A Methodological Comparison: The Advantage of Phosphorimidates in Expanding the Sugar Nucleotide Repertoire

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Introduction

Recent research has revealed that complex glycosylation patterns mediate or modulate a variety of biological processes.[1] Thus, a major focus in glycobiology is on methods to access biologically relevant oligosaccharides via chemical or enzymatic synthesis.[2] Of the available enzymatic methods, glycosyltransferase-catalyzed oligosaccharide synthesis is generally preferred since the reactions are regio- and stereoselective and eliminate the typical multiple protection/deprotection schemes required for chemical synthesis.[3] As a result, access to the nucleotide sugar substrates of glycosyltransferases has become a major limiting factor in glycosyltransferase-catalyzed synthesis, and the chemical or enzymatic syntheses of the eight primary sugar nucleotides utilized by mammalian transferases (UDP−Glc, UDP−GlcNAc, UDP−Gal, UDP−GalNAc, GDP−Man, GDP−Fuc, UDP−GlcUA, and CMP−NeuAc)[4] have been reported.[5]

In contrast, bacterial glycosyltransferases utilize an almost endless repertoire of nucleotide sugars in constructing the cell wall and cell surface antigens as well as for the glycosylation of numerous small bioactive molecules.[6] For example, TDP−Rha (**4**) is the progenitor of L-rhamnose, which is found in numerous Gram-negative bacterial cell surface antigens and is also an integral ligand of a variety of antibiotic/antitumor agents produced by Gram-positive species.[6,7] Unfortunately, **4** is only accessible via a tedious and expensive multienzymatic method.[8] Consequently, in an effort to expand the repertoire of available sugar nucleotides, we report a chemical synthesis of **4** via the one pot generation and reaction of TMP−imidazolide with rhamnose-1-phosphate (**2**). A comparison of the TMP−imidazolide method to two distinct "state of the art" methods[5n,o]—the coupling of TMP−morpholidate with **2** or the reaction of TDP with 2,3,4-tri-*O*-benzyl-L-rhamnopyranosyl bromide (**3**)[9]—reveals that 1,1′-carbonyldiimidazole (CDI)-catalyzed coupling gives rise to reaction times and yields comparable to the morpholidate method with significantly less expensive precursors and more flexibility in the overall reaction parameters.

Results and Discussion

Formation of the activated TMP−imidazolide was accomplished by reacting the triethylammonium salt of TMP (**1**) with 3 equiv of 1,1′-carbonyldiimidazole[5d,g,m,11] in DMF-d_7 or CD_3CN, and the reaction was monitored by ^{31}P NMR. Immediately after the addition of CDI, a complete disappearance of the resonance signal of the starting material (δ 3.31 in DMF-d_7) and the formation of the 5′-phosphocarbonylimidazolide (δ −6.26 in DMF-d_7 and −5.82 in CD_3CN) were observed followed by the eventual disappearance of the TMP 5′-phosphocarbonylimidazolide with concomitant formation of the desired

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(4) Abbreviations: Ara, L-arabinose; CMP, cytidine monophosphate; Fuc, L-fucose; Gal, D-galactose; GDP, guanosine diphosphate; Glc, D-glucose; Glc*N*Ac, 2-*N*-acetyl-D-glucose; GalNAc, 2-*N*-acetyl-D-galactose; GlcUA, D-glucuronic acid; Man, D-mannose; NeuAc, *N*-acetylneuramic acid (sialic acid); NMP, nucleotide monophosphate; Rha, L-rhamnose (6-deoxy-L-mannose); TDP, thymidine diphosphate; TMP, thymidine monophosphate; UDP, uridine diphosphate; UMP, uridine monophosphate.

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(12) Similar intermediates were observed by both ^1H and ^{31}P NMR in the CDI-catalyzed synthesis of NAD analogues.[11e]

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Table 1. Comparative Yields of 4 via the in Situ TMP−Imidazolide Method (Method I), the Coupling of TMP−Morpholidate with 2 (Method II), and the Reaction of TDP with 3 (Method III)

time (h)	method I yield (%)[a,b]	method II yield (%)[a,c]	method III yield (%)[a,d]
5	ND[e]	ND[e]	18.3[f]
12	57.5	53.3	ND[e]
24	71.9	67.7	ND[e]
48	75.5[g]	74.7[g]	ND[e]

[a] Reactants and products were separated by analytical HPLC, and the reported yields were calculated directly from peak integration. [b] Coupling solvent DMF, stoichiometry of TMP−imidazolide and **2** was 1.23:1. [c] Coupling solvent pyridine, stoichiometry of TMP−imidazolide, **2** and 1*H*-tetrazole was 1.33:1:3. [d] Coupling solvent CH_2Cl_2, stoichiometry of TDP and **3** was 1:3.5. [e] Not determined. [f] TLC indicated complete consumption of **3** in less than 5 h. The reported yield was a complex mixture of products based upon ^{31}P NMR. [g] A slight decrease in yield was observed after 48 h attributed to slow product degradation.

TMP−imidazolide (δ −7.51 in DMF-d_7 and −6.75 in CD_3CN).[12] Excess CDI was quenched by the addition of MeOH, and the solvent evacuated followed by the addition of **2**. Based upon analytical HPLC, the coupling of **2** with TMP−imidazolide was complete in less than 24 h. Chromatography provided spectroscopically pure **4** as either the disodium or dipotassium salt in >70% yield. As an alternative, we also examined the coupling of commercially available TMP−morpholidate with **2**, in the presence of 1*H*-tetrazole, using conditions previously described for the synthesis of GDP−Fuc, GDP−Man, and UDP−Gal[5o] and the reaction of TDP with **3** using conditions previously described for the construction of GDP−Fuc, UDP−Ara, and UDP−Gal.[5n] Surprisingly, the CDI method provided reaction times and yields comparable to or slightly better than the morpholidate methodology (Table 1) while both the TMP−imidazolide and −morpholidate coupling reactions were superior in providing **4** to the coupling of TDP with **3**.

Previous studies revealed a large effect upon the addition of an acidic catalyst to morpholidate-catalyzed coupling reactions.[5o] In particular, the extent of stimulation of morpholidate-catalyzed coupling upon the addition of various additives followed the order 1*H*-tetrazole ($pK_a = 4.9$) ≥ 4-(dimethylamino)pyridine hydrochloride (DMAP·HCl, $pK_a = 6.1$) > perchloric acid > *N*-hydroxysuccinimide ($pK_a = 6.1$) ≅ acetic acid ($pK_a = 4.75$) > 1,2,4-triazole ($pK_a = 10.0$), suggesting that 1*H*-tetrazole activates NMP−morpholidate by a combination of protonation of the leaving group nitrogen ($pK_a \cong 8.33$)[13] and nucleophilic catalysis via the formation of a highly reactive phosphotetrazolide. In contrast, we found that permutations of the TDP−imidazolide ($pK_a \cong 6.95$)[13] reaction conditions illustrate this reaction to be much less dependent upon additives or solvent. For example, yields similar to those reported in Table 1 were observed with ethanol, pyridine, DMF, or DMSO as the reaction solvent, while a decrease of approximately 60% was observed in methanol. Furthermore, the addition of 3 equiv of 1*H*-tetrazole to the reaction in either DMF or ethanol resulted in a <2% change in the overall yield, while a slightly larger effect was observed (~10% increase in yield) upon the addition of equivalent amounts of 1*H*-tetrazole in methanol.

To confirm the biological relevance of our synthetic **4**, we examined its ability to serve as a substrate for the in vitro biosynthesis of a precursor for the *Salmonella* group E1 *O* antigen repeat unit (**11**, Scheme 2).[14] The biosynthesis of **11** is initiated by enzyme(Gal-1-*P*T)-catalyzed galactose-1-phosphate transfer from UDP−Gal to undecaprenyl phosphate (**5**),[15] with subsequent loss of UMP to provide **7**, followed by enzyme ($RhaT^{\alpha 3}$)-catalyzed rhamnosyl transfer from TDP−Rha and loss of TDP to give **9**, and subsequent enzyme ($ManT^{\beta 4}$)-catalyzed mannosyl transfer from GDP−Man with loss of GDP.[10] To accomplish the assay, a mixture containing a truncated analogue of **5** (heptaprenyl phosphate, **6**), Gal-1-*P*T, UDP−Gal, $RhaT^{\alpha 3}$, **4**, $ManT^{\beta 4}$, and 0.33 μM GDP−[*U*-^{14}C]Man (15 000 cpm) was allowed to react for 2 h, and the reaction was subsequently quenched by combining with 4 mL of $CHCl_3/CH_3OH$ (2:1). Excess GDP−[*U*-^{14}C]-Man was removed from the glycolipid products by selective extraction,[16] the glycolipids were subsequently dried by evaporation and dissolved in scintillant, and the extent of radioactivity recovered (directly proportional to the amount of **12** produced) was determined by liquid scintillation counting. Under these conditions, significant $ManT^{\beta 4}$-catalyzed mannosyltransfer to provide **12** was observed (819.5 ± 40.5 cpm), and parallel control reactions lacking **4** (164.5 ± 18.1 cpm) clearly demonstrate that chemically synthesized **4** is required to complete the biosynthetic cascade. Control reactions in the absence of Gal-1-*P*T (89.5 ± 13.4 cpm), $RhaT^{\alpha 3}$ (215.0 ± 20.7 cpm), or $ManT^{\beta 4}$ (153.0 ± 17.5 cpm) are also consistent with the requirement of these reagents for the in vitro enzymatic construction of **12**.

Conclusions

In conclusion, this work provides the first direct comparison of the "state of the art" nucleotide sugar synthetic methodologies[5n,o] and reveals the single-flask activation of TMP and subsequent coupling of TMP−imidazolide with rhamnosyl-1-phosphate as the best method for the production of **4**. These results also suggest CDI-catalyzed nucleotide sugar synthesis may prove advantageous in the production of other nucleotide sugars since the choice of solvent is flexible and the method is not limited by the commercial availability of expensive NMP−morpholidates. In addition, by providing significant quantities of **4**, this work sets the stage for our future studies of various glycosyltransferases required for bacterial *O* antigen biosynthesis as well as the unique glycosyltransferases responsible for tailoring steps in calicheamicin biosynthesis.[17] Work is continuing in these exciting areas.

Experimental Section

General Methods. Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrophotometer. ^{1}H NMR spectra were obtained on a Bruker AMX 400 (400 MHz) and

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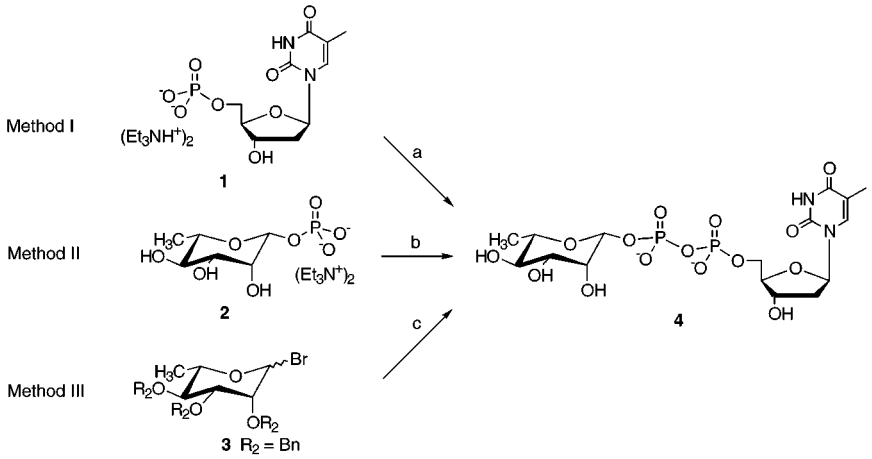
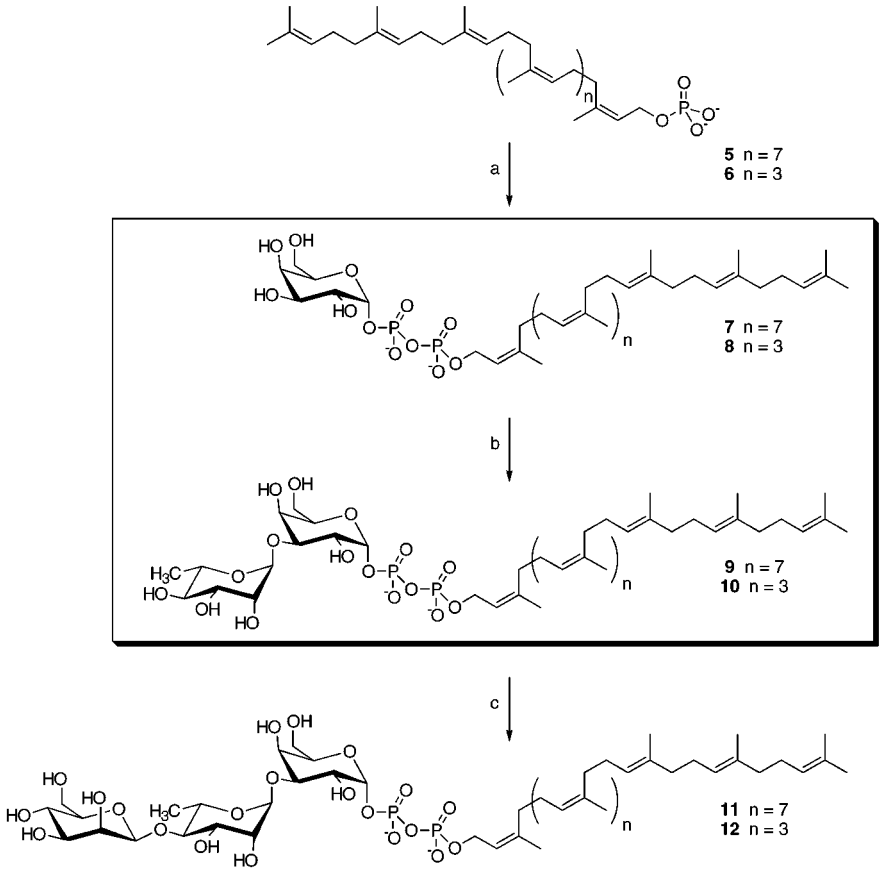
Scheme 1. Synthetic Routes to 4: (a) CDI, CH_3CN; 2, DMF; (b) TMP−Morpholidate, 1*H*-Tetrazole, Pyridine; (c) (i) $TDP(NBu_4)$, CH_2Cl_2; (ii) H_2, PdC



Scheme 2. Schematic Representation of the Enzyme-catalyzed Biosynthesis of the *Salmonella* Group E1 *O* Antigen Precursor 11: (a) Gal-1-*P*T/UDP−Gal; (b) $RhaT^{\alpha 3}$/4; (c) $ManT^{\beta 4}$/GDP-Man[a]



[a] Enzymatic step that requires **4** is highlighted.

are reported in parts per million (δ) relative to either tetramethylsilane (0.00 ppm) or $CDCl_3$ (7.25 ppm) for spectra run in $CDCl_3$, D_2O (4.82 ppm), or CD_3OD (3.35 ppm). Coupling constants (J) are reported in hertz. ^{13}C NMR are reported in δ relative to $CDCl_3$ (77.00 ppm) or CD_3OD (49.05 ppm) as an internal reference, and ^{31}P NMR spectra are reported in δ relative to H_3PO_4 (0.00 ppm in D_2O). Mass spectra were recorded on a PE SCIEX API 100 LC/MS mass spectrometer. Optical rotations were recorded on a Jasco DIP-370 polarimeter using a 1.0 or 0.5 dm cell at room temperature (25 °C) and the reported concentrations. Melting points were measured with an Electrothermal 1A-9100 digital melting point instrument.

Chemicals used were reagent grade and used as supplied except where noted. Analytical TLC was performed on either E. Merck silica gel 60 F_{254} plates (0.25 mm) or Whatman AL Sil G/UV silica gel 60 plates. Compounds were visualized by spraying $I_2/KI/H_2SO_4$ or by dipping the plates in a cerium sulfate−ammonium molybdate solution followed by heating. Liquid column chromatography was performed using forced flow of the indicated solvent on E. Merck silica gel 60 (40−63 μm), and high-pressure liquid chromatography was performed on a RAININ Dynamax SD-200 controlled with Dynamax HPLC software.

Dibenzylphosphoryl-2,3,4-tri-*O*-acetyl-β-L-rhamnopyranoside. Activated 4 Å molecular sieves (powder, 5 g) were added to a solution of dibenzyl phosphate (4.0 g, 14.38 mmol), silver triflate (3.28 g, 12.7 mmol), and 2,4,6-collidine (2.0 g, 16.5 mmol) in dry CH_2Cl_2 (15 mL), and the mixture was stirred at

room temperature for 1.0 h under argon atmosphere in the absence of light. The temperature was decreased to −40 °C, and 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide (4.5 g, 12.7 mmol)[10] in 10 mL of dry CH_2Cl_2 was added in dropwise fashion. The reaction mixture was stirred at −40 °C for 4 h under argon atmosphere and the absence of light and then allowed to warm to room temperature. After approximately 12 h, a few drops of methanol were added to quench unreacted bromide, the mixture was stirred for 1 h and partitioned between $CHCl_3$ (40 mL) and H_2O (40 mL), and the organics were dried over $MgSO_4$, filtered, concentrated, and purified by silica gel chromatography (33%→40% EtOAc/hexanes) to give 5.33 g (76%) of the desired product: $R_f = 0.33$ (40% EtOAc/hexanes); $[\alpha]^{25}_D = -46°$ (*c* 1.0, $CHCl_3$); IR (thin film) 1749 (s), 1372, 1219, 1161, 1015, 957; ^{1}H NMR ($CDCl_3$) δ 7.29 (m, 10H), 5.48 (d, *J* = 6.1 Hz, 1H), 5.21 (m, 1H), 5.16 (s, 1H), 5.07−4.98 (m, 5H), 3.88 (ddd, *J* = 6.2, 12.4, 15.1 Hz, 1H), 2.06 (s, 3H), 1.97 (s, 3H), 1.91 (s, 3H), 1.04 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR δ 169.74, 169.51, 128.58, 128.06, 127.89, 95.18, 70.15, 69.74, 68.92, 68.22, 20.59, 17.04; ^{31}P NMR δ −4.78; MS 573.0 (M + Na).

Triethylammonium β-L-rhamnopyranosylphosphate (2). Dibenzylphosphoryl-2,3,4-tri-*O*-acetyl-β-L-rhamnopyranoside (2.0 g, 3.63 mmol) was hydrogenated over 10% Pd/C (600 mg) in CH_3OH (25 mL) and 1.0 M $NaHCO_3$ (10 mL) under 50 psi hydrogen atmosphere at room temperature until the reaction was determined complete by TLC (7 h). The catalyst was removed by filtering the reaction through Celite, and the filtrate was concentrated and partitioned between CH_2Cl_2 (25 mL) and H_2O (25 mL). The product-containing aqueous layer was isolated and concentrated to 10 mL. This solution was subsequently cooled to 0 °C, and 1.0 M NaOH (15 mL) was added in dropwise fashion while keeping the temperature < 5 °C. After the NaOH had been added, the reaction was stirred and allowed to warm to room temperature (3 h). The pH of the mixture was carefully adjusted to pH 7.5 by the addition of cold 1.0 M HOAc, and the mixture was diluted to 250 mL with H_2O and submitted to anion exchange chromatography (Dowex 1 × 8, HCO_3^-, 3 × 15 cm). The product was eluted with H_2O (250 mL), 0.1 M NH_4HCO_3 (250 mL), 0.2 M NH_4HCO_3 (250 mL), and 0.3 M NH_4HCO_3 (250 mL), during which the desired compound eluted between 0.2 and 0.3 M NH_4HCO_3. The product-containing fractions were pooled, concentrated, and coevaporated several times with water and ethanol to remove remaining NH_4HCO_3 to give 0.81 g (85%) of ammonium β-L-rhamnopyranosylphosphate: $R_f = 0.43$ (33% H_2O/CH_3CN); $[\alpha]^{25}_D = -19°$ (*c* 2.0, H_2O); ^{1}H NMR (D_2O) δ 5.22 (d, *J* = 8.2 Hz, 1H), 3.92 (s, 1H), 3.87 (m, 2H), 3.37 (t, *J* = 9.7 + 9.7 Hz, 1H), 1.23 (d, *J* = 6.1 Hz, 3H);^{13}C NMR (D_2O) δ 95.22, 73.22, 71.02, 70.70, 69.89, 69.02, 17.07; ^{31}P NMR (D_2O) δ −1.26.

A 0.80 g (3.06 mmol) sample of this product was subsequently dissolved in H_2O (15 mL), applied to an AG 50W-X8 cation-exchange column (Et_3NH^+, 2.5 × 15 cm), and eluted with H_2O (150 mL). The eluate was evaporated and coevaporated with methanol (3 × 25 mL) to give the triethylammonium salt of β-L-rhamnopyranosylphosphate (1.01 g, 90%): $[\alpha]^{25}_D = -30°$ (*c*. 2.0, H_2O); ^{1}H NMR (D_2O) δ 5.29 (dd, *J* = 1.7, 7.6 Hz, 1H), 3.63 (t, *J* = 3.0 + 2.1 Hz, 1H), 3.87−3.80 (m, 2H), 3.39 (t, *J* = 9.7 + 9.7 Hz, 1H), 3.16 (m, 10.0H, 1.6 equiv of triethylamine), 1.23 (m, 17H);^{13}C NMR (D_2O) δ 96.06, 72.34, 70.90, 69.99, 69.70, 46.93, 17.08, 8.54; ^{31}P NMR (D_2O) δ −1.36; MS 392.1 (M + Na_2).

Thymidine 5′-diphospho-β-L-rhamnopyranose (4). Method I. The TMP sodium salt was exchanged to triethylammonium salt by passage through a column of Dowex-1 × 8 (Et_3NH^+) in water, and eluent containing 135 mg (419 μmol) of **1** was dried and dissolved in either DMF-d_7 or CD_3CN. Three equivalents of 1,1′-carbonyldiimidazole was added, and the progress of the reaction monitored by ^{31}P NMR. Upon completion (80−90 min), several drops of dry methanol were added to quench the excess CDI. The reaction mixture was concentrated, coevaporated with dry ethanol and toluene, and dried in vacuo, and the crude solid residue was used directly for the coupling reaction without further purification. To accomplish the coupling reaction, a mixture of **2** (118 mg, 342 μmol) and the thymidine 5′-phosphoimidazolide was coevaporated three times with the dry reaction solvent before the solution (concentration of the reactants kept at 0.2−0.3 M) was stirred at room temperature under argon atmosphere. To monitor reactions at various time points, 10 μL aliquots was removed and partitioned between 5 mL of 100 mM $NH_4HCO_3/CHCl_3$ (1:4), and 20 μL of the corresponding aqueous layer was utilized directly for chromatographic analysis. Reactants and products (TMP, 6.5 min; TDP−Rha, 15.0 min; TDP, 22.5 min) were separated by Sphereclone SAX analytical chromatography (0.46 × 250 cm, 1.5 mL min^{-1}, λ = 268 nm) with a mobile phase of H_2O (A) and 200 mM KH_2PO_4, pH 5.5 (B); 20% A (3 min), 20%→40% B (20 min), 40%→100% B (3 min), 100% B (5 min).

Upon completion, the reaction was diluted with 100 mM NH_4HCO_3 and extracted with $CHCl_3$, the aqueous layer concentrated, and the desired product isolated by Sphereclone SAX semipreparative chromatography (1.0 × 250 cm, 7.0 mL min^{-1}, λ = 268 nm) with a mobile phase of H_2O (A) and 200 mM KH_2PO_4, pH 5.5 (B); 20% A (5 min), 20%→40% B (15 min), 40%→100% B (10 min), 100% B (3 min). The product-containing fractions were collected (TMP, 14.2 min; TDP−Rha, 23.6 min; TDP, 30.4 min), concentrated, and desalted upon Sephadex G-10 resin (1.5 × 120 cm) to give the dipotassium salt of the title product as a white solid after lyophilization (mp 147−150 °C). Alternatively, the concentrated reaction mixture was loaded directly upon a Sephadex G-10 column (1.5 × 170 cm), and the product was eluted with water, concentrated, and subsequently crystallized from 1.0 M $NaClO_4$ acetone solution to provide the disodium salt of TDP−Rha (mp 158−161 °C): $R_f = 0.80$ (25% 1.0 M NH_4OAc/2-propanol); $[[\alpha]^{25}_D = -32°$ (*c* 0.5, H_2O); IR (thin film) 3423 (s), 2940, 1701 (s), 1477, 1373, 1249, 1048, 1052, 925; ^{1}H NMR (D_2O) δ 7.68 (s, 1H), 6.31 (t, *J* = 6.9 + 7.0 Hz, 1H), 5.37 (d, *J* = 7.2 Hz, 1H), 4.54 (m, 1H), 4.12 (m, 3H), 3.97 (s, 1H), 3.88−3.78 (m, 2H), 3.39 (t, *J* = 9.7 + 9.7 Hz, 1H), 2.32 (t, *J* = 5.0 + 6.1 Hz, 1H), 1.87 (m, 3H), 1.22 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (D_2O) δ 166.56, 151.71, 137.28, 111.71, 95.52, 85.24, 84.94, 72.80, 72.72, 71.59, 70.95, 69.11, 65.44, 60.23, 38.52, 11.61; ^{31}P NMR (D_2O) δ −10.57, −12.11; MS 663.0 (M + K), 647.1 (M + Na).

Methods II and III. The coupling of commercially available TMP−morpholidate with **2** was accomplished using conditions previously described[5o] for the synthesis of GDP−Fuc, GDP−Man, and UDP−Gal, and the reaction of TDP with **3**[10] was accomplished using conditions previously described[5n] for the construction of GDP−Fuc, UDP−Ara, and UDP−Gal. In each case, the analysis of the reaction progress and final purification was accomplished as described for method I.

Preparation of the Gal-1-*P*T and RhaT$^{\alpha 3}$ Crude Extracts. PCR amplification of *wba*P (Gal-1-*P*T) and *wba*N (RhaT$^{\alpha 3}$) was accomplished from genomic template DNA isolated from *Salmonella* (group LT2) using flanking primers based upon the previously identified sequence.[14a−d] The amplified genes were cloned into T7-driven pET11a-BL21 systems and grown to an $OD_{600} = 0.55$ at 37 °C with shaking (250 rpm). The growth temperature was subsequently reduced to 20 °C, protein expression was induced with 1.0 mM IPTG, rifampicin (75 μg mL^{-1}) was added 2 h after induction, and the cultures were allowed to grow for an additional 12−14 h. Cells were harvested (2000*g*, 15 min), resuspended in 50 mM Tris-HCl and 1 mM EDTA, pH 8.5, and lysed by sonication. The cellular debris was removed by centrifugation (2000*g*, 15 min), and the membrane fractions were isolated from the supernatant by centrifugation (30000*g*, 1 h), resuspended in 50 mM Tris-HCl and 1 mM EDTA, pH 8.5, and stored at −80 °C until used. SDS-PAGE revealed the desired glycosyltransferases as approximately 70−80% of the total membrane associated protein.

Assay for the Production of 12. A mixture containing **6** (6 nmol, Sigma), 25 μL of Gal-1-*P*T crude membrane extracts, 25 μL of RhaT$^{\alpha 3}$ crude membrane extracts, 10 mM UDP−Gal, 10 mM **4**, 50 mM Tris-acetate (pH 8.5), 10 mM $MgCl_2$, 1 mM EDTA, 25 μL of ManT$^{\beta 4}$ crude membrane extracts,[10] and 0.33 μM GDP−[*U*-^{14}C]Man (15 000 cpm) in a final volume of 250 μL was allowed to react at 37 °C for 2 h and subsequently stopped by combining with 4 mL of $CHCl_3/CH_3OH$ (2:1). The organic phase was washed three times with 800 μL of pure solvent upper phase ($CHCl_3$/130 mM NaCl solution/CH_3OH, 3%:48%:49%), dried by evaporation, and dissolved in scintillant, and the extent of radioactivity recovered was determined by liquid scintillation counting.

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Supporting Information Available: ^{1}H, ^{13}C, and ^{31}P NMR spectra for **2** and **4** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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