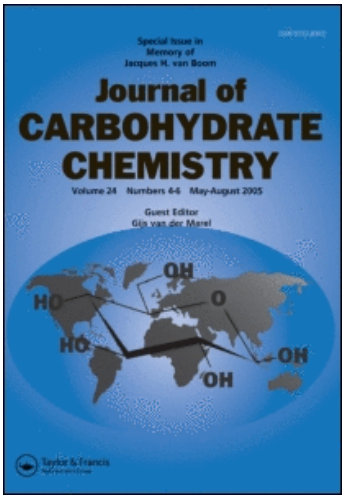

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RAPID CONVERSION OF UNPROTECTED GALACTOSE ANALOGS TO THEIR UDP-DERIVATIVES FOR USE IN THE CHEMO-ENZYMATIC SYNTHESIS OF UNNATURAL OLIGOSACCHARIDES

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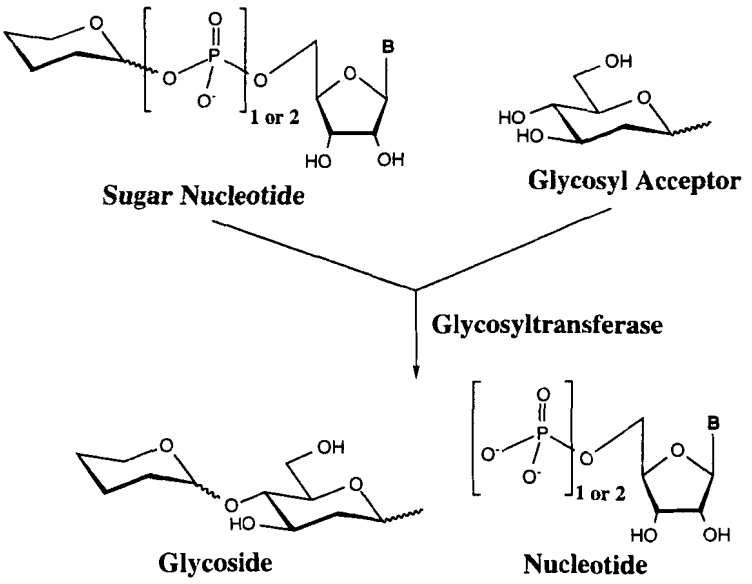
ABSTRACT

The rapid conversion of D-galactose, its 2-deoxy, 3-deoxy, 4-deoxy and 6-deoxy derivatives and L-arabinose to their UDP-derivatives (**2-7**) is described. The procedure involves the *in situ* preparation of the per-*O*-trimethylsilylated glycopyranosyl iodides and their direct reaction with UDP. All six sugar nucleotides were active as substrates for β(1→4)-galactosyltransferase and were used to enzymatically prepare *N*-acetyllactosamine (**8**) and five of its analogs (**9-13**).

INTRODUCTION

Glycosyltransferases transfer sugars from their activated forms (sugar nucleotides) to oligosaccharide acceptors. The use of glycosyltransferases in a combined chemical-enzymatic approach to the preparation of oligosaccharides is becoming an increasingly attractive approach compared with their tedious multistep chemical synthesis. The advantage of using these enzymes in the formation of glycosidic linkages is that they are both stereospecific and regiospecific, thus eliminating the need for laborious protection-deprotection protocols.[1]



Scheme 1. The general reaction catalyzed by glycosyltransferases.

The general reaction catalyzed by a glycosyltransferase is shown in Scheme 1 which summarizes the three key components essential for incorporation of a glycosyltransferase step into a synthetic scheme: 1) an acceptor oligosaccharide substrate must be available, 2) the donor sugar nucleotide is required, and 3) the appropriate glycosyltransferase must be available. In the past, the availability of these enzymes has severely limited their use but now dozens have been cloned[2] and they are therefore becoming increasingly accessible.

The required sugar-nucleotides can be prepared either by chemical synthesis or through the use of enzyme recycling systems.[1] All but two[3] published chemical procedures have involved the multistep synthesis of a pyranosyl phosphate, the activation of either the pyranosyl phosphate or the nucleoside 5'-monophosphate (usually as the morpholidate) and the subsequent formation of the pyrophosphate linkage. This sequence of reactions can take several weeks of work, the product sugar nucleotides are often formed in only low yields (near 5% from the free sugar) and they require extensive purification by ion-exchange chromatography or HPLC. These procedures are difficult to reproduce from laboratory to laboratory when researchers are not experienced in the manipulation of charged yet protected organic compounds. Deoxy analogs of the natural sugar nucleotides

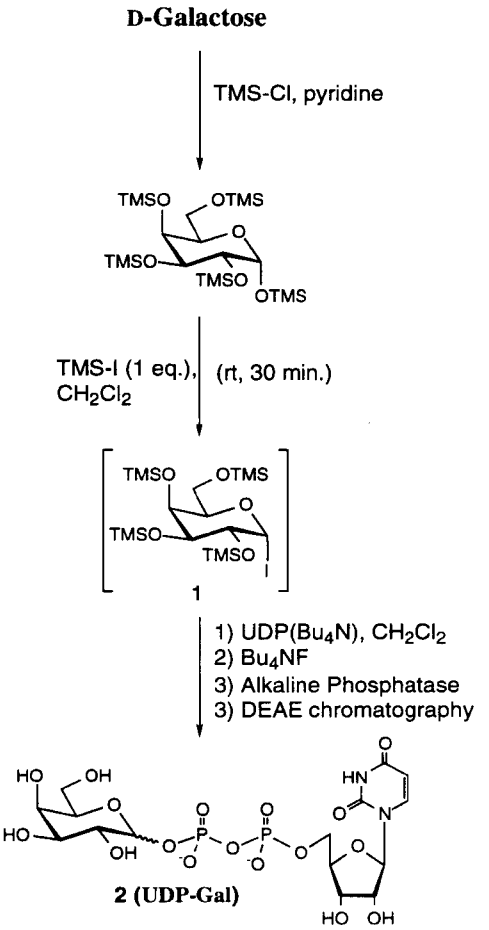
are particularly problematic since they can be very chemically labile and decompose during purification[4] and may therefore be inaccessible for oligosaccharide synthesis.

We have previously reported that a per-*O*-benzylated monosaccharide with a leaving group (Br) at the anomeric carbon can react directly with the readily prepared organic-soluble tetra-*n*-butylammonium salt of UDP to give, on debenzylation, the expected sugar nucleotide.[3b] While effective, this procedure still required the multistep preparation of per-*O*-benzylated sugars bearing a free anomeric OH group, and the product sugar-nucleotide required a hydrogenation step for deprotection. We have now found that use of the readily installed *O*-trimethylsilyl (TMS) group,[5,6] which can be removed by simple solvolysis or fluoride ion, can dramatically simplify the reaction sequence. We report here that per-*O*-trimethylsilylation of unprotected monosaccharides, followed by reaction with trimethylsilyl iodide (TMS-I), yields glycopyranosyl iodides[6] which yield sugar nucleotide diphosphates on simple mixing with UDP. Five commercially available analogs of D-galactose were used as examples to test this reaction sequence.

RESULTS AND DISCUSSION

Reaction of D-galactose (Gal) and five of its analogs; L-arabinose (L-Ara), 2-deoxy-D-galactose (2-deoxy-Gal), 3-deoxy-D-galactose (3-deoxy-Gal), 4-deoxy-D-galactose (4-deoxy-Gal) and 6-deoxy-D-galactose (6-deoxy-Gal) with chlorotrimethylsilane (TMS-Cl) in pyridine gave per-*O*-TMS-derivatives in quantitive yield. For 2-deoxy-Gal and 3-deoxy-Gal, mixtures of pyranose and furanose derivatives were obtained. When 2-deoxy-Gal was silylated using triethylamine (TEA) in CH_2Cl_2, however, only the pyranose derivative was formed. Under the same conditions, 3-deoxy-Gal still gave a 3:2 mixture of pyranose and furanose.

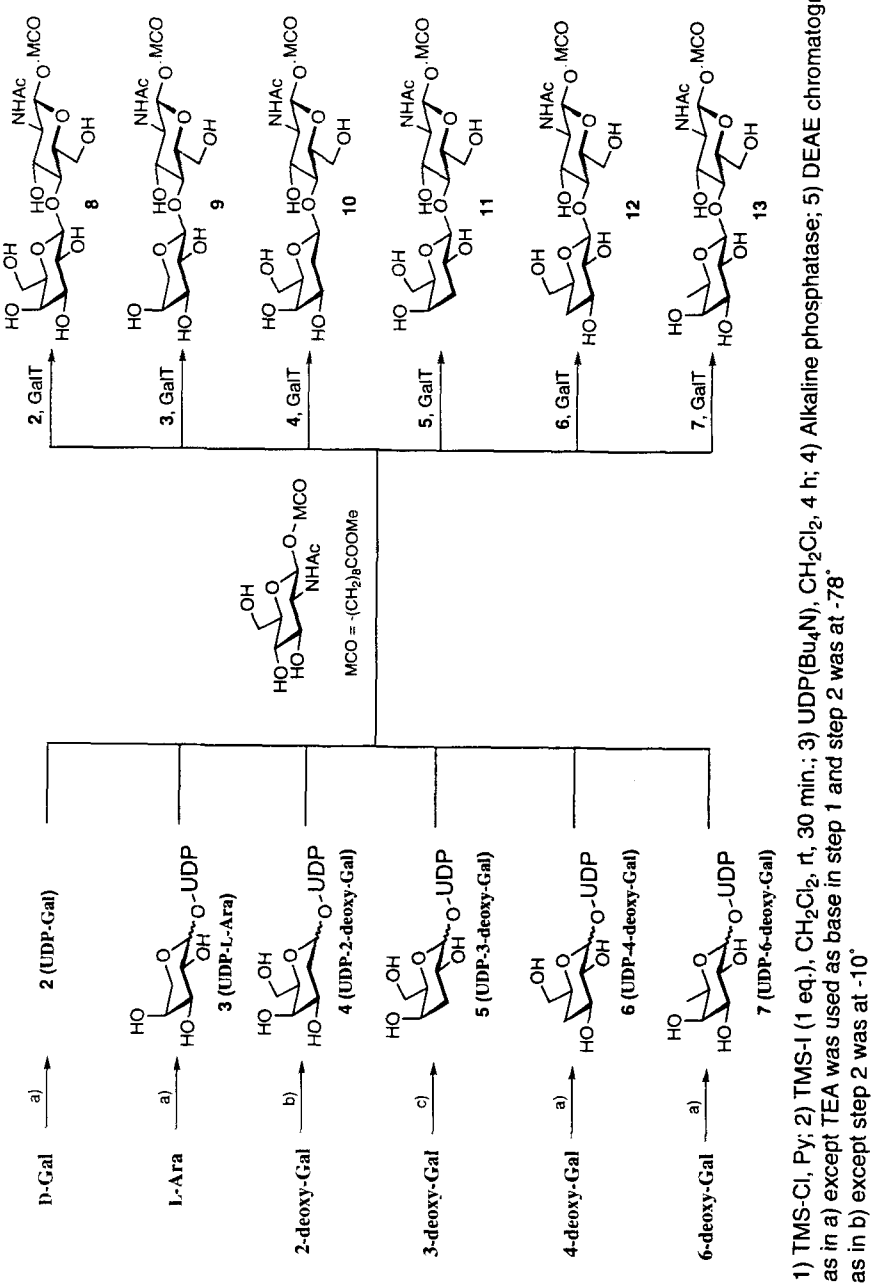
Reaction of the per-*O*-TMS-sugar with TMS-I (1 eq.) in CH_2Cl_2 (Scheme 2) resulted in the essentially quantitive formation of per-*O*-TMS-α-pyranosyl iodide (**1** for Gal) in 30 min.[7] The tetrabutylammonium (*n*-Bu_4N) salt of UDP was then added and, after 4 h, deprotection of TMS groups effected by addition of tetrabutylammonium fluoride (Bu_4NF). After concentration, the reaction mixture was treated with alkaline phosphatase in Tris buffer (pH = 8), which hydrolyzes unreacted UDP to uridine and phosphate, in order to simplify the purification of the sugar-nucleotide by ion exchange chromatography on DEAE. UDP-Gal (**2**) and each of its analogs (**3-7**) were thus obtained as α/β mixtures in yields ranging from 30-49 % (Scheme 3) (Table 1). UMP-Gal was detected as a by-product (~10 %).[8] *The production of α/β mixtures is not viewed as problematic since the*



Scheme 2. Conversion of Gal to UDP-Gal via the per-TMS-pyranosyl iodide **1**.

transferases will select and use only the required anomer. The ^{1}H and ^{31}P NMR spectral data and the MALDI-TOF MS data for each of UDP-Gal analogs are collected in Table 1. Sugar nucleotides **3-7** have previously been prepared by multi-step synthesis.[9]

Commercially available bovine β(1→4)-galactosyltransferase (GalT) is known to transfer each of the galactosyl analogs from their UDP derivatives **2-7**[9] and was used here to demonstrate that the readily produced α/β mixtures of UDP-sugars could function as donors in the synthesis of oligosaccharide analogs. Incubation of sugar nucleotides **2-7** with 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (β-GlcNAc-MCO) andGalT, in the presence of alkaline phosphatase,[10] resulted in complete conversion to *N*-acetyllactosamine and its analogs **8-13** as judged by TLC. The products were



a): 1) TMS-Cl, Py; 2) TMS-I (1 eq.), CH_2Cl_2, rt, 30 min.; 3) UDP(Bu_4N), CH_2Cl_2, 4 h; 4) Alkaline phosphatase; 5) DEAE chromatography
b): as in a) except TEA was used as base in step 1 and step 2 was at -78°
c): as in b) except step 2 was at -10°

Scheme 3. Chemo-enzymatic synthesis of *N*-acetyllactosamine analogs.

Table 1. Chemically synthesized sugar nucleotides **2-7**.

Sugar Nucleotide	α : β ratio[a] (yield)	^{1}H NMR[c] (H–1,δ)	^{31}P NMR[d] (δ)	MALDI TOF[e]	Rf[f]
2 **(UDP-Gal)**	1 : 1 (30 %)	α; 5.65 (dd, J = 7.0, 4.0) β; 4.97 (t; J = 8.0)	P_1; -11.06, -11.23 (each d, J = 21.0) P_2; -12.61, -12.86 (each d, J = 21.0)	566 $[M]^-$	0.33
3 **(UDP-L-Ara)**	4 : 7 (36 %)	α; 5.63 (dd, J = 7.0, 3.5) β; 4.92 (dd, J = 8.0, 7.0)	P_1; -11.15 (d, J = 21.0) P_2; -12.67, -12.97 (each d, J = 21.0)	535 $[M-H^+]^-$	0.33
4 **(UDP-2-deoxy-Gal)**	3 : 1 (40 %)	α; 5.76 (m) β; 5.25 (m)	P_1; -11.42, -11.51 (each d, J = 21.0) P_2; -13.34, -13.54 (each d, J = 21.0)	550 $[M]^-$	0.36
5 **(UDP-3-deoxy-Gal)**	1 : 1 (32 %)[b]	α; 5.62 (dd, J = 7.0, 3.0) β; 5.00 (t; J = 8.0)	P_1; -10.95, -11.02 (each d, J = 21.0) P_2; -12.20, -12.68 (each d, J = 21.0)	550 $[M]^-$	0.40
6 **(UDP-4-deoxy-Gal)**	1 : 3 (49 %)	α; 5.66 (dd, J = 7.0, 3.5) β; 4.97 (t; J = 8.0)	P_1; -11.09, -11.17 (each d, J = 21.0) P_2; -12.63, -12.83 (each d, J = 21.0)	550 $[M]^-$	0.38
7 **(UDP-6-deoxy-Gal)**	1 : 1 (30 %)	α; 5.60 (dd, J = 7.0, 4.0) β; 4.96 (t; J = 8.0)	P_1; -11.13 (d, J = 21.0) P_2; -12.69, -12.88 (each d, J = 21.0)	550 $[M]^-$	0.37

a. Determined by integration based on ^{1}H NMR spectroscopy. b. The product appeared to contain (~30%) UDP-galactofuranose (^{1}H NMR; δ 5.65, d, J = 5.0). c. 360 MHz (D_2O). d. 81 MHz (D_2O). e. 3-Aminoquinoline was used for the matrix and 40 to 80 pmol of sugar nucleotides were analyzed using negative ion detection. f. TLC was performed on Silica gel 60 F_{254} (E. Merck) ($CHCl_3$:MeOH:H_2O:AcOH= 4:5:2:1, v/v/v/v) with detection by quenching of fluorescence or by charring with *p*-anisaldehyde reagent (MeOH:*p*-anisaldehyde: H_2SO_4=85:10:5, v/v/v).

isolated by adsorption on C-18 silica followed by purification on silica gel. The disaccharide analogs were characterized by ^{1}H NMR and MALDI-TOF MS (Table 2) data.

In summary, a rapid chemical synthesis of sugar nucleotide diphosphates from the free sugar has been developed. The sugar nucleotides thus prepared were used in the enzymatic synthesis of *N*-acetyllactosamine and five of its analogs.[11] The procedure does

Table 2. Enzymatically synthesized disaccharide analogs **8-13**.

	β(1→4) GalT units (hr)[a] yield[b]	Enzymatic product		
		reporter protons [c]	MALDI TOF [d]	Rf [e]
8	1 (2) 75 %	**GlcNAc** 4.52 (d, J = 8.0) **Gal** 4.47 (d, J = 8.0)	576 $[M+Na]^+$	0.30
9	10 (18) 69 %	**GlcNAc** 4.52 (d, J = 8.0) **L-Ara** 4.39 (d, J = 8.0)	546 $[M+Na]^+$	0.38
10	5 (2) 62 %	**GlcNAc** 4.51 (d, J = 8.0) **2-deoxy-Gal** 4.72 (dd, J = 10.0, 2.0) 2.09 ($H\text{-}2_{eq.}$, ddd, J = 12.0, 5.0, 2.0) 1.70 ($H\text{-}2_{ax.}$, dt, J = 10.0, 12.0)	560 $[M+Na]^+$	0.39
11	15 (36) 88 %	**GlcNAc** 4.53 (d, J = 8.0) **3-deoxy-Gal** 4.47 (d, J = 8.0) 2.22 ($H\text{-}3_{eq.}$, ddd, J = 14.0, 5.0, 3.0) 1.75 ($H\text{-}3_{ax.}$, ddd, J = 14.0, 12.0, 3.0)	560 $[M+Na]^+$	0.44
12	10 (18) 72 %	**GlcNAc** 4.52 (d, J = 8.0) **4-deoxy-Gal** 4.45 (d, J = 8.0) 1.98 ($H\text{-}4_{eq.}$, ddd, J = 13.0, 5.0, 2.0) 1.45 ($H\text{-}4_{ax.}$, dt, J = 13.0, 13.0)	560 $[M+Na]^+$	0.46
13	10 (18) 90 %	**GlcNAc** 4.52 (d, J = 8.0) **6-deoxy-Gal** 4.43 (d, J = 8.0) 1.25 (H-6, d, J = 6.0)	560 $[M+Na]^+$	0.48

a. Total amount of GalT and time for enzymatic reaction. b. Isolated yield after chromatography. c. 500 MHz (D_2O). d. 2,5-Dihydroxybenzoic acid was used as the matrix. e. TLC was performed on Silica gel 60 F_{254} (E. Merck) ($CHCl_3$:MeOH:H_2O = 7:3:0.5, v/v/v).

not involve a complex organic synthesis but is designed such that it can be routinely performed in a biochemical laboratory.

EXPERIMENTAL

Preparation of uridine 5'-diphosphate-tetrabutyl ammonium (UDP Bu_4N) salt from uridine 5'-diphosphate sodium (UDP Na) salt. UDP (Na salt) (Sigma, 0.1 mmol) was dissolved in water (1 mL) and the solution was passed through Dowex 50W X8 (H^+, 200-400 mesh, 0.75 cm x 4 cm) and fractions (10 mL, pH < 2) containing protonated UDP were titrated using aqueous $Bu_4N(OH)$ (40 %) to pH 6. The resulting solution was freeze-dried to yield UDP•Bu_4N salt as a white powder.[3b]

General procedure for the preparation of per-*O*-TMS sugars. Chlorotrimethylsilane (1.5 eq per OH group) was added at 0 °C to a solution of free sugar (0.1 mmol) in pyridine (0.5 mL). After 1 h, pentane (6 mL) was added. The resulting mixture which was then extracted with cold water (5 x 1 mL). The organic layer was dried (Na_2SO_4), and solvent evaporated to yield the per-*O*-TMS sugar (quantitive): ^{1}H NMR (360 MHz, $CDCl_3$) for anomeric Hδ: per-*O*-TMS-Gal 5.03 (d, J = 2.0 Hz, α-pyranose); per-*O*-TMS-L-Ara 4.99 (d, J = 2.0 Hz, α-pyranose); per-*O*-TMS-2-deoxy-Gal 4.69 (dd, J = 10.0, 2.0 Hz, β-pyranose); per-*O*-TMS-3-deoxy-Gal 5.13 (br s, β-furanose, 25 %), 5.03 (d, J = 4.0 Hz, 15 %) and 5.00 (d, J = 3.0 Hz, 15 %) for α-pyranose and α-furanose, 4.41 (d, J = 7.0 Hz, β-pyranose, 45 %); per-*O*-TMS-4-deoxy-Gal 5.01 (d, J = 3.0 Hz, α-pyranose, 5 %), 4.39 (d, J = 7.0 Hz, β-pyranose, 95 %); per-*O*-TMS-6-deoxy-Gal 5.01 (br s, α-pyranose).

General procedure for the preparation of sugar nucleotides. To a stirred solution of per-*O*-TMS-sugar (0.1 mmol) in CH_2Cl_2 (0.5 mL), iodotrimethylsilane (14 μL, 0.1 mmol) was added at either room temperature, -10 °C, or -78 °C (see Scheme 3) and the reaction mixture was stirred for 30 min. The reaction mixture was then added to a mixture of UDP Bu_4N salt (0.1 mmol) in dry CH_2Cl_2 (2 mL) and stirred for 4 h. A solution of BuN_4F (1.0 M in tetrahydrofuran, 0.2 mL) was added to the reaction mixture which was stirred for 1 h. The mixture was concentrated and the residue was dissolved in Tris buffer (0.05 M, pH = 8, 3 mL). Alkaline phosphatase (50 units, Boehringer Mannheim, from calf intestine) was added to the solution which was kept at rt for 16 h. The enzyme reaction mixture was diluted with water (25 mL) and applied to DEAE Sephacel (Pharmacia, 2.5 cm x 15 cm, packed in 30 mM NH_4HCO_3), and eluted using a linear gradient from 30 mM to 400 mM NH_4HCO_3 (2 mL/min., 6 mL for each fraction, monitoring at 254 nm). The

appropriate fractions were pooled and concentrated to about 10 mL (pH = 8 to 8.5). Dowex 50W X8 (H^+, 200-400 mesh) was then added slowly until the pH of the solution reached 6.5-7.0. The mixture was stirred at that pH for 5 min at 4 °C. The resin was removed by filtration, and the filtrate was passed through a column of Dowex 50W X8 (Na^+, 20-50 mesh, 1.5 cm x 5 cm) with water. The eluate was freeze-dried to give the sugar nucleotides as white powders.

Enzymatic synthesis. Solutions of 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (β-GlcNAc-MCO, 1.40 mg, 3.6 μmol), galactosyltransferase (EC 2.4.1.22 from bovine milk, Sigma, 1-15 units), alkaline phosphatase (100 units), and sugar nucleotides **2-7** (containing 2 eq.-4 eq. of the active α-anomer) in 100 mM sodium cacodylate buffer (5 mL, pH = 7.5) containing 5 mM $MnCl_2$ were kept at room temperature for 2-36 h. TLC examination of the mixtures in $CHCl_3$:MeOH:H_2O (40:10:1) showed complete conversion into new products of lower Rf. The mixtures were diluted with water (20 mL) and loaded directly onto SepPak C-18 cartridges that had been prewashed with 20 mL each of MeOH, $CHCl_3$:MeOH (1:1), MeOH:H_2O (1:1), and finally water. The cartridges were washed with water (20 mL) and the products were eluted with MeOH (20 mL). Evaporation of solvents left residues that were purified by chromatography ($CHCl_3$:MeOH:H_2O = 40:10:1) on Iatrobeads to give the pure disaccharides.

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7. Data for 2,3,4,6-tetra-*O*-tetramethylsilyl-α-D-galactopyranosyl iodide: ^{1}H NMR (360 MHz, $CDCl_3$) δ 6.80 (d, J = 4.0 Hz, H-1), 2.87 (dd, J = 9.0, 4.0 Hz, H-2); conversion to per-*O*-TMS-galactosyl bromide (δ 6.38, d, J = 3.5 Hz, H-1) using TMS-Br (1 eq.) required over 2 days at rt.
8. Data for the UMP-Gal by-product: ^{1}H NMR (360 MHz, D_2O) δ 5.56 (dd, J = 7.0, 3.0 Hz, H-1 for α form), 4.88 (t, J = 7.5 Hz, H-1 for β form), ^{31}P NMR (81 MHz, D_2O) δ -1.33 and -1.56 (each s), MALDI-TOF MS 486 $[M]^-$, Rf 0.44 ($CHCl_3$:MeOH:H_2O:AcOH = 4:5:2:1).
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11. The sugar nucleotides **2**-**7** have also been used as glycosyl donors, α(1→3)-galactosyltransferase, α(1→4)-galactosyltransferase and blood group B-galactosyltransferase. Compounds **2**, **4** and **7** could be transferred at preparatively useful rates by all three galactosyltransferases. The details will be published elsewhere.