The First Chemical Synthesis of UDP[6-^{3}H]-α-D-galactofuranose

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Galactofuranose metabolism is a good target for the development of novel chemotherapeutic agents for the treatment of some microbial infections. This is a valid objective because galactofuranose is absent in mammals. Two enzymes are involved in the biosynthesis of molecules containing galactofuranose: a mutase, which catalyzes the interconversion of UDP-Gal*p* and UDP-Gal*f*, and D-galactofuranosyltransferases. The mechanism of action of the mutase and its inhibition is currently being investigated, whereas studies on the galactofuranosyltransferases have been hampered by the lack of a labeled galactofuranose nucleotide. In the present work we describe the chemical synthesis of UDP-α-D-[6-^{3}H]Gal*f* and we prove its effectiveness for incorporation of radioactive galactofuranose into a natural acceptor. This is the first report on the chemical synthesis of a labeled donor of galactofuranose with the potential for studying the galactofuranosyltransferases independently from the UDP-Gal*p* mutase.

Introduction

The metabolic pathways involved in the incorporation of galactofuranose into microbial glycoconjugates are attractive targets for the development of chemotherapeutic agents, since the furanose galactose is absent in mammalian cells. β-D-Galactofuranosyl residues are constituents of pathogenic microorganisms such as the bacteria *Mycobacterium tuberculosis* and *M. leprae*,[1,2] trypanosomatids like *Trypanosoma cruzi* and *Leishmania*,[3–5] and fungi like *Paracoccidioides brasiliensis*.[6] In the latter species, the rare α-D-galactofuranosyl linkage has also been detected, depending on the phase of the fungus.

At least two enzymes are necessary for the construction of the galactofuranosyl linkage: a mutase, UDP-galactopyranosylmutase (EC 5.4.99.9),[7,8] which converts UDP-α-D-Gal*p* into UDP-α-D-Gal*f*, and a UDP-galactofuranosyl transferase, which is responsible for the incorporation of the sugar into the glycan. The mechanism of action of the mutase is currently under investigation by several groups.[9–11] However, few studies have been reported for the transferase.[12,13] It has been only described in *Mycobacterium tuberculosis*, using synthetic glycosides of Gal*f* disaccharides as acceptor substrates.[12] The transferase is a bifunctional enzyme that catalyzes the formation of β(1,5) and β(1,6) linkages (gene product of Rv 3808c).[12] The Gal*f* trisaccharides resulting from the enzymatic reaction have been characterized by fast atom bombardment mass spectrometry. The sugar donor used for the reaction was UDP-[^{14}C]Gal*p*, which must be converted to UDP-[^{14}C]Gal*f* before transference.

The availability of labeled substrates is important for the detection of galactofuranose intermediates and the enzymes involved in Gal*f* metabolism. We have previously described the synthesis of methyl β-D-[6-^{3}H]galactofuranoside, which is useful for the detection of β-D-galactofuranosidase.[14]

A drawback of the preparation of UDP-Gal*f* from labeled UDP-Gal*p* by using the mutase in vitro is the low yield of UDP-Gal*f*, as only 7% of the furanose nucleotide is present at equilibrium.[15] A microtiter assay for the mutase is based on the release of tritiated formaldehyde by periodate oxidation of the UDP-[6-^{3}H]Gal*f* formed from UDP-[6-^{3}H]Gal*p*.[16]

The synthesis of nonlabeled UDP-Gal*f*[17,18] from α-D-Gal*f*-1-phosphate (**1**), which was synthesized for the first time in our laboratory, has been described.[19] In the present report we describe a chemical synthesis of α-D-[6-^{3}H]Gal*f*-1-phosphate (**1***) and UDP-[6-^{3}H]Gal*f* (**3***) from the precursor **5**.[14] The radioactive donor substrate **3*** should be useful for discrimination of inhibitors of both the UDP-α-D-Gal*p* mutase and the galactofuranosyl transferases in different species. The α-D-galactofuranose 1,2-cyclic phosphate (**4**), a product usually formed by decomposition of UDP-Gal*f*,[20] was also prepared from the monophosphate **1** for the identification of the reaction products. Radioactive galactose from the UDP-[6-^{3}H]Gal*f* (**3***) was incorporated into the peptidophosphogalactomannan by a membrane preparation of *Penicillium fellutanum*.

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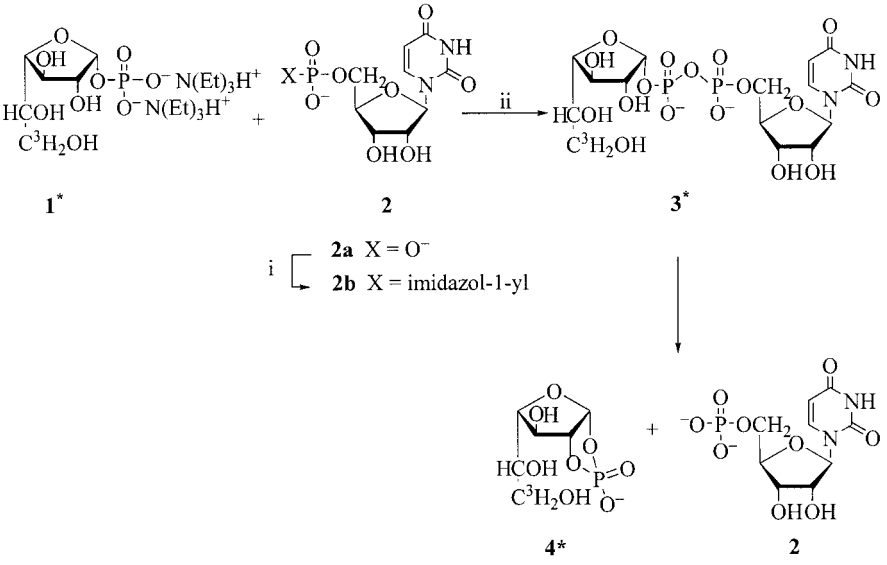
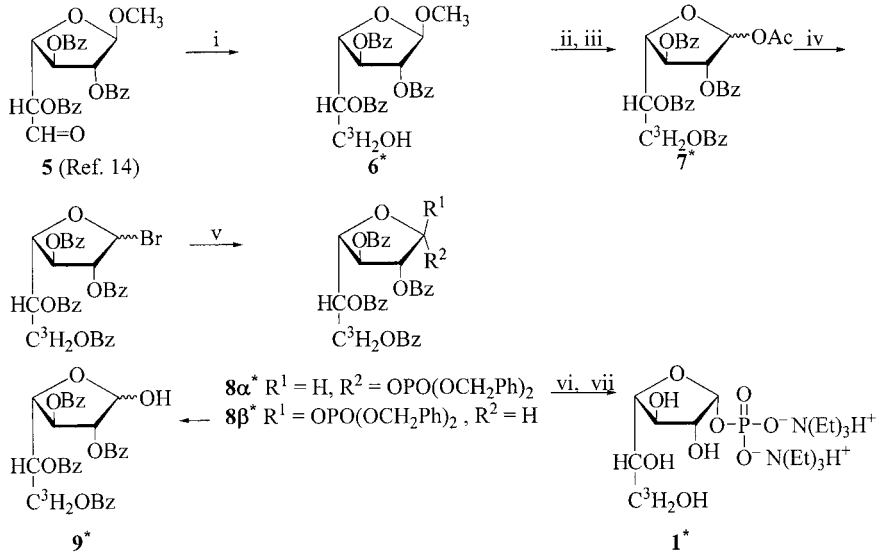
Results and Discussion

The synthesis of UDP-Gal*f* (**3**) relies on the coupling of α-D-Gal*f*-1-P (**1**) with an activated derivative of 5′-UMP (**2**) (Scheme 1). The tritium-labeled Gal*p* analog, namely UDP-[6-^{3}H]Gal*p*, was synthesized from the cold nucleotide by treatment with galactose oxidase followed by reduction with NaB^3H_4.[21] The introduction of a tritium label directly in UDP-Gal*f* (**3**), or in **1**, was not possible, first because there is no enzyme available for oxidation of C-6 in Gal*f*, and secondly due to the extreme lability of UDP-Gal*f*. In a previous study, we have developed a strategy for the specific tritium labeling of the 6-position of methyl β-D-galactofuranoside,[14] and now we extended it to the synthesis of UDP-[6-^{3}H]Gal*f* (**3***) starting from compound **5** (Scheme 2). All the reactions were performed in parallel with the nonlabeled compounds. The structures for the target, UDP-Gal*f*, and for intermediate and degradation products were confirmed by the chemical shifts for the diagnostic signals in the NMR spectra (Table 1), which were in agreement with those reported in the literature.[17,19,20,28] Standard acetolysis of **6** caused partial isomerization to the pyranosic forms, according to the ^{13}C NMR spectrum of the nonlabeled product. Ring expansion was avoided by previous benzoylation of *O*-6 and mild acetolysis in CH_2Cl_2 solution to afford 1-*O*-acetyl-2,3,5,6-tetra-*O*-benzoyl-D-galactofuranose (**7**).[22] Compound **7*** was used for the preparation of α-D-[6-^{3}H]Gal*f*-phosphate (**1***) following the procedure previously described in our laboratory,[19] with slight modifications for the nonradioactive analog. Thus, treatment of **7*** with bromotrimethylsilane and condensation with dibenzyl phosphate in the presence of triethylamine



Scheme 1. i) 1,1′-carbonyldiimidazole, DMF; ii) DMF.



Scheme 2. i) NaB^3H_4, MeOH; ii) BzCl, Py; iii) 2.5% H_2SO_4/Ac_2O in CH_2Cl_2 (1:20); iv) $Si(CH_3)_3Br$, CH_2Cl_2; v) $(BnO)_2PO_2H$, Et_3N, toluene; vi) H_2 (35 psi), Pd/C, EtOAc, NEt_3; vii) NEt_3/H_2O/MeOH (1:2:5).

Table 1. Chemical shifts for the diagnostic signals of compounds **1**, **3**, **4**, **8α**, **8β**, and **10**.

Compounds	^{1}H NMR				^{13}C NMR		^{31}P NMR	Ref.
	H-1		H-2		C-1	C-2		
	($J_{1,2}$)	($J_{H,P}$)	($J_{2,3}$)	($J_{H,P}$)	($J_{C,P}$)	($J_{C,P}$)		
α-D-Gal*f* 1-P (**1**)[a]	5.42		4.00[c]		96.9	77.9	+2.5	[19]
	(4.5)	(4.9)	(m)		(5.3)	(7.6)		
UDP-Gal*f* (**3**)[a] Gal*f*	5.45		4.03		98.0	77.0	–10.1, –12.0	[17]
	(4.4)	(5.1)	(8.5)	(2.1)	(5.5)	(8.1)		
Rib*f*	5.85				88.6			
	(4.5)							
α-D-Gal*f* 1,2-P (**4**)[a]	5.93		4.75		102.6	85.7	+16.9	[20]
	(4.5)	(14.0)	(2.1)	(6.9)	(4.1)			
8α[b]	6.34		5.73		97.6	76.7	–3.7	[19]
	(4.5)	(5.8)			(4.4)	(6.0)		
8β[b]	6.13		5.53		103.3	82.1		[19]
	(<0.5)	(4.9)	(<0.5)		(5.5)			
α-D-Gal*p* 1,2-P (**10**)	5.80		4.32		98.7	79.0	+10.4	[28]
	(8.9)		(4.7)	(19.1)	(8.8)	(2.7)		

[a] In D_2O. [b] In $CDCl_3$. [c] Center of a complex multiplet.

led to the anomeric mixture **8***, which was separated by column chromatography. The fractions were counted, pooled, and analyzed by TLC and fluorography (Figure 1, A). The first radioactive fraction eluted from the column contained a mixture of **8β***, 2,3,5,6-tetra-*O*-benzoyl-D-[6-^{3}H]galactofuranose (**9***), and traces of **8α*** (Figure 1, part A, lane 1). The second radioactive fraction (Figure 1A, lane 2) contained **8α*** (R_f = 0.16). The double spots shown are due to partial transesterification during the previous acetolysis step, as proved by NMR experiments carried out on the compounds isolated from the corresponding nonlabeled synthesis, which showed the presence of more than one acetate group. This preparation was also analyzed for comparison (Figure 1, B). We again confirmed by ^{13}C and ^{1}H NMR spectroscopy (Table 1) that the β-anomer (**8β**) spontaneously decomposes to the stable tetrabenzoate **9** (R_f = 0.24).[19] This observation has been contested,[18] probably based on the fact that the β-phosphate **8β** and its decomposition product, **9**, could not be distinguished (Figure 1 part B, lane 3). Compound **8α*** was obtained in 42% yield from **7*** and could be stored at –20 °C for several months. The yield could be improved by further acetylation of **9***, to obtain **7***.

Conditions for the removal of the protecting groups in **8α*** were optimized for minimum decomposition. Thus, debenzylation of **8α*** was performed in a shorter time than previously described,[19] and a pressure of 35 psi was used, followed by debenzoylation under mild conditions. α-D-[6-^{3}H]Gal*f* 1-phosphate, as the triethylamonium salt (**1***), showed the same retention time (R_t = 9.75, Condition 1, Figure 2, A) as the nonradioactive compound when analyzed by HPAEC-PAD. The peak of galactose at 1.5 min could be due to partial decomposition of the radioactive sample **1*** under the basic conditions of the analysis.

For the coupling of **1*** with UMP, activation with 1,1′-carbonyldiimidazole, as described previously for the nonlabeled nucleotide,[17] gave better yields (26% for nonlabeled **3** and 13% for the radioactive nucleotide **3***) than morpholidate activation[23] (Scheme 1). Thus, the triethylammonium

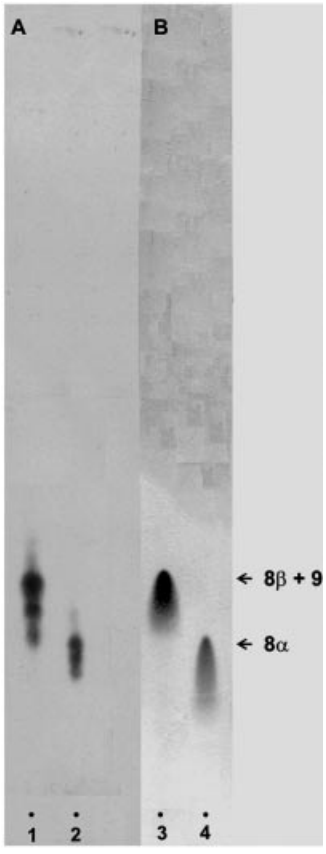


Figure 1. A) Thin layer chromatography and fluorography of fractions obtained from column chromatography purification of **8α***. Lane 1: fractions 15–35, corresponding to 2,3,5,6-tetra-*O*-benzoyl-D-[6-^{3}H]galactofuranose (**9***), dibenzyl 2,3,5,6-tetra-*O*-benzoyl-β-D-[6-^{3}H]galactofuranosylphosphate (**8β***), and dibenzyl 2,3,5,6-tetra-*O*-benzoyl-α-D-[6-^{3}H]galactofuranosylphosphate (**8α***). Lane 2: fractions 36–43, corresponding to dibenzyl 2,3,5,6-tetra-*O*-benzoyl-α-D-[6-^{3}H]galactofuranosylphosphate (**8α***). B) Column chromatography of unlabeled **8α**. Lane 3: 2,3,5,6-tetra-*O*-benzoyl-α,β-D-galactofuranose (**9**) and dibenzyl 2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosylphosphate (**8β**). Lane 4: dibenzyl 2,3,5,6-tetra-*O*-benzoyl-α-D-galactofuranosylphosphate (**8α**). TLC was performed on silica gel 60 plates with solvent A and detected as described in the Experimental Section.

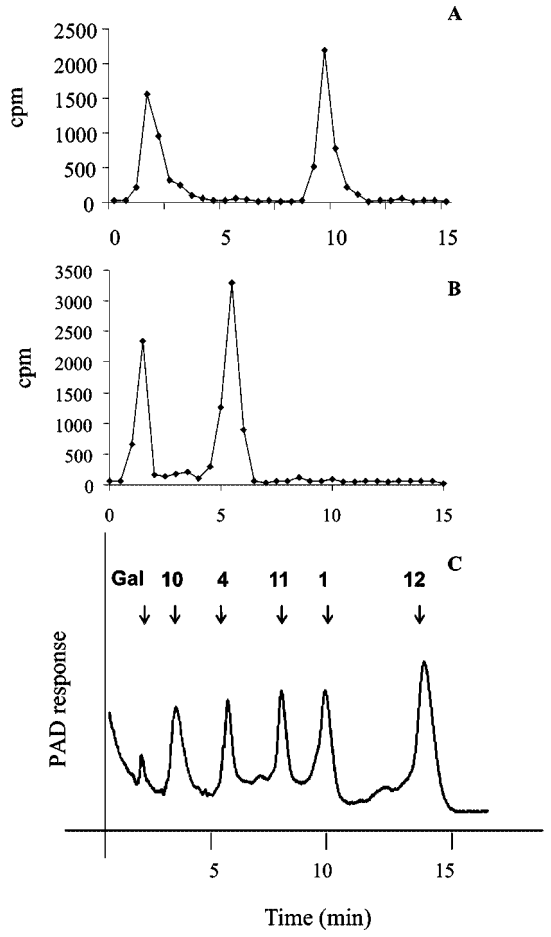


Figure 2. HPAEC-PAD analysis of isomeric galactose phosphates. A) Preparation of α-D-[6-^{3}H]Gal*f* 1-P (**1***). B) Included fraction B (Figure 3) from the Sephadex purification of UDP-[6-^{3}H]Gal*f*. C) Authentic samples of α-D-Gal*p* 1,2-P (**10**), α-D-Gal*f* 1,2-P (**4**), α-D-Gal*p* 1-P (**11**), α-D-Gal*f* 1-P (**1**), and D-Gal 2-P (**12**). The separation was performed on a CarboPac PA-10 column under Condition 1, as described in the Experimental Section.

salt of nucleotide **2a** was converted into the activated UMP-imidazolide **2b**. For the characterization of UDP-[6-^{3}H]Gal*f* (**3***), the nonradioactive analog **3** was prepared under the same reaction conditions for the coupling and was characterized by NMR spectroscopy as described previously.[17] Attempts to analyze the reaction product by TLC using several eluent systems were unsuccessful due to the instability of UDP-[6-^{3}H]Gal*f* (**3***). Gel chromatography (Sephadex G-10) of the reaction mixture showed the profile depicted in Figure 3. Three fractions were obtained: the excluded volume (A) with 12% of the radioactivity, and two included peaks slightly resolved at 70 mL (B, 45%) and 77 mL (C, 43%). The fractions were counted, pooled, and analyzed by HPLC as described in the Experimental Section. Fraction A from a radioactive and nonlabeled preparation was analyzed by reversed-phase C-18 HPLC using UDP-Gal*p* as internal standard (R_t = 4.6 min, Figure 4). A radioactive peak with R_t = 5.2 min was coincident with UDP-Gal*f*, as detected by UV spectroscopy (Figure 4). UMP (R_t = 6.6 min, Figure 4) was also present in fraction A. The specific activity of **3*** (50 μCi μmol^{-1}) was calculated by using the absorbance of UDP-Gal*p* uridine at 260 nm as internal standard to determine the molar concentration, and scintillation counting to determine the radioactivity of the sample separated. Fractions B and C (Figure 3), when analyzed by high pressure anion exchange chromatography with pulse amperometric detection (HPAEC-PAD), showed a peak coincident with galactose and another peak at 5.45 min (Condition 1, Figure 2, B). The latter was identified as the α-D-galactofuranose 1,2-cyclic phosphate **4** as described below.

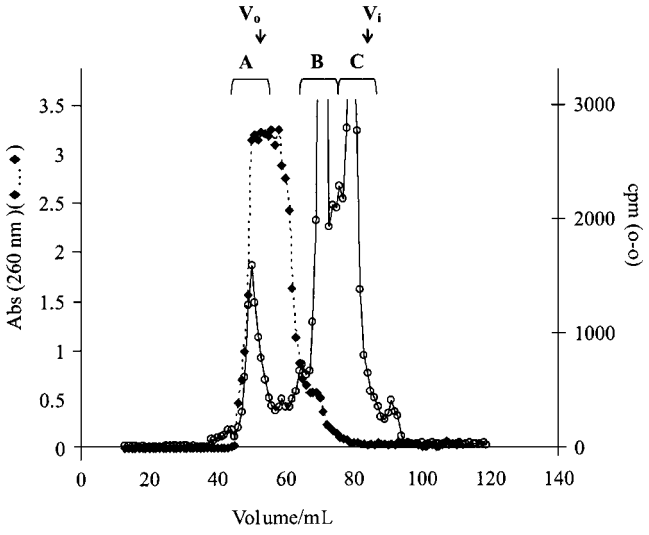


Figure 3. Sephadex G-10 chromatography of UDP-[6-^{3}H]Gal*f* and byproducts of the coupling reaction. The reaction was performed as described in the Experimental Section and chromatographed on a column of Sephadex G-10. Fractions of 1 mL were collected and analyzed by UV at 260 nm (♦) and liquid scintillation counting (o).

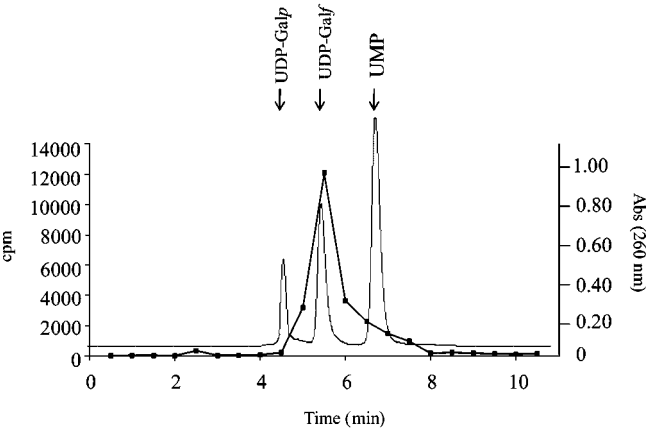


Figure 4. HPLC purification of UDP-[6-^{3}H]Gal*f*. The excluded fraction from Sephadex G-10, was chromatographed on an RP-18 column, as described in the Experimental Section. An internal standard of UDP-Gal*p* was used. (■) Liquid scintillation counting; (—) UV detection at 260 nm.

The degradation of **3** to **4** and 5′-UMP (Scheme 1), or glycosidic bond cleavage to afford D-galactose and 5′-UDP, are the main problems when handling the furanose nucleotide **3**.[17,20] The α-D-Gal*f* 1,2-cyclic phosphate **4*** could be

formed during the coupling step or by decomposition of the nucleotide in the course of its purification. With the aim of confirming the identity of **4***, an authentic sample was prepared starting from compound **1**. α-D-Gal*f* 1-P (**1**) was treated with dicyclohexylcarbodiimide, as described for D-fructose 1-phosphate.[24] TLC examination showed total conversion into a compound with a higher R_f value. No cyclization was obtained upon treatment of **1** with carbonyldiimidazole under the reaction conditions used for the coupling of **1** with UMP, thus indicating that cyclic compound **4** is formed from nucleotide **3**. We also proved that the peak at 5.45 min observed during HPAEC-PAD analysis of fraction B (Figure 2, B) corresponds to the formation of **4** as a decomposition product from **3**. The NMR spectroscopic data of **4** (Table 1) are in agreement with the signals assigned by Köplin et al. in the spectrum of crude UDP-Gal*f* obtained enzymatically.[20] The chromatographic resolution of α-D-Gal*p* 1,2-P (**10**), α-D-Gal*f* 1,2-P (**4**), α-D-Gal*p* 1-P (**11**), α-D-Gal*f* 1-P (**1**), and D-Gal 2-P (**12**) by HPAEC-PAD is shown in Figure 2, part C; their retention times are 3.05, 5.45, 7.93, 9.75, and 13.35 min, respectively. The furanosic derivatives eluted later than the pyranosic analogs, as previously observed for other compounds.[25] Compound **12** can be prepared from either of the cyclic phosphates by mild acid hydrolysis, as described in the Experimental Section. We proved that the synthesized UDP-[6-^{3}H]Gal*f* (**3***) is a good donor of [6-^{3}H]Gal*f* by using a membrane preparation of *Penicillium fellutanum* and added peptidophosphogalactomannan (pPGM)[26] obtained from a three day culture, as described in the Experimental Section. To avoid decomposition of the furanosic nucleotide, which is extremely labile,[17,18,20] we used the radioactive fraction A (Figure 3) containing the donor UDP-[6-^{3}H]Gal*f* obtained from the Sephadex separation without further purification. The incubated reaction mixture was dialyzed for removal of the donor substrate and the labeled pPGM was separated on a BioGel P6 column (Figure 5). Incubations with radioactive α-D-Gal*f* 1,2-P (**4***) in the presence of UDP-glucose with membranes or with a lysate of mycelium did not show incorporation of radioactivity.

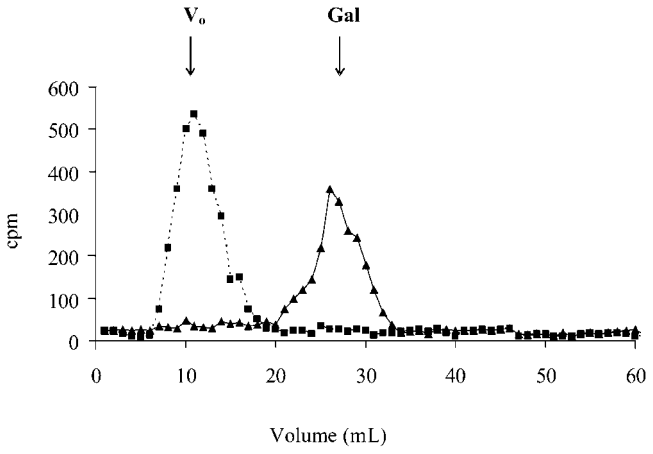


Figure 5. BioGel P-6 chromatography of radiolabeled pPGM. (■), and its mild acid hydrolysis product, (▲). Samples were obtained as indicated in the Experimental Section and chromatographed on a column of BioGel P-6. Fractions of 1 mL were collected and aliquots were analyzed by liquid scintillation counting.

In order to confirm the incorporation of Gal*f*, the excluded radioactive fraction from the column was hydrolyzed with 20 mM trifluoroacetic acid under conditions that lead to hydrolysis of galactofuranosyl linkages. All the radioactivity eluted as an included peak (Figure 5) which corresponded to galactose when analyzed by HPAEC-PAD. Galactose eluted at 12.7 min (Condition 2, data not shown).

In conclusion, the present synthesis of a labeled donor substrate of galactofuranose provides a tool for studies on galactofuranosyltransferases, which are valuable targets for the inhibition of the biosynthesis of glycoconjugates essential to the growth of microbial pathogens.

Experimental Section

General: Thin layer chromatography (TLC) was performed on 0.2 mm silica gel 60 F_{254} aluminum-supported plates (Merck), and developed using the following solvent systems: (A) toluene/ethyl acetate (9:1, v/v); (B) 1-propanol/concd. aqueous NH_3/water (7:1:2, v/v). The radioactivity in the fractions was determined with a Rack-beta Wallac liquid scintillation counter using a scintillation cocktail (Optiphase "Hisafe" 3, LKB). Radiolabeled compounds were detected by fluorography. The TLC plates were sprayed with EN^{3}HANCE (New England Nuclear) and were exposed to Kodak X-Omat A-R5 films at –70 °C. The nonradioactive standards were detected by exposure to UV light or by spraying with 5% (v/v) sulfuric acid in ethanol and charring. Column chromatography was performed on silica gel 60 (230–400 mesh). The ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker AM 500 spectrometer. The chemical shift reference for ^{31}P was that of external phosphoric acid (85%) in D_2O set at 0.0 ppm.

Protein concentrations were obtained by the Lowry method adapted for membrane proteins.[27] Solutions were concentrated by rotary evaporation with a bath temperature below 40 °C.

α-D-Gal*p* 1-P (sodium salt) was purchased from Sigma and converted into the triethylammonium salt by passage through Dowex AG 50W-X12 (triethylammonium form). All other reagents, unless otherwise stated, were purchased from Sigma–Aldrich.

HPLC Analysis of UDP-Gal*f*: Nucleoside mono- and diphosphates and nucleotide sugars were analyzed by reversed-phase HPLC with a Phenomenex SphereClone 5 μm ODS(2) column (250 × 4.60 mm, RP-18) and 36 mM KH_2PO_4 as eluent (flow rate = 0.6 mL min^{-1}). The eluted fractions were analyzed by UV or liquid scintillation counting.

HPAEC-PAD Analysis: Analysis by HPAEC-PAD was performed with a Dionex DX 300 HPLC system equipped with a CarboPac PA-10 anion-exchange analytical column (4 × 250 mm), and a guard column PA-10 (4 × 50 mm). The following conditions were used:

Condition 1: 2 mM NaOH and 150 mM sodium acetate, isocratically at a flow rate of 1 mL min^{-1}.

Condition 2: 20 mM NaOH, isocratically, at a flow rate of 1 mL min^{-1}.

The pulsed sensitivity in both cases was set at 30 nA and the pulsed potentials were as follows: E_1 = +0.05 V, E_2 = +0.60 V, and E_3 = –0.60 V.

1-*O*-Acetyl-2,3,5,6-tetra-*O*-benzoyl-D-[6-^{3}H]galactofuranose (7*): Compound **5** (3 mg, 6 μmol), prepared from 1,2,3,5,6-penta-*O*-benzoyl-α,β-D-galactofuranose as described previously,[14] was reduced in methanol (0.3 mL) with 10 mCi of NaB^3H_4 in 0.1 M KOH and, after 3 h at room temperature, solid $NaBH_4$ (3 mg, 80 μmol) was added. The mixture was left overnight at 4 °C and the excess of reagent was destroyed with acetone (100 μL). The solution was stirred for 15 min and deionized by elution through a column of Bio-Rad AG 50W-X12 resin (H^+ form) and an Amberlite MB-3A (mixed form) column to eliminate the boric acid. Compound **6*** (R_f = 0.52, solvent A) was benzoylated in the 6-position with benzoyl chloride (0.4 mL) in pyridine (0.5 mL) during 4 h. The reaction was quenched by addition of cold water and stirred for 30 min. After extraction with CH_2Cl_2, the organic phase was washed with $NaHCO_3$ and water. The organic extracts were dried over anhydrous $MgSO_4$, filtered, and taken to dryness (42 μCi).The acetolysis step was performed in dry CH_2Cl_2 (1 mL) with Ac_2O/H_2SO_4 (40:1, 52 μL) for 16 h at 30 °C. The reaction was stopped by dilution with CH_2Cl_2 (10 mL) and addition of cold water. After stirring for 30 min, the organic phase was washed and treated as above. Crude compound **7*** (35 μCi, R_f = 0.46, solvent A) was obtained by evaporation of the solvent.

The yield of compound **7** obtained from nonlabeled compound **5** (25 mg) was 84%.

Dibenzyl 2,3,5,6-Tetra-*O*-benzoyl-α-D-[6-^{3}H]galactofuranosylphosphate (8α*): Compound **7*** (35 μCi) was dissolved in dry CH_2Cl_2 (1 mL) and bromotrimethylsilane (0.2 mL, 1.5 mmol) was added, with external cooling (0 °C). After 24 h of stirring at room temperature, the solution was concentrated in vacuo, and co-evaporated several times with toluene to remove the excess of reagent. The resulting bromide was dissolved in anhydrous toluene (1.2 mL) and treated with dibenzylphosphate (20 mg, 70 μmol) and triethylamine (15 μL, 105 μmol). After 4 h of stirring, the solution was filtered, and the filtrate was concentrated in vacuo at room temperature and chromatographed on a silica gel column (4 × 1.5 cm) with toluene/EtOAc (95:5) as eluent. Fractions (1 mL) were collected and aliquots were taken to estimate the radioactivity by liquid scintillation counting. Fractions containing the compound of R_f = 0.16 (solvent A, Figure 1) were evaporated to dryness to afford 14 μCi. A mixture of compounds **8β** and the tetrabenzoate **9** (R_f = 0.24) was obtained from fractions 15–35 (20 μCi).

The yield of compound **8α** obtained from nonlabeled compound **7** (20 mg) was 49%.

α-D-[6-^{3}H]Galactofuranosyl Bis(triethylammonium)phosphate Salt (1*): A solution of **8α*** (14 μCi) in EtOAc (1 mL) containing triethylamine (20 μL, 144 μmol) and 10% Pd/C (1 mg) was hydrogenated at 35 psi during 4 h. The mixture was filtered through Celite and, after evaporation of the solvent, the resulting syrup was dried under vacuum and debenzoylated with 2 mL of 0.07 M NaOMe in 1:1 CH_2Cl_2/MeOH, under argon. After 30 min, the solution was diluted with MeOH (5 mL) and the solvents evaporated. Water was added and the solution was washed with toluene (1 mL) to eliminate methyl benzoate. Both fractions were analyzed by liquid scintillation counting, and no radioactivity was found in the toluene fraction. The aqueous solution was lyophilized, and compound **1*** (10.5 μCi) was identified by comparison with an authentic cold sample[19] by HPAEC-PAD (Condition 1, Figure 2A).

The yield of compound **1** obtained from nonlabeled compound **8α** (10 mg) was 88%.

Uridine 5′-(α-D-[6-^{3}H]Galactofuranosyl Diphosphate) Di(triethylammonium) Salt (3*): The procedure described for the nonradioactive nucleotide[17] was essentially followed. Thus, 1,1′-carbonyldiimidazole (1.3 mg, 9 μmol) was added to a solution of uridine 5′-monophosphate (2 mg, 4 μmol) in DMF (1 mL). The solution was kept under argon for 3 h at room temperature and then evaporated to obtain the imidazolide **2b** as described previously.[17] A mixture of α-D-[6-^{3}H]galactofuranosyl bis(triethylammonium) phosphate salt (**1***, 10.5 μCi) and the imidazolide **2b**, previously dried by co-evaporation with pyridine, was redissolved in DMF (1 mL) and kept under argon at room temperature for 19 h. The reaction was stopped by adding cold water (8 mL) and rapidly extracted with chloroform (2 × 3 mL). The aqueous solution was coevaporated several times with water in order to eliminate DMF, concentrated by lyophilization, and purified by size-exclusion chromatography (1.4 μCi, Sephadex G-10 column, 1.8 × 80 cm; eluent: water, flow: 1 mL min^{-1}, Figure 3).

The yield of compound **3** obtained from nonlabeled compound **1** (9 mg) was 26%.

α-D-Galactofuranosyl 1,2-Cyclic Phosphate (4) and D-Galactose 2-Phosphate (12): Triethylamine (28 μL, 0.2 mmol), dicyclohexylcarbodiimide (140.3 mg, 0.6 mmol), and water (0.1 mL) were added to a solution of compound **1** (31.4 mg, 0.078 mmol, R_f = 0.1, solvent B) in pyridine (2 mL). This solution was stirred at 30 °C for 20 h. TLC examination showed total conversion of the starting material into a compound with a higher R_f (R_f = 0.4, solvent B). Water (8 mL) was added and cyclohexylurea was filtered off. The aqueous filtrate was extracted three times with diethyl ether, and the combined ether extracts were washed once with water. The aqueous solution was lyophilized to obtain **4** (26.8 mg, 86%). ^{1}H NMR (500 MHz, D_2O): δ = 5.93 (dd, $^3J_{1,2}$ = 4.5, $^3J_{H,P}$ = 14.0 Hz, 1 H, H1), 4.75 (dd, $^3J_{2,3}$ = 2.1, $^3J_{H,P}$ = 6.9 Hz, 1 H, H2) ppm. The spectroscopic data were in agreement with those previously reported.[20]

The same procedure was followed to prepare α-D-galactopyranosyl 1,2-cyclic phosphate (**10**, R_f = 0.29, 90%), whose structure was confirmed by spectroscopic data, which were found to be coincident with those described in the literature.[28]

Compound **4** (15 mg, 0.044 mmol) was treated with 20 mM TFA (100 μL) at 100 °C during 15 min. TLC examination showed total conversion of the starting material into a product showing a lower R_f value (R_f = 0.13, solvent B). Triethylamine was added, and after evaporation of the reagents, D-galactose 2-phosphate (**12**, 75%, 8.5 mg, 0.033 mmol) was obtained as an anomeric mixture. ^{1}H NMR (500 MHz, D_2O): δ = 5.32 (d, $^3J_{1,2}$ = 3.8 Hz, 1 H, H1 α anomer), 4.58 (d, $^3J_{1,2}$ = 7.6 Hz, 1 H, H1 β anomer) ppm. ^{13}C NMR (125 MHz, D_2O): β anomer: δ = 97.01 (d, $J_{C,P}$ = 5.9 Hz, C1), 76.22 (d, $J_{C,P}$ = 5.1 Hz, C5), 73.90 (d, $J_{C,P}$ = 2.5 Hz, C3), 72.58 (d, $J_{C,P}$ = 5.1, C2), 69.41 (C4), 61.8 (C6) ppm; α anomer: δ = 92.38 (d, $J_{C,P}$ = 3.4 Hz, C1), 71.20 (C5), 69.97 (C4), 69.72 (d, $J_{C,P}$ = 3.4 Hz, C2), 69.61 (C3), 62.00 (C6) ppm.

The same product was obtained from α-D-galactopyranosyl 1,2-cyclic phosphate (**10**).

Preparation of Cell-Free Extracts and Membranes of *P. fellutanum*: Cultures of *Penicillium fellutanum*[26] were obtained using 0.5% galactose as carbon source.[29] The cultures were filtered after 3 d of growing and the mycelial pads were finely ground in a mortar previously cooled to –10 °C. For one standard pad (52 g wet weight), Al_2O_3 (20 g) was added and the mass was ground vigorously for 2–4 min at 4 °C, whereupon 12 mL of 0.1 M sodium phosphate buffer (pH 6.8, Buffer A) was added and the grinding continued for a further 3 min. A total of 25 mL buffer (approx. 1 mL per gram of wet weight of mycelium) was used in the grinding and in washing out the mortar.

The homogenate was centrifuged at 8000 g for 30 min at 4 °C to remove Al_2O_3 and cell debris. The supernatant containing 0.5 mg mL^{-1} of protein (pH 6.5–6.8) was centrifuged at 200000 g for 1 h at 4 °C to sediment the membranes, which were resuspended in buffer A and again centrifuged. The final suspension of membranes was obtained at a protein concentration of 3 mg mL^{-1}. This membrane suspension can be stored at –70 °C for at least 6 months without significant loss of activity.

Preparation of Peptidophosphogalactomannan (pPGM): Cultures of *P. fellutanum*[26] using 0.5% galactose as carbon source[29] were filtered after 4 d of growing, and the filtrates were dialyzed for 72 h against distilled water. The pPGM[26] was precipitated with four volumes of ethanol. The precipitate was redissolved in distilled water and lipids were removed by three extractions with $CHCl_3$. The aqueous phase was lyophilized and stored at –20 °C.

Galactofuranosyl Transfer Assays: The procedure was optimized by incubation of the membrane preparation (45 µL, 3 mg mL^{-1} protein) with UDP-[6-^{3}H]Gal*p* (500000 cpm, 6.59 GBq mmol^{-1}, Amersham Pharmacia) before incorporation from UDP-[6-^{3}H]Gal*f* (**3***).

pPGM (2 mg) was added to a mixture of buffer B (0.2 M sodium phosphate buffer pH 6.8, containing 30 mM $FeSO_4$ and 20 mM $MgCl_2$, 500 µL) and membrane suspension (45 µL, 3 mg mL^{-1} of protein). The mixture was incubated at 25 °C for 30 min before adding UDP-[6-^{3}H]Gal*f* (**3***, 400000 cpm) to a final volume of 1 mL and, after 1 h at 25 °C, the reaction mixture was centrifuged at 200000 g at 4 °C. The pellet was suspended in 1% Triton X-100 and recentrifuged. The supernatants and the washing supernatant were separately dialyzed (Spectra/Por membrane, MWCO 3,500, Thomas Scientific) against distilled water for 24 h in order to eliminate the radiolabeled donor in excess.

The solutions were concentrated by lyophilization to a final volume of 0.5 mL and applied to a BioGel P-6 column (30 mL), eluting with water. Fractions (1 mL) were collected and counted in a liquid scintillation counter. The labeled excluded fractions of the gel chromatography were combined and lyophilized to a final volume of 0.2 mL. In order to confirm the incorporation of radioactivity as galactofuranose into pPGM, a sample was hydrolyzed under mild acid conditions (20 mM TFA, 100 °C, 2 h) and analyzed by Biogel P-6. Included radioactive fractions were pooled, concentrated by lyophilization to a final volume of 0.5 mL, and analyzed by HPAEC-PAD chromatography using Condition 2; they were found to be identical to galactose.

Abbreviations: UDP-Gal*p*: Uridine 5′-(α-D-galactopyranosyl diphosphate); UDP-Gal*f*: Uridine 5′-(α-D-galactofuranosyl diphosphate); α-D-Gal*p* 1-P: α-D-galactopyranosyl bis(triethylammonium) phosphate salt; α-D-Gal*f* 1-P: α-D-galactofuranosyl bis(triethylammonium) phosphate salt; D-Gal 2-P: galactose 2-phosphate; α-D-Gal*f* 1,2-P: α-D-galactofuranose 1,2-cyclic phosphate; α-D-Gal*p* 1,2-P: α-D-galactopyranose 1,2-cyclic phosphate; pPGM: peptidophosphogalactomannan; HPAEC: High pressure anion exchange chromatography; PAD: pulse amperometric detection.

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