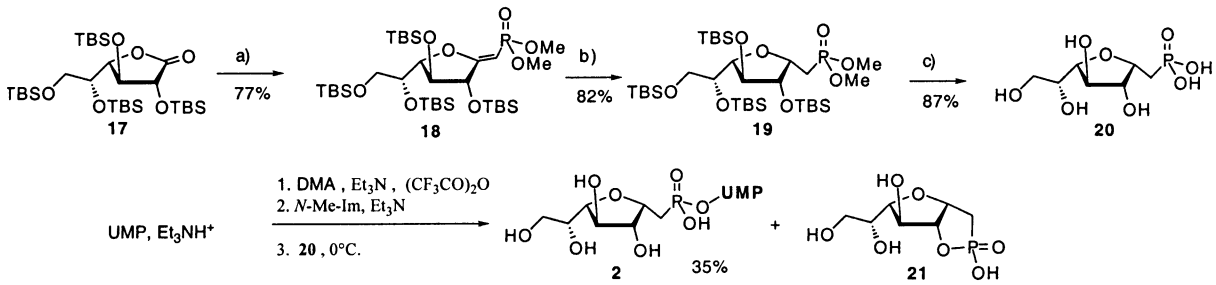
The phosphonylated glycal **10** readily cyclized in the presence of CSA in refluxing CH_2Cl_2 to give the constrained structure **15** in 88% yield. The $^{1,4}B$ conformation was confirmed by comparison with ^{1}H NMR data of analogous compounds,[25–27] with typically small coupling constants between H-2, H-3, H-4, and H-5 ($0.9\,Hz < J < 1.2\,Hz$). The three *tert*-butyldimethylsilyl (TBDMS) groups of intermediate **15** were removed with tetrabutylammonium fluoride (TBAF) in THF at $-5\,^\circ C$. Deprotection was completed by hydrogenolysis under standard conditions, affording **16** in 78% yield for the two deprotection steps.

Coupling to UMP: To circumvent the often problematic coupling of hindered phosphonates to UMP-morpholidate, we explored a procedure recently developed by Bogachev for the large-scale synthesis of nucleoside triphosphates.[28] This methodology was first exploited by Kiessling et al., for the synthesis of UDP-Gal*f*, and then by our group for the synthesis of GDP-hexanolamine.[29, 30] The procedure consists of the sequential activation of UMP by trifluoroacetic anhydride (TFAA) followed by the addition of an excess of *N*-Me-imidazole, affording a reactive phosphoimidazolium intermediate in situ. This undergoes a fast nucleophilic substitution upon phosphonate addition (Scheme 4). This reaction usually gives good to excellent yields with reaction times within hours, comparing very favorably with those required for classical morpholidate couplings. Under optimized conditions, coupling of phosphonate **16** with the activated UMP afforded the desired UDP-*C*-1,4-anhydrogalactopyranose **1** in 76% yield after size exclusion chromatography. From ^{31}P NMR spectra performed on aliquots of crude reaction mixture, we estimated that the conversion into **1** was 90% after 12 h. The only observed side product was the expected UMP dimer (UppU), in proportions (typically 5%) highly dependant on the proper drying of the reaction mixture. We have thus demonstrated the efficiency of this coupling methodology, allowing the preparation of **1** in good yield from a hindered neopentyl phosphonate.

A new synthesis of UDP-*C*-Gal*f*: UDP-*C*-Gal*f*, the phosphonate analogue of UDP-Gal*f* (Scheme 5), was first synthesized in our laboratory in eight steps by exploiting, as the key cyclization step, an α-stereospecific iodoetherification.[25] Here we report a shorter and still stereospecific sequence to this target molecule. Persilylated $(TBS)_4$-1,4-galactonolactone **17** was subjected to a one-pot sequence affording *exo*-glycal **18** in 77% yield. Hydrogenation over Pearlman's catalyst in AcOEt gave the desired α-phosphonate **19** in 82% yield (no β product could be isolated or even detected by ^{31}P NMR on the final crude reaction mixture). The α configuration at the 1-position could be confirmed by ^{1}H NMR (NOEs between H-1 and H-4 could be observed in molecules **19** and **20**) as well as by comparison with the previously characterized phosphonate **20**.[25] The source of the selectivity is probably the steric hindrance of the TBDMS group at the 2-position. Excess TMSI in CCl_4 quantitatively removed all the protecting groups. Phosphonate **20** was then coupled to UMP to afford pure UDP-*C*-Gal*f* in five steps from commercial 1,4-galactonolactone (Scheme 5). We observed that UDP-*C*-Gal*f* **2** decomposes through an intramolecular cyclization during the course of the reaction and the purification, to afford UMP and the bicyclic phosphonate **21**. This side reaction explains the poorer yield in relation to the same coupling method affording **1** (Scheme 4), despite almost total consumption of



Scheme 4. Synthesis of nucleotide-sugar **1**. DMA = *N*,*N*-dimethylaniline.



Scheme 5. Synthesis of UDP-*C*-Gal*f* **2**. Reagents and conditions: a) $(RO)_2POCH_2Li$, THF, concentrated then THF/Py, $(CF_3CO)_2O$; b) H_2, $Pd(OH)_2/C$, AcOEt; c) TMSI, CCl_4.

starting phosphonate **20**. This tendency was also observed in the preparation of other cyclic 1,2-*syn*-hydroxy-pyrophosphates such as UDP-Galf[29] and ADP-β-mannoheptopyranose[31].

Enzymatic assay: Two methods to evaluate UDP-galactose mutase kinetics have been described: a HPLC method and the use of radiolabeled UDP-Gal*f**.[9] The HPLC method was first described by McNeil et al., and was based on the separation of UDP-Gal*p* (used as the substrate) and UDP-Gal*f* (as the product of the enzymatic reaction).[32] Because of the small amount of furanose formed at the equilibrium (8%), this method was improved by Liu et al.,[15, 33] by following the reaction in the reverse direction (Gal*f* to Gal*p*). In this study we followed Liu's procedure as strictly as possible to compare the binding properties of our molecules with those of the described fluorinated inhibitors.[15] UDP-Gal mutase was overexpressed and purified by an improved procedure, similar to that described by Liu et al.,[33] but with the concomitant expression of chaperones that allowed an increase in the yield of soluble protein. A very intriguing and important observation was that fluorinated molecules were found to be inhibitors of UDP-Gal mutase when the native enzyme was used, and substrates of the enzyme when reductive conditions were employed (upon addition of freshly prepared sodium dithionite).[15] Furthermore, Zhang and Liu showed that their inhibitors did not display the same inhibition properties if the inhibitors were preincubated with the enzyme.[15] This binding and catalytic behavior might suggest significant changes in the conformation and/or the chemical state of the UDP-galactose binding pocket, when the enzyme is maintained (or not) in a reduced form. To provide a complete comparative inhibition pattern, we measured the residual mutase activities in the presence of our molecules under both native and reductive conditions, with and without preincubation. The results are summarized in Table 1. As in the case of 2-fluoro and 3-fluoro analogues of UDP-Gal*f*, compounds **1** and **2** displayed better inhibition properties when the native enzyme was used. The most significant information provided by this study is in the relative inhibition levels observed from native to reduced conditions: strong inhibition by UDP-*C*-Gal*f* **2** (81 to 91%) was observed when the native enzyme was used, whereas the inhibition—with or without preincubation of the inhibitor—collapsed to 2–14% under reductive conditions. Under the same assay conditions, the boat-locked molecule **1** displayed a moderate decrease in the inhibition level on going from the native enzyme (42–53%) to the reduced one (32–34%), making **1** a better inhibitor than **2** when the enzyme is maintained in its optimal catalytic efficiency. These results corroborate the previously described inhibition levels of UDP-2F-Gal*f* and UDP-3F-Gal*f* (Table 1), with which strong inhibition was observed when the native enzyme was used whereas these fluorinated molecules were either substrates or poor inhibitors of the reduced enzyme.[15] The inhibitory activity of the fluorinated analogues of UDP-Gal*f* on the native enzyme could be explained by the irreversible inactivation of the enzyme, whereas such a phenomenon is not likely to occur with ether-phosphonates such as **1** and **2**. Thus, our results clearly demonstrate that UDP-galactose mutase displays two different conformational states, a "native" and a "reduced" state, characterized by high and low affinity, respectively, for the galactofuranose moiety, and comparable affinities for the 1,4-anhydrogalactopyranose substructure.

The activity of the native enzyme, without dithionite addition, is typically very low compared to that of the reduced one. Photoreduction of the flavin cofactor has been proposed to explain the residual activity of the enzyme when no chemical reductant is added.[9] The kinetic and binding properties of the same enzyme under reducing or native conditions are very different: the K_m values for UDP-Gal*f* were determined to be 22 μM and 194 μM under reduced and native conditions, respectively.[15, 33] Similar values could be reproduced in our hands. A recent report showed that the flavosemiquinone $FADH^{\cdot}$ is stabilized in the presence of

Table 1. Inhibition percentages of UDP-galactose mutase at [UDP-Gal*f*] = 1 mM and [inhibitor] = 1 mM.

	UDP-1,4-anh-Gal*p* (**1**) [%]	UDP-*C*-Gal*f* (**2**) [%]	UDP-2F-Gal*f*[15] [%]	UDP-3F-Gal*f*[15] [%]
native enzyme, no preincubation[a]	42	81	nd[c]	87
native enzyme, preincubated[b]	53	91	61	98.6
reduced enzyme, no preincubation	34	14	nd	10
reduced enzyme, preincubated	32	2	nd	nd

[a] For conditions, see Experimental Section. [b] The inhibitor was incubated with the enzyme for 1 h at 21 °C before addition of UDP-Gal*f*. [c] Not determined.

UDP-galactose and that the fully reduced flavin is the anionic $FADH^-$ rather than $FADH_2$, suggesting a crypto-redox process.[16] Nevertheless, no redox turnover of the flavin cofactor has ever been detected during the UDP-Gal*p*/UDP-Gal*f* interconversion, which is also the case for several other flavoenzymes where the FAD has no apparent redox activity.[34] The exact role of the FAD cofactor therefore remains obscure, two different mechanistic assumptions being possible: 1) the flavin acts as a conformational switch, its bent conformation, when reduced as $FADH^-$, inducing a conformational change in the enzyme catalytic pocket, and 2) single-electron transfers are part of an activation of the galactose moiety at the anomeric center, triggering the cycle contraction.

At first sight, our data suggest that a $^{1,4}B$ conformation is not adopted by the galactose moiety at the transition state(s); otherwise stronger inhibition levels would have been observed in both native and reduced conditions. If a 1,4-anhydrogalactopyranose **A** is involved as a low-energy intermediate (mechanism depicted in Scheme 1), it might have been expected that **1** and **2** should bind to the enzyme with similar affinities; that is, with inhibition levels of similar magnitudes. The fact that a stronger inhibition level was observed with the boat-locked molecule **1** than with the corresponding furanose-locked **2** suggests that such a mechanism is possible. However, one cannot conclude solely on the basis of our inhibition study that the postulated mechanism[17] gives a complete picture of the furanose/pyranose interconversion catalyzed by UDP-galactose mutase. An alternative explanation would be that the distortion of the galactose moiety during the cycle contraction transiently brings the carbohydrate into a conformation close to a $^{1,4}B$ boat to allow nucleophilic substitutions at the anomeric position from the 4- and the 5-hydroxy groups. Co-crystallization of the mutase with molecules **1** and **2** might provide a better understanding of the conformational changes involved in this enzymatic reaction.

Conclusion

In summary, this study describes the straightforward synthesis of two C-glycosidic UDP-galactose derivatives: analogue **1**, presenting a galactose moiety locked in a bicyclic $^{1,4}B$ boat conformation, and analogue **2**, in which the galactose residue is locked in the conformation of the UDP-Gal mutase substrate. The two molecules were found to be inhibitors of the mutase at levels depending on the redox state of the enzyme. UDP-*C*-Gal*f* **2** is a strong inhibitor of the native enzyme but a poor inhibitor of the reduced one, while **1** displayed intermediate inhibition levels under both native and reducing conditions. These data indicate a significant conformational difference in the mutase binding pocket on going from the reduced enzyme to the native one, the enzyme switching from a low Gal*f*-affinity (reduced enzyme) to a strong one (native enzyme). It is remarkable that the mutase in its two conformational states binds the boat-locked analogue **1** with related affinities. These results support a mechanism involving the formation of 1,4-anhydrogalactopyranose as a low-energy intermediate.

Experimental Section

Materials and procedures: All chemicals were purchased from Sigma, Aldrich, or Fluka and were used without further purification. Tetrahydrofuran, diethyl ether, and toluene were freshly distilled over sodium benzophenone, dichloromethane over P_2O_5, and acetonitrile over CaH_2. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded with Bruker AC 250 and AMX 400 spectrometers. All compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR, as well as by ^1H,^1H and ^1H,^{13}C correlation experiments. Specific optical rotations were measured on a Perkin Elmer 241 polarimeter in a 1 dm cell. Melting points were determined with a Büchi 535 apparatus. Column chromatography was performed on silica gel (Kieselgel Si 60, 40–63 μm). Size exclusion chromatography was carried out on a Sephadex G15 column (2.5 × 60 cm) on a Pharmacia Biotech Äkta FPLC apparatus, fractions containing uridine derivatives being detected by UV. When required, purifications of nucleotide-sugars were achieved by semi-preparative HPLC, by use of a Waters Delta prep 4000 chromatography system fitted with a NovaPack C18 (1 × 10cm) column (eluent triethylammonium acetate 50 mM, pH = 6.8). For enzymatic assays, a Waters 600 E analytical apparatus fitted with a Zorbax C18-SB column (25 × 0.46 cm, 5 μm) was used (eluent triethylammonium acetate 50 mM, pH = 6.8). UDP-Gal*f* was prepared by known procedures.[29, 33]

Bacterial strains, plasmid vectors, and growth conditions: The *E. coli* strains DH5α (Life Technologies, Inc.) and BL21(DE3)pLysS (Novagen) were used as hosts for plasmids and for the preparation of the overproduced Glf enzyme. The pREP4groESL plasmid allowing overproduction of the bacterial chaperones was obtained from K. Amrein.[35] Unless otherwise noted, 2YT (Miller) was used as a rich medium and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. For strains carrying drug resistance genes, antibiotics were used at the following concentrations ($\mu g\,ml^{-1}$): ampicillin (100), kanamycin (35), and chloramphenicol (25).

General DNA techniques: DNA restriction and modification enzymes were obtained from New England Biolabs and oligonucleotides were obtained from MWG-Biotech. Small- and large-scale plasmid isolations from *E. coli* cells were carried out by the alkaline lysis method, and standard procedures were used for endonuclease digestions, ligation, and agarose electrophoresis.[36] *E. coli* cells were made suitable for transformation with plasmid DNA by treatment with $CaCl_2$.[37]

Construction of plasmids: A plasmid allowing expression of the *glf* gene from *E. coli* in the 6xHis-tagged form under control of the T7 promoter was constructed as follows. Firstly, a vector pET2160 that derived from pET21d (Novagen) was constructed by removal (filling-in) of its unique *Bgl*II site and replacement of the *Nco*I-*Hin*dIII polylinker by that from the pQE60 vector (Qiagen), as described previously for the construction of p*Trc*His60.[38] Polymerase chain reaction (PCR) primers were designed to incorporate a *Bsp*LU11I site (in bold) 5′ at the initiation codon (underlined) of the *glf* gene (5′-GAGGATT**AC*ATG*T**ACGATTATATCATTGTTGGTTC-3′), and a *Bgl*II site (in bold) 3′ to the gene and replacing the stop codon (5′-ATAGA**AGATCT**ATCCGTACTCATTATATTTTTCAC-3′). PCR amplification from the *E. coli* chromosome was performed in a Thermocycler 60 apparatus (Bio-med) by use of the polymerase Expand-Fidelity from Roche. The resulting 1.1 Kb product was purified by use of the Wizard PCR Preps DNA purification kit (Promega), treated with *Bsp*LU11I and *Bgl*II, and cloned between the compatible sites *Nco*I and *Bgl*II of vector pET2160, giving rise to plasmid pMLD200. DNA sequencing was performed to check that the sequence of the cloned fragment was correct.

Preparation of crude enzyme: Cells of BL21(DE3)pLysS carrying the two plasmids pREP4groESL and pMLD200 were grown exponentially at 37 °C in 2YT-ampicillin medium (1 L cultures). When the optical density of the culture reached 1, the temperature of the culture was decreased to 22 °C and expression of both the chaperones and the Glf protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Growth was continued for 16 h at 22 °C and cells were

harvested and washed with 40 mL of cold 20 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM $MgCl_2$ and 0.1% β-mercaptoethanol (buffer A). The cell pellet was then resuspended in 20 mL of the same buffer and disrupted by sonication (sonicator VibraCell, Bioblock, Illkirch, France) for 10 min in the cold. The resulting suspension was centrifuged at 4 °C for 30 min at 200 000 × *g* and the supernatant (20 mL, 350 mg of proteins) designated as crude enzyme was stored at −20 °C. SDS-PAGE analysis of proteins was performed as previously described, by use of 12% polyacrylamide gels.[39] Protein concentrations were determined by the method of Bradford,[40] with bovine serum albumin as standard.

Purification of the 6xHis-tagged Glf enzyme: The one-step purification procedure was carried out under native conditions, basically by following the steps in the manufacturer's (Qiagen, Santa Clarita, Calif.) recommendations: binding of 6xHis-Glf on Ni^{2+}-nitrilotriacetate-agarose (Ni^{2+}-NTA), washing with buffer A containing 200 mM KCl and 20 mM imidazole to remove impurities, and elution of the protein with increasing concentrations of imidazole added to buffer A (from 40 to 300 mM; the 6xHis-Glf protein eluted in 100 mM and 200 mM). The purified protein was then concentrated to 10 mL on PM10 membranes (Millipore) and dialyzed overnight against buffer A at 4 °C. The 6xHis-tagged enzyme prepared in this manner was more than 95% pure, as estimated by SDS-PAGE. Glycerol (15%) was added for its storage at −20 °C.

Enzyme kinetics and inhibition assays: The conditions described by Liu et al. were followed.[15] All assays were performed with a potassium phosphate buffer (100 mM, pH = 6.8) at T = 21 °C. Under reductive conditions, freshly prepared sodium dithionite solutions were used to allow a final concentration of 20 mM. When native mutase was used (without sodium dithionite), reactions were conducted in the dark under aerobic conditions. All inhibition studies consisted of measurement of the conversion of pure starting UDP-Gal*f* into UDP-Gal*p* (compared with a commercially available sample) by analytical HPLC (C18 column, elution by triethylammonium acetate 50 mM, pH = 6.8, detection at 262 nm). Substrate and inhibitor concentrations were adjusted at 1 mM from titrated mother solutions. Incubation times were always 1 h at 21 °C. As a control, the relative concentrations of any species in the reaction mixtures could be titrated with UMP as an internal standard. Enzyme concentrations were adjusted to allow a conversion of UDP-Gal*p* between 5 and 15%. Reactions were stopped by freezing the solution in liquid nitrogen. Residual enzyme activities were then measured in the presence of inhibitors at three different times, compared with the same experiment conducted without inhibitors, and averaged.

Atom and position numberings: We have systematically numbered the phosphonate methylene group as 1′ and adopted the usual numbering for carbohydrates from 1 to 6, with 1 for the anomeric position. For nucleotide-sugars, we used the conventional ribose and pyrimidine numberings: 1′ for the anomeric position, 1″ for the nitrogen atom linked to the ribose.

Uridine diphosphate-*C*-α-D-1,4-anhydrogalactopyranose (1): 5′UMP (triethylammonium salt, 59.6 mg, 0.14 mmol) was suspended in a mixture of freshly distilled MeCN (750 μL), *N*,*N*-dimethyl-aniline (70 μL, 0.56 mmol), and Et_3N (40 μL, 0.28 mmol), cooled to 0 °C and stirred under argon. Trifluoroacetic anhydride (118 μL, 0.84 mmol) was slowly added dropwise to the flask containing 5′-UMP. The reaction mixture was stirred for a few minutes at room temperature, after which a red-brown coloration was obtained. Excess trifluoroacetic anhydride and trifluoroacetic acid were removed from the reaction mixture under vacuum (by use of an oil pump and a liquid N_2 trap). In a separate flask, a mixture of *N*-Me-imidazole (56 μL, 0.70 mmol) in anhydrous MeCN (150 μL) and Et_3N (118 μL, 0.84 mmol) was prepared, cooled to 0 °C, and then added to the flask containing the mixed phosphoryl anhydride. The reaction mixture was stirred for 5–10 min at 0 °C, after which a bright yellow solution was obtained. In the meantime, a flask containing **16** (tributylammonium salt, 73 mg, 0.117 mmol) and preactivated 4 Å molecular sieves in MeCN (800 μL) was stirred for 30 min at 0 °C. The solution of UMP-*N*-methylimidazolide was then added dropwise to the solution containing **16**. The resulting mixture was stirred at 0 °C under Ar for 2 h and then for 13 h at room temperature. The reaction was quenched with cold aqueous ammonium formate (3 mL, 250 mM, pH 7). After filtration through a celite pad, the amines were extracted from the aqueous phase with CH_2Cl_2 (3 mL). The organic phase was washed with cold aqueous ammonium formate (2 mL, 250 mM, pH = 7) and the combined aqueous phases were pooled and lyophilized. They were purified by size exclusion chromatography (Sephadex G15) eluted with water. The appropriate fractions were pooled and lyophilized. Compound **1** was further purified by HPLC on a C_{18} column with 1% acetonitrile in 50 mM triethylammonium acetate buffer pH = 6.8 as eluent and a flow rate of 1 mL min^{-1}. The retention time of **1** under these conditions was 12.6 min. This protocol afforded **1** as a white solid (triethylammonium salt, 61 mg) in 76% yield.

^{1}H NMR (400 MHz, D_2O): δ = 7.95 (d, J(5″,6″) = 8.0 Hz, 1H; H-6″), 5.97 (d, J(1′,2′) = 4.1 Hz, 1H; H-1′), 5.95 (d, J(5″,6″) = 8.0 Hz, 1H; H-5″), 4.49 (d, J(3,4) = 1.0 Hz, 1H; H-4), 4.36 (m, 2H; H-2′, H-3′), 4.27 (m, 1H; H-4′), 4.22–4.18 (m, 2H; H-5′a, H-5′b), 3.95 (br s, 1H; H-3), 3.83 (dd, 1H; H-5), 3.80 (d, J(2,3) = 0.6 Hz, 1H; H-2), 3.60 (syst. ABX, J(5,6a) = 4.4 Hz, J(6a,6b) = 12.0 Hz, 1H; H-6a), 3.53 (ABX, J(5,6b) = 5.6 Hz, J(6a,6b) = 12.0 Hz, 1H; H-6b), 3.18 (q, J = 7.3 Hz; CH_2 (Et_3N)), 2.51 (ABX, J = 19.1 Hz, J(H,P) = 19.0 Hz, 2H; CH_2P), 1.26 (t, J = 7.3 Hz; CH_3 (Et_3N)) ppm; ^{13}C NMR (NH_4^+ salt, 100 MHz, D_2O): δ = = 168.48 (C-4″), 153.58 (C-2″), 141.76 (C-6″), 106.99 (d, J(1,P) = 4.0 Hz; C-1), 103.00 (C-5″), 89.02 (C-1′), 84.33 (d, J(3,P) = 5.2 Hz; C-3), 83.78 (C-4), 83.32 (d, J(4′,P) = 9.0 Hz; C-4′), 78.03 (C-2), 76.87 (C-5), 74.07 (C-3′), 62.58 (C-2′), 65.18 (d, J(5′,P) = 5.5 Hz; C-5′), 62.58 (C-6), 29.59 (d, J(C,P) = 138.5 Hz; CH_2P) ppm; ^{31}P NMR (101 MHz, D_2O): δ = 8.95 (d, J = 24.7 Hz), −11.43 (d, J = 24.8 Hz) ppm; MS (ESI): m/z (%): 561 (100) $[M-H]^+$, 280 (64) $[\langle B(M-2H)/2\rangle]^+$, 583 (21) $[M-2H^+ + Na]^+$, 599 (21) $[M-2H^+ + K]^+$; HRMS: found 561.05049; $C_{16}H_{23}N_2O_{16}P_2$ calcd (%) 561.05228

Uridine diphosphate *C*-α-D-galactofuranose (2): The procedure for the preparation of **1** was followed. Compound **20** and activated UMP were allowed to react for 1.5 h at 0 °C. The eluent for size exclusion chromatography was 50 mM triethylammonium acetate buffer (pH = 6.8) to prevent decomposition of the desired nucleotide-sugar. This protocol afforded **2** as a viscous solid (triethylammonium salt, 35% yield). ^{1}H, ^{13}C, ^{31}P NMR and mass spectra were in agreement with those published.[25]

1,4-Anhydro-2,3,6-tri-*O*-*tert*-butyldimethylsilyl-1-methyl-α-D-galactopyranose (6): Tebbe's reagent (0.4 M in toluene, 0.55 mL) was added dropwise at 0 °C to a solution of lactone **4**[20] (49 mg, 94 μmol) in an anhydrous mixture of toluene (1 mL), THF (1 mL), and pyridine (0.4 mL). This dark red solution was stirred for 1 h at 0 °C and for 30 min at room temperature. The reaction was stopped by dropwise addition of NaOH (1N, 2.0 mL) at 0 °C. The resulting mixture was filtrated through a short pad of celite and concentrated under vacuum. The resulting enol ether decomposed during the flash chromatography on silica gel (eluent cyclohexane/AcOEt 9:1) to give **6** (28 mg, 57%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.34 (d, J(3,4) = 1.3 Hz, 1H; H-4), 3.79 (br t, J(2,3) = J(3,4) = 1.2 Hz, 1H; H-3), 3.70 (d, J(2,3) = 1.0 Hz, 1H; H-2), 3.66 (dd, J(5,6a) = 4.4 Hz, J(5,6b) = 9.7 Hz, 1H; H-5), 3.56 (dd, J(5,6a) = 4.4 Hz, J(6a,6b) = 9.7 Hz, 1H; H-6a), 3.38 (t, J(5,6b) = J(6a,6b) = 9.7 Hz, 1H; H-6b), 1.52 (s, 3H; CH_3), 0.95 (s, 9H; Si–*t*Bu), 0.94 (s, 9H; Si–*t*Bu), 0.91 (s, 9H; Si–*t*Bu), 0.13 (s, 6H; 2 × Si–Me), 0.12 (s, 6H; 2 × Si–Me), 0.08 (s, 6H; 2 × Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 107.13 (C-1), 87.38 (C-3), 83.65 (C-4), 80.82 (C-2), 76.53 (C-5), 62.32 (C-6), 25.79, 25.66, 25.55, 18.02, 17.95, 16.11 (CH_3), −4.23, −4.49, −4.66, −4.71, −5.45, −5.47 ppm; MS (DCI-NH_3): m/z (%): 536 (100%) $[M+NH_4]^+$.

1,4-Anhydro-2,3,6-tri-*O*-*tert*-butyldimethylsilyl-1-iodomethyl-α-D-galactopyranose (7): Tebbe's reagent (0.4 M in toluene, 1.4 mL) was added dropwise at 0 °C to a solution of lactone **4**[20] (226 mg, 435 μmol) in an anhydrous mixture of toluene (4 mL), THF (2 mL), and pyridine (0.7 mL). This dark red solution was stirred for 1 hour at 0 °C and for 30 min at room temperature. The reaction was stopped by dropwise addition of NaOH (1N, 2.0 mL) at 0 °C. The resulting mixture was filtrated through a short pad of celite and concentrated under vacuum. The crude material was dried twice by azeotropic evaporation with anhydrous toluene, and then dissolved in anhydrous CH_2Cl_2 (5 mL). Molecular sieves (4 Å, 300 mg) were added to this solution, followed by NIS (170 mg, 756 μmol, 1.7 equiv) at 0 °C. The resulting mixture was stirred for 45 min at 0 °C, and then filtrated through celite, diluted with CH_2Cl_2 (15 mL), and washed twice with sodium dithionite (0.5N, 10 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography, eluted with cyclohexane/AcOEt (2:1) to give **7** (174 mg, 62% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.42 (d, J(3,4) = 1.3 Hz, 1H; H-4), 4.04 (br t, 1H; H-3), 3.87 (d, J(2,3) = 0.9 Hz, 1H; H-2), 3.82 (dd, J(5,6a) = 4.5 Hz, J(5,6b) = 9.9 Hz, 1H; H-5), 3.61 (dd, J(5,6a) = 4.5 Hz, J(6a,6b) =

9.8 Hz, 1H; H-6a), 3.52 (t, $J(5,6b)=J(6a,6b)=9.8$ Hz, 1H; H-6b), 3.47 (AB, $J(AB)=11.5$ Hz, 2H; CH_2–I), 0.94 (s, 9H; Si–*t*Bu), 0.93 (s, 9H; Si–*t*Bu), 0.91 (s, 9H; Si–*t*Bu), 0.14 (s, 3H; Si–Me), 0.13 (s, 6H; 2 × Si–Me), 0.12 (s, 3H; Si–Me), 0.09 (s, 3H; Si–Me), 0.08 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\Delta=105.44$ (C-1), 84.89 (C-3), 83.66 (C-4), 81.80 (C-2), 77.20 (C-5), 61.72 (C-6), 25.76, 25.74, 18.05, 17.93, 17.89, 0.52 (CH_2–I), −4.19, −4.46, −4.71, −4.79, −5.43, −5.44 ppm; MS (DCI-NH_3): *m*/*z* (%): 662 (100) $[M+NH_4]^+$.

1-(Dibenzyloxyphosphoryl)methyl-2,3,6-tri-*O*-*tert*-butyldimethylsilyl-α,β-D-galactofuranose (8): Butyllithium (3.75 mL, 9.37 mmol, of a 2.5 M solution in hexane) was added under Ar to a cooled (−70 °C) solution of dibenzyl methylphosphonate (2.59 g, 9.37 mmol) in anhydrous THF (18 mL), followed after 20 min by a solution of 2,3,6-tri-*O*-*tert*-butyldimethylsilyl-D-galactono-1,4-lactone **4**[20] (1.95 g, 3.75 mmol) in anhydrous THF (4 mL). The temperature was maintained at −70 °C for 10 min, and the reaction mixture was then allowed to come to −40 °C over a 1 h period. The solution was then diluted with phosphate buffer (1M, 70 mL, pH = 7) and extracted with CH_2Cl_2 (2 × 180 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography eluted with cyclohexane/AcOEt (4:1) to give **8** (2.42 g, 81% yield) as a white solid. α/β ratio: 20:80.

^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.40-7.33$ (m, 12.5H; H arom.), 5.14–5.05 (m, 4H; CH_2Ph$_\beta$), 5.00–4.93 (m, 1H; CH_2Ph$_\alpha$), 4.84 (br s, 1H; OH-1$_\beta$), 4.57 (br s, 0.25H; OH-1$_\alpha$), 4.42 (d, $J(2,3)=2.4$ Hz, 0.25H; H-2$_\alpha$), 4.25 (dd, 0.25H; H-3$_\alpha$), 4.24 (d, $J(3,4)=4.0$ Hz, $J(4,5)=1.8$ Hz, 1H; H-4$_\beta$), 4.15 (t, $J(3,4)=4.0$ Hz, $J(2,3)=3.8$ Hz, 1H; H-3$_\beta$), 4.10 (d, $J(2,3)=3.8$ Hz, 1H; H-2$_\beta$), 4.07 (t, $J(3,4)=J(4,5)=2.6$ Hz, 0.25H; H-4$_\alpha$), 3.71–3.53 (m, 3.75H; H-5$_{\alpha,\beta}$, H-6a$_{\alpha,\beta}$, H-6b$_{\alpha,\beta}$), 3.28 (d, $J(5,OH)=7.3$ Hz, 1H; OH-5$_\beta$), 3.08 (d, $J(5,OH)=5.5$ Hz, 0.25H; OH-5$_\alpha$), 2.70 (ABX, $J(1'a,1'b)=15.3$ Hz, $J(1'a,P)=18.4$ Hz, 0.25H; H-1'a$_\alpha$), 2.55 (ABX, $J(1'a,1'b)=15.3$ Hz, $J(1'b,P)=19.0$ Hz, 0.25H; H-1'b$_\alpha$), 2.52 (ABX, $J(1'a,1'b)=15.4$ Hz, $J(1'a,P)=19.2$ Hz, 1H; H-1'a$_\beta$), 2.29 (ABX, $J(1'a,1'b)=15.4$ Hz, $J(1'b,P)=17.9$ Hz, 1H; H-1'b$_\beta$), 0.97 (s, 2.25H; Si–*t*Bu), 0.93 (s, 9H; Si–*t*Bu), 0.92 (2 × s, 11.25H; 2 × Si–*t*Bu), 0.90 (s, 9H; Si–*t*Bu), 0.89 (s, 2.25H; Si–*t*Bu), 0.23 (2 × s, 1.5H; 2 × Si–Me), 0.17 (s, 3H; Si–Me), 0.16 (s, 3H; Si–Me), 0.15 (2 × s, 7.5H; 4Si–Me), 0.12 (s, 0.75H; Si–Me), 0.06 (2 × s, 6H; 2 × Si–Me), 0.04 (s, 0.75H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=136.61$ (d, $J(C,P)=6.5$ Hz; C^q arom.$_\alpha$), 136.26 (d, $J(C,P)=6.4$ Hz; C^q arom.$_\beta$), 136.20 (d, $J(C,P)=6.5$ Hz; C^q arom.$_\alpha$), 136.01 (d, $J(C,P)=6.5$ Hz; C^q arom.$_\beta$), 128.81–127.60 (CH arom.), 104.60 (d, $J(1,P)=6.0$ Hz; C-1$_\beta$), 102.72 (d, $J(1,P)=6.0$ Hz; C-1$_\alpha$), 83.65 (C-4$_\alpha$), 82.79 (C-4$_\beta$), 82.45 (d, $J(2,P)=7.7$ Hz; C-2$_\beta$), 80.15 (d, $J(2,P)=4.2$ Hz; C-2$_\alpha$), 78.50 (C-3$_\alpha$), 78.27 (C-3$_\beta$), 71.09 (C-5$_\alpha$), 70.45 (C-5$_\beta$), 67.84 (d, $J(C,P)=5.9$ Hz; CH_2Ph_α), 67.64 (d, $J(C,P)=6.0$ Hz; CH_2Ph_β), 67.26 (d, $J(C,P)=6.3$ Hz; CH_2Ph_β), 66.50 (d, $J(C,P)=6.3$ Hz; CH_2Ph_α), 63.58 (C-6$_\beta$, C-6$_\alpha$), 34.20 (d, $J(1',P)=137$ Hz; C-1'$_\alpha$), 32.90 (d, $J(1',P)=138.5$ Hz; C-1'$_\beta$), 26.26, 26.07, 25.87, 25.80, 25.78, 25.72, 18.21, 17.89, 17.77, −4.30, −4.42, −4.44, −4.47, −4.57, −4.64, −5.38, −5.41, −5.44 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta=29.36$ (major diast.), 26.58 (minor diast.) ppm; MS (DCI-NH_3): *m*/*z* (%): 797 (25) $[M+H]^+$, 814 (37) $[M+NH_4]^+$, 779 (100) $[M+H-H_2O]^+$; elemental analysis calcd (%) for $C_{39}H_{69}O_9PSi_3$: C 58.76, H 8.72; found: C 58.61, H 8.84.

2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-1-(dimethoxyphosphoryl)methyl-α,β-D-galactofuranose (9): The procedure used for the preparation of **8** was followed. The residue was purified by silica gel chromatography eluted with cyclohexane/AcOEt (2:1) to give **9** (65% yield) as a colorless syrup. α/β ratio: 35:65.

^{1}H NMR (400 MHz, $CDCl_3$): $\delta=4.32$ (d, $J(2,3)=2.5$ Hz, 0.5H; H-2$_\alpha$), 4.25 (dd, $J(3,4)=3.8$ Hz, $J(4,5)=1.9$ Hz, 1H; H-4$_\beta$), 4.23 (t, $J(2,3)=J(3,4)=2.5$ Hz, 0.5H; H-3$_\alpha$), 4.16 (tdd, $J(2,3)=2.7$ Hz, $J(3,4)=3.8$ Hz, $J(3,P)=0.8$ Hz, 1H; H-3$_\beta$), 4.07 (d, $J(2,3)=2.7$ Hz, 1H; H-2$_\beta$), 4.05 (m, 0.5H; H-4$_\alpha$), 3.82 (d, $J(H,P)=11.2$ Hz, 3H; $OCH_{3\beta}$), 3.81 (d, $J(H,P)=11.1$ Hz, 1.5H; $OCH_{3\alpha}$), 3.79 (d, $J(H,P)=10.9$ Hz, 3H; $OCH_{3\beta}$), 3.74 (d, $J(H,P)=10.9$ Hz, 1.5H; $OCH_{3\alpha}$), 3.74–3.58 (m, 4.5H; H-5$_{\alpha,\beta}$, H-6a$_{\alpha/\beta}$, H-6b$_{\alpha/\beta}$), 2.56 (ABX, $J(1'a,1'b)=15.3$ Hz, $J(1'a,P)=18.5$ Hz, 0.5H; H-1'a$_\alpha$), 2.46 (ABX, $J(1'a,1'b)=15.3$ Hz, $J(1'b,P)=19.1$ Hz, 0.5H; H-1'b$_\alpha$), 2.45 (ABX, $J(1'a,1'b)=15.4$ Hz, $J(1'a,P)=19.1$ Hz, 1H; H-1'a$_\beta$), 2.25 (ABX, $J(1'a,1'b)=15.4$ Hz, $J(1'b,P)=18.1$ Hz, 1H; H-1'b$_\beta$), 0.96 (s, 4.5H; Si–*t*Bu), 0.95 (s, 9H; Si–*t*Bu), 0.93 (s, 4.5H; Si–*t*Bu), 0.92 (s, 9H; Si–*t*Bu), 0.91 (s, 9H; Si–*t*Bu), 0.90 (s, 4.5H; Si–*t*Bu), 0.23 (s, 3H; 2 × Si–Me), 0.22 (s, 1.5H; Si–Me), 0.20 (s, 3H; Si–Me), 0.19 (s, 3H; Si–Me), 0.15 (s, 4.5H; 2 × Si–Me), 0.14 (s, 3H; Si–Me), 0.12 (s, 1.5H; Si–Me), 0.08 (s, 4,5H; Si–Me), 0.07 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=104.80$ (d, $J(1,P)=5.9$ Hz; C-1$_\beta$), 102.48 (d, $J(1,P)=2.8$ Hz; C-1$_\alpha$), 83.59 (C-4$_\beta$), 83.55 (C-4$_\alpha$), 82.36 (d, $J(2,P)=7.8$ Hz; C-2$_\beta$), 80.43 (d, $J(2,P)=5.3$ Hz; C-2$_\alpha$), 78.68 (C-3$_\beta$), 78.53 (C-3$_\alpha$), 70.99 (C-5$_\alpha$), 70.64 (C-5$_\beta$), 63.75 (C-6$_\beta$), 63.49 (C-6$_\alpha$), 52.97 (d, $J(C,P)=6.2$ Hz, $OCH_{3\beta}$), 52.83 (d, $J(C,P)=6.3$ Hz, $OCH_{3\alpha}$), 52.24 (d, $J(C,P)=6.2$ Hz, $OCH_{3\alpha}$), 51.81 (d, $J(C,P)=6.3$ Hz, $OCH_{3\beta}$), 33.27 (d, $J(1',P)=136.8$ Hz; C-1'$_\alpha$), 31.39 (d, $J(1',P)=139.3$ Hz; C-1'$_\beta$), 25.87, 25.83, 25.77, 25.71, 25.66, 18.24, 18.20, 17.92, 17.91, 17.75, −4.30, −4.38, −4.43, −4.48, −4.57, −4.68, −4.70, −5.39, −5.44, −5.49 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta=30.59$ (major diast.), 28.19 (minor diast.) ppm; MS (DCI-NH_3): *m*/*z* (%): 645 (17) $[M+H]^+$, 662 (100) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{27}H_{61}O_9PSi_3$: C 50.28, H 9.53; found: C 50.27, H 9.61.

(1(1′)*Z*)-1-(Dibenzyloxyphosphoryl)methylidene-2,3,6-tri-*O*-*tert*-butyldimethylsilyl-D-galactofuranose (10): Pyridine (510 μL, 6.32 mmol) and trifluoroacetic anhydride (440 μL, 3.16 mmol) were added at 0 °C to a solution of **8** (503 mg, 0.63 mmol) in anhydrous THF (20 mL). After 3 h at 0 °C, the reaction was stopped by addition of saturated aqueous $NaHCO_3$, and the mixture was extracted with AcOEt (100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated at reduced pressure. Purification by silica gel chromatography, eluted with cyclohexane/AcOEt (2:1), afforded **10** in (378 mg, 77% yield) as a colorless syrup.
$[\alpha]_D^{23}$ +55.4 (c = 1.2, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.38-7.30$ (m, 10H; H arom.), 5.09–5.01 (m, 4H; CH_2Ph), 4.75 (dd, $J(1',2)=1.5$ Hz, $J(1',P)=12.4$ Hz, 1H; H-1′), 4.53 (ddd, $J(1',2)=1.5$ Hz, $J(2,P)=4.1$ Hz, $J(2,3)=6.6$ Hz, 1H; H-2), 4.44 (d, $J(3,4)=6.3$ Hz, 1H; H-4), 4.35 (dd, $J(2,3)=6.6$ Hz, $J(3,4)=6.3$ Hz, 1H; H-3), 3.73–3.64 (m, 3H; H-5, H-6a, H-6b), 0.95 (s, 9H; Si–*t*Bu), 0.93 (s, 9H; Si–*t*Bu), 0.91 (s, 9H; Si–*t*Bu), 0.17 (s, 3H; Si–Me), 0.14 (2 × s, 6H; 2 × Si–Me), 0.12 (s, 3H; Si–Me), 0.06 (s, 6H; 2 × Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=174.29$ (d, $J(1,P)=2.0$ Hz; C-1), 136.50 (d, $J(C,P)=7.2$ Hz; C^q arom.), 136.40 (d, $J(C,P)=7.2$ Hz; C^q arom.), 128.45–127.75 (10 × CH arom.), 84.35 (C-4), 83.81 (d, $J(1',P)=193.5$ Hz; C-1′), 78.38 (d, $J(2,P)=12.5$ Hz; C-2), 75.67 (C-3), 70.36 (C-5), 67.20 (d, $J(C,P)=5.2$ Hz; CH_2Ph), 66.90 (d, $J(C,P)=5.2$ Hz; CH_2Ph), 62.82 (C-6), 25.81, 25.78, 25.70, 18.12, 17.83, 17.81, −4.01, −4.1, −4.28, −4.56, −5.44, −5.53 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta=20.69$ ppm; MS (DCI-NH_3): *m*/*z*: 779 $[M+H]^+$; elemental analysis calcd (%) for $C_{39}H_{67}O_8PSi_3$: C 60.12, H 8.67; found: C 60.03, H 8.76.

(1(1′)*Z*)-2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-1-(dimethoxyphosphoryl)methylidene-D-galactofuranose (11): Triethylamine (320 μL, 2.28 mmol) and trifluoroacetic anhydride (475 μL, 3.42 mmol) were added at 0 °C to a solution of **9** (489 mg, 0.76 mmol) in anhydrous pyridine (10 mL). The resulting solution was allowed to reach room temperature for a period of 2 h. Solvents were evaporated, and the crude mixture was diluted with CH_2Cl_2 (20 mL) and washed with saturated aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$, filtered, and concentrated at reduced pressure. The purification by silica gel chromatography, eluted with cyclohexane/AcOEt (1:1), afforded **11** (300 mg, 63%) as a white solid.

M.p. 95–96 °C; $[\alpha]_D^{24}$ +50.3 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=4.68$ (dd, $J(1',2)=1.5$ Hz, $J(1',P)=12.0$ Hz, 1H; H-1′), 4.55 (ddd, $J(1',2)=1.5$ Hz, $J(2,P)=4.0$ Hz, $J(2,3)=6.3$ Hz, 1H; H-2), 4.45 (d, $J(3,4)=5.9$ Hz, 1H; H-4), 4.35 (t, $J(2,3)=J(3,4)=6.3$ Hz, 1H; H-3), 3.76 (d, $J(H,P)=11.6$ Hz, 3H; OCH_3), 3.74 (d, $J(H,P)=11.3$ Hz, 3H; OCH_3), 3.81–3.69 (m, 3H; H-5, H-6a, H-6b), 0.98 (s, 9H; Si–*t*Bu), 0.93 (s, 9H; Si–*t*Bu), 0.92 (s, 9H; Si–*t*Bu), 0.18 (s, 3H; Si–Me), 0.17 (s, 3H; Si–Me), 0.14 (s, 3H; Si–Me), 0.09 (s, 9H; 3Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=174.31$ (d, $J(1,P)=2.0$ Hz; C-1), 84.71 (C-4), 82.82 (d, $J(1',P)=193.3$ Hz; C-1′), 78.46 (d, $J(2,P)=12.5$ Hz; C-2), 75.80 (C-3), 70.43 (C-5), 62.91 (C-6), 52.44 (d, $J(C,P)=5.6$ Hz, OCH_3), 51.99 (d, $J(C,P)=5.5$ Hz, OCH_3), 25.82, 25.77, 25.69, 18.16, 17.86, 17.82, −4.05, −4.19, −4.29, −4.58, −5.44, −5.57 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta=22.60$ ppm; MS (DCI-NH_3): *m*/*z* (%): 627 (100) $[M+H]^+$, 644 (70) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{27}H_{59}O_8PSi_3$: C 51.72, H 9.48; found: C 51.57, H 9.64.

2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-5-*O*-pivaloyl-D-galactono-1,4-lactone (12): 2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-D-galactono-1,4-lactone **4**[20] (1 g, 1.92 mmol) was dissolved in anhydrous pyridine (15 mL) under Ar atmosphere. Excess pivaloyl chloride (0.9 mL, 7.29 mmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP) (94 mg, 0.77 mmol) in anhydrous pyridine (1.5 mL) were slowly added to this solution, and the resulting mixture was stirred for an additional 12 h at room temperature.

The solution was washed with saturated aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$. After solvent evaporation, the residue was purified by silica gel chromatography eluted with cyclohexane/AcOEt (96:4) to give **12** (1.12 g, 96.5% yield) as a white solid.

m.p. 35–36 °C; $[\alpha]_D^{24}$ +2.5 (c = 1.01, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.01 (ddd, J(4,5) = 1.1 Hz, J(5,6a) = 7.1 Hz, J(5,6b) = 7.3 Hz, 1H; H-5), 4.48 (dd, J(4,5) = 1.1 Hz, J(3,4) = 7.0 Hz, 1H; H-4), 4.43 (d, J(2,3) = 7.0 Hz, 1H; H-2), 4.13 (t, J(2,3) = J(3,4) = 7.0 Hz, 1H; H-3), 3.74–3.72 (m, 2H; H-6a, H-6b), 1.23 (s, 9H; Piv), 0.95 (s, 9H; Si–tBu), 0.93 (s, 9H; Si–tBu), 0.91 (s, 9H; Si–tBu), 0.24 (s, 3H; Si–Me), 0.18 (s, 3H; Si–Me), 0.13 (s, 3H; Si–Me), 0.12 (s, 3H; Si–Me), 0.10 (s, 3H; Si–Me), 0.09 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.49 (C=O , Piv), 172.88 (C-1), 78.78 (C-4), 76.53 (C-2), 75.42 (C-3), 70.03 (C-5), 59.59 (C-6), 39.0, 27.09, 25.71, 25.64, 18.13, 18.06, 17.76, −4.19, −4.29, −4.77, −5.49, −5.57 ppm; MS (DCI-NH_3): m/z (%): 622 (100) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{29}H_{60}O_7Si_3$: C 57.57, H 10.00; found: C 57.70, H 10.00.

2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-1-(dimethoxyphosphoryl)methyl-5-*O*-pivaloyl-α,β-D-galactofuranose (13): The procedure used for the preparation of **8** was applied. The residue was purified by silica gel chromatography eluted with cyclohexane/AcOEt (2:1) to give **13** (511 mg, 65% yield) as a colorless oil. α/β ratio: 45:55.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.31 (dt, J(4,5) = 9.1 Hz, J(5,6a,b) = 3.9 Hz, 0.8H; H-5α), 5.08 (q, J(4,5) = 4.7 Hz, J(5,6a,b) = 5.0 Hz, 1H; H-5$_\beta$), 4.39 (s, 0.8H; H-2$_\alpha$), 4.31 (t, J(3,4) = J(4,5) = 4.7 Hz, 1H; H-4$_\beta$), 4.08 (d, J(3,4) = 0.9 Hz, 0.8H; H-3$_\alpha$), 4.07 (d, J(2,3) = 2.9 Hz, 1H; H-2$_\beta$), 4.02 (d, J(4,5) = 9.1 Hz, 0.8H; H-4$_\alpha$), 3.96 (ddd, J(2,3) = 2.9 Hz, J(3,4) = 4.6 Hz, J(3,P) = 0.7 Hz, 1H; H-3$_\beta$), 3.83 (d, J(H,P) = 11.1 Hz, 3H; $OCH_{3\beta}$), 3.79 (d, J(H,P) = 11.3 Hz, 3H; $OCH_{3\beta}$), 3.76 (d, J(H,P) = 11.2 Hz, 2.4H; $OCH_{3\alpha}$), 3.72 (d, J(H,P) = 10.9 Hz, 2.4H; $OCH_{3\alpha}$), 3.81–3.72 (m, 5.4H; H-5$_{\alpha/\beta}$, H-6a$_{\alpha/\beta}$, H-6b$_{\alpha/\beta}$), 2.51 (AX, J(1′,P) = 18.3 Hz, 1.6H; H-1′a$_\alpha$, H-1′b$_\alpha$), 2.30 (ABX, J(1′a,1′b) = 15.4 Hz, J(1′a,P) = 18.1 Hz, 1H; H-1′a$_\beta$), 2.24 (ABX, J(1′a,1′b) = 15.4 Hz, J(1′b,P) = 18.1 Hz, 1H; H-1′b$_\beta$), 1.25 (2 × s, 16.2H; Piv$_{\alpha/\beta}$), 0.98 (s, 7.2H; Si–tBu), 0.95 (s, 7.2H; Si–tBu), 0.93 (s, 7.2H; Si–tBu), 0.92 (s, 9H; Si–tBu), 0.91 (2 × s, 18H; 2 × Si–tBu), 0.19 (s, 2.4H; Si–Me), 0.17 (s, 2.4H; Si–Me), 0.16 (s, 3H; Si–Me), 0.14 (2 × s, 8.4H; 3Si–Me), 0.13 (2 × s, 5.4H; 2 × Si–Me), 0.09 (2 × s, 5.4H; 2 × Si–Me), 0.08 (s, 5.4H; 2 × Si–Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.47 (C=O , Piv$_\beta$), 177.64 (C=O, Piv$_\alpha$), 104.82 (d, J(1,P) = 6.2 Hz; C-1$_\beta$), 104.12 (C-1$_\alpha$), 84.24 (d, J(2,P) = 8.7 Hz; C-2$_\beta$), 83.04 (C-4$_\alpha$), 81.69 (C-4$_\beta$), 79.32 (d, $J_{(2,P)}$ = 2.9 Hz; C-2$_\alpha$), 78.71 (C-3$_\alpha$), 78.67 (C-3$_\beta$), 72.69 (C-5$_\alpha$), 71.99 (C-5$_\beta$), 62.40 (C-6$_\alpha$), 61.66 (C-6$_\beta$), 53.11 (d, J(C,P) = 5.9 Hz, $OCH_{3\beta}$), 52.89 (d, J(C,P) = 6.3 Hz, $OCH_{3\beta}$), 51.71 (d, J(C,P) = 6.0 Hz, $OCH_{3\alpha}$), 51.65 (d, J(C,P) = 6.0 Hz, $OCH_{3\alpha}$), 38.91, 35.21 (d, J(1′,P) = 136.4 Hz; C-1′$_\alpha$), 31.80 (d, J(1′,P) = 137.2 Hz; C-1′$_\beta$), 27.29, 27.25, 25.85, 25.83, 25.76, 25.74, 25.63, 18.26, 18.20, 17.98, 17.96, 17.83, 17.81, −4.26, −4.37, −4.40, −4.49, −4.72, −4.74, −4.78, −4.88, −5.39, −5.41, −5.47 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): δ = 31.07 (major diast.), 28.18 (minor diast.) ppm; MS (DCI-NH_3): m/z (%): 746 (100) $[M+NH_4]^+$, 728 (45) $[M+NH_4-H_2O]^+$; elemental analysis calcd (%) for $C_{32}H_{69}O_{10}PSi_3$: C 52.71, H 9.54; found: C 52.53, H 9.71.

(1(1′)*Z*)-2,3,6-Tri-*O*-*tert*-butyldimethylsilyl-1-(dimethoxyphosphoryl)methylidene-5-*O*-pivaloyl-D-galactofuranose (14): Trifluoroacetic anhydride (266 μL, 1.91 mmol) was added to a solution of **13** (278 mg, 0.38 mmol) in anhydrous pyridine (4 mL). The resulting solution was stirred at 0 °C overnight. Solvents were evaporated and the crude mixture was diluted with CH_2Cl_2 (10 mL) and washed with saturated aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$, filtered, and concentrated at reduced pressure. Purification by silica gel chromatography, eluted with cyclohexane/AcOEt (2:1), afforded **14** (108 mg, 40%) as a colorless oil.

$[\alpha]_D^{24}$ +34.8 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.10 (ddd, J(4,5) = 3.5 Hz, J(5,6a) = 7.3 Hz, J(5,6b) = 4.9 Hz, 1H; H-5), 4.59 (dd, J(3,4) = 4.8 Hz, J(4,5) = 3.5 Hz, 1H; H-4), 4.58 (d, J(1′,P) = 10.2 Hz, 1H; H-1′), 4.55 (dt, J(1′,2) = J(2,P) = 1.2 Hz, J(2,3) = 4.8 Hz, 1H; H-2), 4.07 (t, J(2,3) = J(3,4) = 4.8 Hz, 1H; H-3), 3.82 (ABX, J(5,6a) = 7.3 Hz, J(6a,6b) = 9.9 Hz, 1H; H-6a), 3.76 (ABX, J(5,6b) = 4.9 Hz, J(6a,6b) = 9.9 Hz, 1H; H-6b), 3.74 (d, J(H,P) = 11.4 Hz, 3H; OCH_3), 3.73 (d, J(H,P) = 11.4 Hz, 3H; OCH_3), 1.23 (s, 9H; Piv), 0.95 (s, 9H; Si–tBu), 0.93 (s, 9H; Si–tBu), 0.91 (s, 9H; Si–tBu), 0.24 (s, 3H; Si–Me), 0.18 (s, 3H; Si–Me), 0.13 (s, 3H; Si–Me), 0.12 (s, 3H; Si–Me), 0.10 (s, 3H; Si–Me), 0.09 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.67 (C=O, Piv), 172.50 (d, J(1,P) = 2.5 Hz; C-1), 85.80 (C-4), 81.67 (d, J(1′,P) = 194.3 Hz; C-1′), 79.97 (d, J(2,P) = 13.3 Hz; C-2), 76.31 (C-3), 71.74 (C-5), 60.47 (C-6), 52.13 (d, J(C,P) = 5.1 Hz, OCH_3), 51.99 (d, J(C,P) = 5.3 Hz, OCH_3), 29.69, 27.10, 25.78, 25.73, 25.65, 18.15, 17.80, −4.21, −4.27, −4.31, −4.41, −5.47, −5.59 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): δ = 21.16 ppm; MS (DCI-NH_3): m/z (%): 711 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{32}H_{67}O_9PSi_3$: C 54.05, H 9.50; found: C 53.81, H 9.80.

1,4-Anhydro-1-(dibenzyloxyphosphoryl)methyl-2,3,6-tri-*O*-*tert*-butyldimethylsilyl-α-D-galactopyranose (15): Camphorsulfonic acid (272 mg, 1.17 mmol) was added to a solution of **10** (537 mg, 0.69 mmol) and molecular sieves (4 Å, 900 mg) in dry dichloromethane (21 mL). The reaction mixture was heated at reflux under Ar for 17 hours, and then filtered through a pad of celite. The filtrate was washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. The residue was chromatographed on silica gel with cyclohexane/AcOEt (4:1) as eluent. The product **15** was obtained as a colorless oil (596 mg, 74% yield), along with **15′** (65 mg, 14% yield), the product of monodesilylation at the 6-position. Compounds **15** and **15′** could be pooled for the removal of the TBDMS groups.

$[\alpha]_D^{20}$ +26.6 (c = 1.2, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38–7.30 (m, 10H; H arom.), 5.16–5.03 (2ABX, 4H; CH_2Ph), 4.46 (d, J(3,4) = 1.5 Hz, 1H; H-4), 4.12 (t, J(2,3) = J(3,4) = 1.3 Hz, 1H; H-3), 3.69 (brs, 1H; H-2), 3.66 (dd, J(5,6a) = 4.5 Hz, J(5,6b) = 9.9 Hz, 1H; H-5), 3.53 (ABX, J(5,6a) = 4.5 Hz, J(6a,6b) = 9.8 Hz, 1H; H-6a), 3.45 (ABX, J(5,6b) = J(6a,6b) = 9.8 Hz, 1H; H-6b), 2.50 (ABX, J(1′a,1′b) = 15.7 Hz, J(1′a,P) = 19.5 Hz, 1H; H-1′a), 2.43 (ABX, J(1′a,1′b) = 15.7 Hz, J(1′b,P) = 19.4 Hz, 1H; H-1′b), 0.93 (s, 9H; Si–tBu), 0.92 (s, 9H; Si–tBu), 0.90 (s, 9H; Si–tBu), 0.13 (s, 6H; 2 × Si–Me), 0.11 (s, 3H; Si–Me), 0.05 (s, 9H; 3Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 136.54 (d, J(C,P) = 6.5 Hz; C^q arom.), 136.38 (d, J(C,P) = 6.5 Hz; C^q arom.), 128.46–127.88 (10 × CH arom.), 105.32 (d, J(1,P) = 4.3 Hz; C-1), 86.72 (d, J(3,P) = 6.4 Hz; C-3), 84.09 (C-4), 79.99 (C-2), 76.49 (C-5), 67.58 (d, J(C,P) = 5.9 Hz; CH_2Ph), 67.05 (d, J(C,P) = 6.1 Hz; CH_2Ph), 61.94 (C-6), 27.57 (d, J(1′,P) = 141.7 Hz; C-1′), 25.76, 25.75, 25.72, 18.03, 17.90, 17.86, −4.21, −4.52, −4.66, −4.77, −5.46 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): δ = 24.66 ppm; MS (DCI-NH_3): m/z (%): 779 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{39}H_{67}O_8PSi_3$: C 60.12, H 8.67; found: C 60.01, H 8.76.

Analytical data for molecule 15′: $[\alpha]_D^{20}$ +84.6 (c = 1.9, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39–7.35 (m, 10H; H arom.), 5.19–5.02 (2 × ABX, 4H; CH_2Ph), 4.57 (d, J(3,4) = 1.4 Hz, 1H; H-4), 3.94 (ABX, J(5,6a) = 2.0 Hz, J(6a,6b) = 12.9 Hz, 1H; H-6a), 3.79 (brs, 1H; H-3), 3.78 (brt, J(5,6a,b) = 2.0 Hz, 1H; H-5), 3.68 (brs, 1H; H-2), 3.64 (ABX, J(5,6b) = 2.0 Hz, J(6a,6b) = 12.9 Hz, 1H; H-6b), 2.61 (ABX, J(1′a,1′b) = 15.7 Hz, J(1′a,P) = 19.4 Hz, 1H; H-1′a), 2.45 (ABX, J(1′a,1′b) = 15.7 Hz, J(1′b,P) = 18.1 Hz, 1H; H-1′b), 0.95 (s, 9H; Si–tBu), 0.93 (s, 9H; Si–tBu), 0.15 (s, 3H; Si–Me), 0.14 (s, 3H; Si–Me), 0.12 (s, 3H; Si–Me), 0.09 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 136.19 (d, $J_{(C,P)}$ = 6.7 Hz; C^q arom.), 136.17 (d, J(C,P) = 6.6 Hz; C^q arom.), 128.49–128.04 (10 × CH arom.), 104.97 (d, J(1,P) = 9.7 Hz; C-1), 87.70 (d, J(3,P) = 12.6 Hz; C-3), 84.33 (C-4), 80.41 (d, J(2,P) = 3.2 Hz; C-2), 77.37 (C-5), 67.63 (d, J(C,P) = 6.1 Hz; CH_2Ph), 67.39 (d, J(C,P) = 6.0 Hz; CH_2Ph), 63.73 (C-6), 27.78 (d, J(1′,P) = 142.3 Hz; C-1′), 25.66, 17.86, 17.83, −4.17, −4.48, −4.73, −4.76 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): δ = 27.26 ppm; MS (DCI-NH_3): m/z (%): 665 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{33}H_{53}O_8Psi_2$: C 59.61, H 8.03; found: C 59.41, H 8.23.

(1,4-Anhydro-α-D-galactopyranosyl)methyl phosphonic acid (16): A solution of **15** (186 mg, 0.24 mmol) in THF (10 mL) was cooled to −20 °C. Tetrabutylammonium fluoride (226 mg, 0.72 mmol) was added, and the solution was stirred for 16 h at −5 °C. The solvent was then removed from the reaction mixture under reduced pressure. The residue was first purified by flash chromatography on silica gel with AcOEt/EtOH (9:1) as eluent and secondly repurified by flash chromatography with acetone/CH_2Cl_2 (8:2) to afford **16a** (86 mg, 83%) as a colorless oil.

$[\alpha]_D^{24}$ +33.7 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.56–7.52 (m, 10H; H arom.), 5.29–5.18 (2 × ABX, 4H; CH_2Ph), 4.64 (d, J(3,4) = 1.5 Hz, 1H; H-4), 4.00 (t, J(2,3) = J(3,4) = 1.3 Hz, 1H; H-3), 3.97 (dd, J(5,6a) = 6.0 Hz, J(5,6b) = 4.9 Hz, 1H; H-5), 3.88 (d, 1H; H-2), 3.72 (ABX, J(5,6a) = 6.0 Hz, J(6a,6b) = 11.6 Hz, 1H; H-6a), 3.67 (ABX, J(5,6b) = 4.9 Hz, J(6a,6b) = 11.6 Hz, 1H; H-6b), 2.84 (ABX, J(1′a,1′b) = 15.9 Hz, J(1′a,P) = 19.2 Hz, 1H; H-1′a), 2.80 (ABX, J(1′a,1′b) = 15.9 Hz,

$J(1'b,P) = 19.0$ Hz, 1H; H-1′b) ppm; ^{13}C NMR (100 MHz, CD_3OD): $\delta =$ 137.90 (d, $J(C,P) = 6.0$ Hz; C^q arom.), 137.87 (d, $J(C,P) = 6.3$ Hz; C^q arom.), 129.91–129.45 (10 CH arom.), 107.08 (d, $J(1,P) = 5.9$ Hz; C-1), 86.98 (d, $J(3,P) = 8.1$ Hz; C-3), 85.84 (C-4), 79.86 (d, $J(2,P) = 1.6$ Hz; C-2), 78.86 (C-5), 69.36 (d, $J(C,P) = 6.5$ Hz; CH_2Ph), 69.28 (d, $J_{(C,P)} = 6.5$ Hz; CH_2Ph), 63.79 (C-6), 28.36 (d, $J(1',P) = 141.8$ Hz; C-1′) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 26.94$ ppm; MS (DCI-NH_3): m/z (%): 437 (100) $[M+H]^+$, 454 (20) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{21}H_{25}O_8P$: C 57.80, H 5.77; found: C 57.74, H 5.94.

Compound **16a** (55 mg, 0.13 mmol) in a mixture of MeOH (5 mL) and diisopropylamine (18 μL, 0.13 mmol) was stirred overnight at room temperature under H_2 atmosphere (1 bar) with 10% activated Pd/C (16 mg) as catalyst. After being filtered through a celite pad, the solution was concentrated to dryness to yield **16** as its monodiisopropylammonium salt (43 mg, 94% yield).

$[\alpha]_D^{20}$ +28.0 (c = 1.0, H_2O); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 4.44$ (br s, 1H; H-4), 4.07 (br s, 1H; H-3), 3.92 (t, $J(5,6a) = J(5,6b) = 6.1$ Hz, 1H; H-5), 3.87 (d, $J(2,3) = 0.7$ Hz, 1H; H-2), 3.66–3.64 (d, $J(5,6a,b) = 6.1$ Hz, 2H; H-6a, H-6b), 3.63 (sept, $J = 6.5$ Hz, 2H; $(CH_3)_2CH$), 2.51 (ABX, $J(1'a,1'b) = 14.7$ Hz, $J(1'a,P) = 18.1$ Hz, 1H; H-1′a), 2.45 (ABX, $J(1'a,1'b) = 14.7$ Hz, $J(1'b,P) = 18.7$ Hz, 1H; H-1′b), 1.51 (d, $J = 6.5$ Hz, 12H; $(CH_3)_2CH$) ppm; ^{13}C NMR (100 MHz, CD_3OD): $\delta = 108.77$ (d, $J(1,P) = 1.4$ Hz; C-1), 86.69 (C-3), 84.59 (C-4), 80.31 (C-2), 78.60 (C-5), 64.17 (C-6), 48.66, 48.59, 33.07 (d, $J(1',P) = 129.4$ Hz; C-1′), 19.66, 19.65 ppm; ^{31}P NMR (101 MHz, D_2O): $\delta = 15.80$ ppm; MS (negative ESI): m/z (%): 255 (100) $[M-H]^+$; HRMS found 255.02751; $C_7H_{12}O_8P$ calcd 255.02698.

2,3,5,6-Tetra-*O*-*tert*-butyldimethylsilyl-D-galactono-1,4-lactone (17): D-Galactono-1,4-lactone (2.69 g, 15.1 mmol) was dissolved in DMF (40 mL) and treated with imidazole (7.3 g, 107 mmol) and *tert*-butyldimethylsilyl chloride (13.4 g, 89.2 mmol) at 70 °C for 5 h under argon. The solution was then allowed to warm to room temperature and stirred overnight. The reaction mixture was concentrated to dryness under vacuum, diluted with CH_2Cl_2 (800 mL), washed with water (2 × 100 mL), and dried over $MgSO_4$. After solvent evaporation, the residue was purified by flash chromatography with cyclohexane/AcOEt (25:1 to 23:1) as eluent to give **17** (9.39 g, 98% yield) as a white solid.

M.p. 49–50 °C; $[\alpha]_D^{23}$ −11.4 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.45$ (t, $J(2,3) = J(3,4) = 6.1$ Hz, 1H; H-3), 4.38 (d, $J(2,3) = 6.1$ Hz, 1H; H-2), 4.35 (dd, $J(3,4) = 6.1$ Hz, $J(4,5) = 1.7$ Hz, 1H; H-4), 3.85 (ddd, $J(4,5) = 1.7$ Hz, $J(5,6a) = 7.7$ Hz, $J(5,6b) = 5.8$ Hz, 1H; H-5), 3.70 (ABX, $J(5,6a) = 7.7$ Hz, $J(6a,6b) = 9.7$ Hz, 1H; H-6a), 3.66 (ABX, $J(5,6b) =$ 5.8 Hz, $J(6a,6b) = 9.7$ Hz, 1H; H-6b), 0.95 (s, 9H; Si–*t*Bu), 0.92 (s, 9H; Si–*t*Bu), 0.91 (s, 9H; Si–*t*Bu), 0.90 (s, 9H; Si–*t*Bu), 0.23 (s, 3H; Si–Me), 0.18 (s, 3H; Si–Me), 0.16 (s, 3H; Si–Me), 0.15 (s, 3H; Si–Me), 0.14 (s, 3H; Si–Me), 0.11 (s, 3H; Si–Me), 0.09 (s, 6H; 2 × Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) = 173.51 (C-1), 81.53 (C-4), 76.91 (C-2), 75.59 (C-3), 71.52 (C-5), 63.04 (C-6), 25.83, 25.76, 25.74, 25.62, 18.18, 18.15, 18.08, 17.77, −3.42, −4.13, −4.16, −4.32, −4.71, −4.85, −5.45, −5.49 ppm; MS (DCI-NH_3): m/z (%): 652 (100) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{30}H_{66}O_6Si_4$: C 56.73, H 10.47; found: C 56.87, H 10.58.

(1(1′)*Z*)-2,3,5,6-Tetra-*O*-*tert*-butyldimethylsilyl-1-(dimethoxyphosphoryl)-methylidene-D-galactofuranose (18): Butyllithium (2.5 M solution in hexane, 7.9 mL, 19.8 mmol) was added under argon to a cooled (−70 °C) solution of freshly distilled dimethyl methylphosphonate (2.24 mL, 20.7 mmol) in anhydrous THF (41 mL), followed after 20 min by a solution of **17** (5.24 g, 8.26 mmol) in anhydrous THF (8 mL). The temperature was maintained at −70 °C for 10 min, and the mixture was then allowed to come to −40 °C over a 1 h period. The solution was then diluted with phosphate buffer ((1 M, pH = 7, 190 mL) and extracted with CH_2Cl_2 (2 × 400 mL). The combined organic phases were dried over $MgSO_4$, filtered, concentrated, and dried overnight in vacuo. The crude product was then dissolved at 0 °C in anhydrous THF (58 mL), and pyridine (6.7 mL, 82.6 mmol) and trifluoroacetic anhydride (5.8 mL, 41.7 mmol) were added. The resulting solution was stirred at 0 °C for 3 h, quenched by addition of saturated aqueous $NaHCO_3$, and extracted with AcOEt (400 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated at reduced pressure. Purification by silica gel chromatography, eluted with cyclohexane/AcOEt (3:1), afforded **18** (4.7g, 77%) as a white solid.

m.p 60–61 °C; $[\alpha]_D^{19}$ +9.4 (c = 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.51$ (ddd, $J(1',2) = 1.3$ Hz, $J(2,3) = 3.8$ Hz, $J(2,P) = 4.6$ Hz, 1H; H-2), 4.48 (dd, $J(1',2) = 1.3$ Hz, $J(1',P) = 10.3$ Hz, 1H; H-1′), 4.39 (t, $J(3,4) =$ $J(4,5) = 3.8$ Hz, 1H; H-4), 4.33 (t, $J(2,3) = J(3,4) = 3.8$ Hz, 1H; H-3), 3.89 (ddd, $J(4,5) = 3.8$ Hz, $J(5,6a) = 7.4$ Hz, $J(5,6b) = 5.0$ Hz, 1H; H-5), 3.74 (ABX, $J(5,6a) = 7.4$ Hz, $J(6a,6b) = 10.0$ Hz, 1H; H-6a), 3.70 (d, $J(H,P) =$ 11.6 Hz, 3H; OMe), 3.69 (d, $J(H,P) = 11.4$ Hz, 3H; OMe), 3.65 (ABX, $J(5,6b) = 5.0$ Hz, $J(6a,6b) = 10.0$ Hz, 1H; H-6b), 0.92 (s, 9H; Si–*t*Bu), 0.90 (s, 9H; Si–*t*Bu), 0.89 (s, 9H; Si–*t*Bu), 0.88 (s, 9H; Si–*t*Bu), 0.15 (s, 3H; Si–Me), 0.14 (s, 6H; 2 × Si–Me), 0.11 (s, 3H; Si–Me), 0.10 (s, 3H; Si–Me), 0.09 (s, 3H; Si–Me), 0.08 (s, 3H; Si–Me), 0.07 (s, 3H; Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 173.12$ (d, $J(1,P) = 2.8$ Hz; C-1), 89.26 (C-4), 80.99 (d, $J(2,P) = 13.7$ Hz; C-2), 80.39 (d, $J(1',P) = 194.9$ Hz; C-1′), 76.47 (C-3), 72.55 (C-5), 63.99 (C-6), 52.00 (d, $J(C,P) = 5.4$ Hz, OMe), 51.86 (d, $J(C,P) = 5.6$ Hz, OMe), 25.88, 25.77, 25.65, 25.63, 18.25, 18.09, 17.79, 17.71, −3.72, −3.88, −4.32, −4.37, −5.00, −5.46, −5.53 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 22.01$ ppm; MS (DCI-NH_3): m/z (%): 741 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{33}H_{73}O_8PSi_4$: C 53.47, H 9.93; found: C 53.51, H 9.93.

Dimethyl (1-deoxy-2,3,5,6-tetra-*O*-*tert*-butyldimethylsilyl-α-D-galactofuranosyl)methyl phosphonate (19): Compound **18** (1 g, 1.35 mmol) was dissolved in ethyl acetate (105 mL) and was vigorously stirred at room temperature under H_2 atmosphere (1.5 bar) with palladium hydroxide (470 mg, 20% Pd on carbon, wet) as catalyst. After 24 h, the catalyst was removed by filtration through a pad of celite and the filtrate was concentrated. The residue was purified by chromatography on silica gel with cyclohexane/AcOEt (4:1) as eluent. Product **19** was obtained as a colorless oil (826 mg, 82% yield).

$[\alpha]_D^{19}$ −10.6 (c = 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.32$ (tdd, $J(1,2) = 2.8$ Hz, $J(1,1'a,b) = 6.3$ Hz, $J(1,P) = 9.7$ Hz, 1H; H-1), 4.05 (d, $J(3,4) = 1.1$ Hz, 1H; H-3), 3.88 (d, $J(1,2) = 2.8$ Hz, 1H; H-2), 3.79 (m, 1H; H-5), 3.78 (d, $J(H,P) = 10.9$ Hz, 3H; OMe), 3.77 (d, $J(H,P) = 10.8$ Hz, 3H; OMe), 3.72 (ABX, $J(5,6a) = 3.7$ Hz, $J(6a,6b) = 10.5$ Hz, 1H; H-6a), 3.69 (dd, $J(3,4) = 1.1$ Hz, $J(4,5) = 6.9$ Hz, 1H; H-4), 3.59 (ABX, $J(5,6b) =$ 5.4 Hz, $J(6a,6b) = 10.5$ Hz, 1H; H-6b), 2.17 (AXX′, $J(1,1'a,b) = 6.3$ Hz, $J(1',P) = 18.0$ Hz, 2H; H-1′a, H-1′b), 0.95 (s, 9H; Si–*t*Bu), 0.92 (s, 18H; 2 × Si–*t*Bu), 0.89 (s, 9H; Si–*t*Bu), 0.15 (s, 3H; Si–Me), 0.14 (s, 3H; Si–Me), 0.12 (s, 3H; Si–Me), 0.11 (s, 6H; 2 × Si–Me), 0.10 (s, 3H; Si–Me), 0.08 (s, 6H; 2 × Si–Me) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\Delta = 88.55$ (C-4), 79.41 (d, $J(2,P) = 8.0$ Hz; C-2), 78.80 (C-3), 75.26 (C-1), 74.50 (C-5), 63.43 (C-6), 52.38 (d, $J(C,P) = 6.5$ Hz, OMe), 52.22 (d, $J(C,P) = 6.4$ Hz, OMe), 26.10, 26.02, 25.90, 25.62, 25.25 (d, $J(1',P) = 141.4$ Hz; C-1′), 18.52, 18.30, 18.08, 17.73, −4.15, −4.25, −4.31, −4.36, −4.49, −4.90, −5.17, −5.28 ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 32.52$ ppm; MS (DCI-NH_3): m/z (%): 743 (40) $[M+H]^+$, 760 (100) $[M+NH_4]^+$; elemental analysis calcd (%) for $C_{33}H_{75}O_8PSi_4$: C 53.33, H 10.17; found: C 53.29 10.30.

(1-Deoxy-α-D-galactofuranosyl)methyl phosphonic acid (20): Trimethylsilyl iodide (0.9 mL, 6.14 mmol) was added at 0 °C to a solution of **19** (304 mg, 0.41 mmol) in anhydrous carbon tetrachloride (24 mL). The solution was stirred for 4 h at 0 °C and was then allowed to come to room temperature for a period of 1 h. The solvent was evaporated and the residue was dried overnight in vacuo. The residue was dissolved in water and extracted three times with ether, and the combined aqueous phases were pooled and lyophilized. The resulting product was dissolved in a minimum amount of water and applied to a Dowex 50WX8–200 column (Bu_3NH^+ form). The appropriate fractions (detected by TLC EtOH/NH_4OH/H_2O 5:3:1) were pooled and lyophilized to give **20** as a white hygroscopic solid (bistributylammonium salt, 223 mg, 87% yield). Analysis corresponded to those previously published.[25]

Acknowledgement

This work was supported by the Centre National de la Recherche Scientifique (CNRS) as well as by the Ministère Délégué à la Recherche et aux Nouvelles Technologies (Ph.D. grant to A.C.).

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Received: May 15, 2003 [F5141]