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A very simple synthesis of GlcNAc-α-pyrophosphoryl-decanol: A substrate for the assay of a bacterial galactosyltransferase

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Abstract—Lipid-linked sugar pyrophosphates, such as GlcNAc-pyrophosphoryl undecaprenol, are important intermediates in the biosynthesis of cell-surface bacterial polysaccharides. It was recently demonstrated that much simpler lipids could substitute for undecaprenol while retaining biological activity, thus making efficient synthetic access to this class of compounds highly desirable. In order to facilitate the synthesis of pure substrates for bacterial glycosyltransferases, we have developed a simple 'two-pot' synthesis which we demonstrate here for GlcNAc-α-pyrophosphoryl-decanol (**4**). GlcNAc pyrophosphate, produced by mild periodate oxidation/β-elimination of commercial UDP-GlcNAc, is alkylated using 1-iododecane to yield the target compound **4** in 39% yield. Compound **4** is shown to be an efficient acceptor for a bacterial galactosyltransferase.

Sugar pyrophosphates are ubiquitous in nature where they are used as building blocks for the assembly of oligosaccharides and polysaccharides. The Leloir[1] sugar nucleotides, such as UDP-GlcNAc (**1**), have been the most intensively studied as they are critical to the biosynthesis of mammalian glycoconjugates. Numerous chemical syntheses have been reported for this class of compounds where the key step has been the coupling of two monophosphates to form the pyrophosphate linkage. As an example, GlcNAc-1-phosphate and uridine monophosphate (usually activated as either the morpholidate[2] or imidazolide[3]) are condensed to form UDP-GlcNAc (Fig. 1). In a few cases,[4] a nucleotide pyrophosphate has been used as the nucleophile in the displacement of an anomeric leaving group on the sugar. Despite being laborious, many analogs have also been prepared by these chemical methods.[5] More recently, the use of enzymes for both the anomeric phosphorylation of sugars and their condensation to form sugar nucleotides has become increasingly applied.[6]

Less synthetic attention has been paid to lipid-linked sugar pyrophosphates which can serve both as glycosyl acceptors and donors. Prominent examples include the oligosaccharyl dolicholpyrophosphates involved in the biosynthesis of N-linked glycans[7] and the sugar-pyrophosphoryl polyprenols involved in the assembly of bacterial cell-surface polysaccharides[8].

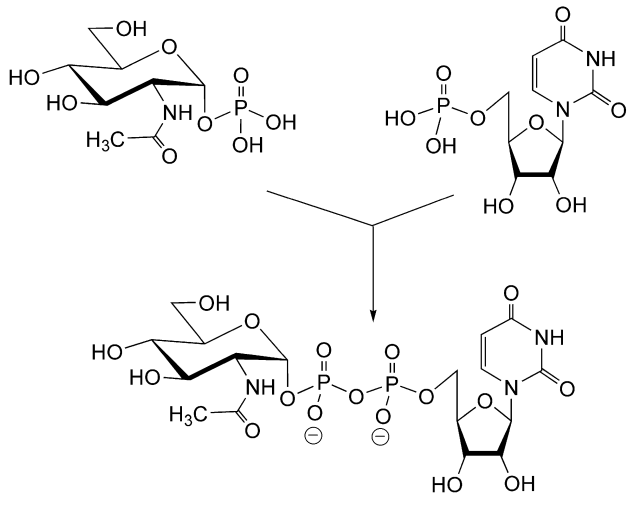

Figure 1. Traditional chemical synthesis of a sugar-pyrophosphoryl derivative: UDP-GlcNAc as example.

Keywords: Glycosyl acceptor; Galactosyltransferase; Sugar pyrophosphate; GlcNAc-pyrophosphoryl-lipid.
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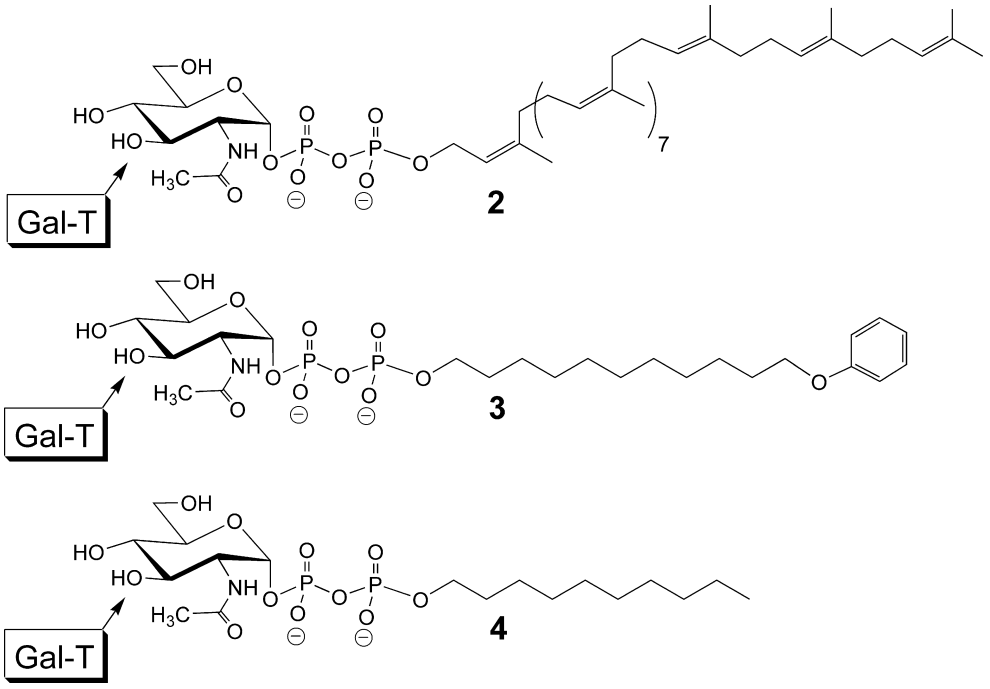


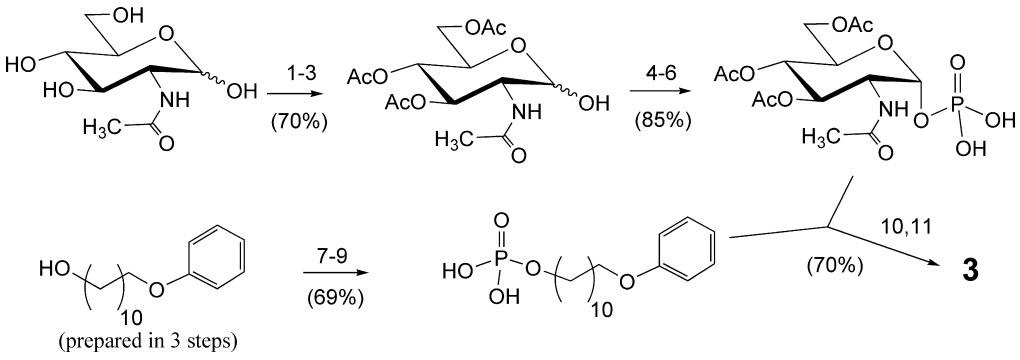
Figure 2. Reported acceptors for a bacterial galactosyltransferase and the synthetic target **4**.

Montoya-Peleaz et al.[9] recently reported the identification of a UDP-Gal: GlcNAc-R galactosyltransferase activity in *Escherichia coli* that used GlcNAc-α-pyrophosphoryl-undecaprenol (**2**) as the natural acceptor substrate for the second step in the synthesis of the O7 antigen repeat unit (Fig. 2). Since compound **2** is very difficult to access synthetically and from natural sources, they prepared compound **3** which was demonstrated to be an excellent acceptor for the target galactosyltransferase (Gal-T) that transfers to the 3-OH group as shown.[10] The simple lipophilic undecane group was therefore demonstrated to functionally substitute for the much more complex branched undecaprenol. The transfer reaction was quantitated using a radiochemical assay measuring the incorporation of [^{3}H]-labeled Gal, from UDP-[^{3}H]Gal, into lipophilic acceptor **3**. The terminal phenoxy group was included to permit UV-detection in HPLC. It was not reported if this group contributed to the observed enzyme activity. Yi et al.[11] later used the same synthetic strategy to prepare the corresponding GalNAc-α-pyrophosphoryl-lipid which was shown to be an acceptor for an α-GalNAc-transferase in a different *E. coli* strain. In their case, they found that the phenyl group at the end of an aliphatic chain of 11 carbons was not essential for activity.

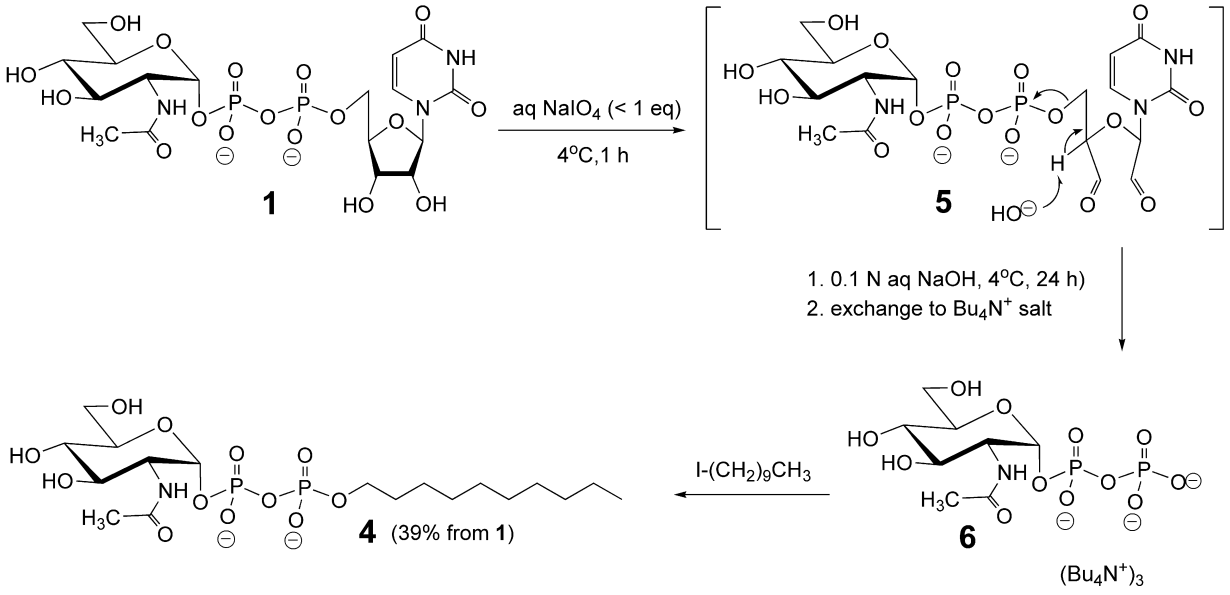
More GlcNAc-α-pyrophosphoryl-lipid acceptors were required to continue the studies of Montoya-Peleaz et al.,[9] and we contemplated repeating the reported synthesis of **3**. Though very efficient (Scheme 1), this would have required 11 reactions with multiple extensive purification steps without contributing new methodology to facilitate the preparation of other potential substrates with different sugars and different lipids. Such substrates would be of value in the assay of other glycosyltransferases involved in bacterial polysaccharide synthesis and utilizing sugar-pyrophosphate-lipids as natural substrates,[8,11] including the functional characterization of glycosyltransferases of many different bacterial strains that have until now been described as 'putative glycosyltransferase' based solely on DNA and protein sequence information.

Since Yi et al.[11] found that the phenoxy group in **3** was not essential for GalNAc-transferase activity, we decided to devise a simple rapid synthesis of a Gal-transferase acceptor functionally equivalent to **3**, that is, **4**, which would not involve either protecting group manipulations or coupling of phosphates to give the pyrophosphate linkage. The synthesis developed is shown in Scheme 2 and has two key steps. We reasoned that if we could provide ready access to GlcNAc-α-pyrophosphate (**6**), which is not commercially available, it could be alkylated using a long-chain alkyl halide (or tosylate) as demonstrated for other pyrophosphates by Poulter.[12] The final step in the synthesis would therefore be the conversion of **6** to the target **4** (Scheme 2). There remained to devise a simple preparation of **6** from a commercial starting material and for this we took advantage of the known preference of periodate to cleave cyclic *cis*-diols over cyclic *trans*-diols.[13] If UDP-GlcNAc (**1**) were the starting material, limited periodate oxidation should therefore produce the dialdehyde intermediate **5** (Scheme 2) which, by analogy with other nucleosides and nucleotides like ATP, should readily undergo β-elimination[14] to the desired **6**.

UDP-GlcNAc (**1**, ca. 1 mg/USD, from Sigma) was reacted in the cold with less than one equivalent of periodate to produce in situ the dialdehyde **5** formed by selective cleavage of the ribose ring. Addition of base then led to a β-elimination to directly produce **6**. After



Scheme 1. Reported synthesis of **3**, adapted from Ref. 9. Reagents: (1) BnBr, NaH, DMF; (2) Ac_2O/Pyr; (3) H_2, Pd/C; (4) $^{i}Pr_2NP(OBn)_2$, tetrazole; (5) ^{m}CPBA; (6) H_2, Pd/C; (7) $^{i}Pr_2NP(OBn)_2$, tetrazole; (8) ^{m}CPBA; (9) H_2, Pd/C; (10) carbonyldiimidazole; (11) NaOMe/MeOH.



Scheme 2. 'Two-pot' synthesis of target compound **4**.

ion exchange to produce the nucleophilically reactive tris-tetrabutylammonium salt, reaction with 1-iododecane gave the target **4**, which was purified by ion exchange and reverse-phase adsorption/elution from solid-phase extraction cartridges.[15] The overall yield from **1** to **4** was 39%. HPLC analysis of **4** using an analytical C18 column and acetonitrile/water (9:91) as the mobile phase at 1 mL/min showed a single peak eluting at 12 min with the expected mass ($M-H^+ = 520$). That the decyl and GlcNAc groups were attached to different phosphate groups was evident from the two-dimensional $^{1}H/^{31}P$ correlations in NMR.[15]

The activity of **4** as an acceptor substrate for WbbD, UDP-Gal: GlcNAc-R β3-galactosyltransferase, activity in *E. coli* VW187[10] was compared with that of known **3**. The new compound **4** was a very good acceptor for WbbD Gal-transferase.[16] At 0.5 mM concentration in the assay, an activity of 115 nmol/h/mg was obtained, corresponding to 70% of the activity obtained with **3**. The nano-electrospray-MS (negative ion mode) showed *m/z* of 520 for **4** and *m/z* of 682 for the enzyme product of WbbD, indicating the addition of Gal to **4**. This shows that the decyl chain as a lipid moiety in the acceptor is sufficient for sugar transfer, and that a phenyl group may enhance activity but is not essential.

In contrast, **3** was a poor substrate for bovine β4Gal-transferase. At 0.5 mM concentration in the assay, **3** showed only 3% of the activity obtained with 0.5 mM GlcNAcβ-Bn substrate. This indicates that the bacterial and mammalian Gal-transferases differ significantly in binding to their acceptor substrates although they both bind GlcNAc-R and UDP-Gal, and require Mn^{2+} as a cofactor. Thus, in contrast to the bacterial Gal-transferase, the mammalian enzyme cannot easily accommodate the pyrophosphate group in the acceptor binding site and has a distinct specificity for GlcNAc in β-configuration.[17]

In summary, we have reported a very simple synthesis of target compound **4** which was demonstrated to be an acceptor for a bacterial galactosyltransferase. The procedure involved the facile generation of GlcNAc-pyrophosphate **6** from UDP-GlcNAc and its subsequent alkylation by 1-iododecane. Since a large number of pri-

mary alkyl halides and tosylates (or their precursor alcohols), as well as nucleotide sugars, are commercially available, this method should provide rapid access to a panel of lipid-linked sugar pyrophosphates for the functional characterization of bacterial glycosyltransferases. The key step of the synthesis relies on the facile periodate oxidation of the *cis*-diol of a ribofuranose over the *trans*-diol of a glucopyranose. Beyond this selectivity, we have not yet defined the scope and limitations of the method, in particular whether it can be extended to sugar nucleotides containing pyranoses with *cis*-diols, such as UDP-Gal and GDP-Man.

Acknowledgment

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15. To a solution of UDP-GlcNAc di-sodium salt (**1**, 50 mg, 77 μmol), at 4 °C, was added $NaIO_4$ (402 μL of a 0.187 M solution, 0.98 equiv) and the reaction tube was vortexed for 1 min and then placed at 4 °C for 1 h. The reaction was quenched by the addition of glycerol (20 μL). After 15 min, 1 M aq NaOH (80 μL) was added, the sample vortexed for 1 min, and placed at 4 °C for 24 h.
The reaction mixture was diluted to 10 mL with cold H_2O and applied to a column of Dowex-H^+ (4 × 2 cm id) and eluted with cold H_2O (80 mL) into a beaker containing 1 M tetrabutylammonium hydroxide (305 μL) in H_2O (10 mL). The pH of the resulting solution was found to be ca. 6–7 and was increased up to ca.7–8 by addition of 1 M tetrabutylammonium hydroxide (40 μL). The solution was lyophilized, re-dissolved in H_2O (1.5 mL), transferred to a microfuge tube, and re-lyophilized. The brownish oily residue was again re-dissolved in dry acetonitrile (300 μL) and split into three 100 μL portions.
To one of the 100 μL portions was added 1-iododecane, (13.6 μL, 64 μmol, 2.5 equiv) and the reaction tube was vortexed for 1 min and placed on a shaker-table. After 48 h the reaction was diluted with H_2O (1 mL), then loaded onto a 10 × 0.5 cm column of Dowex-50 (NH_4^+ form). The column was washed with H_2O. A total of 50 mL was collected, and analyzed by ESI-MS which showed **4** as the major product. In addition, the mass spectra verified that all tetrabutylammonium salts had been removed which otherwise hampered the following separation. The water fraction was lyophilized, re-dissolved in H_2O (3 mL), and added onto 2 preactivated C18 Sep-Pak cartridges connected in series. After the pass-through, the cartridges were washed and fractions (1.5 mL) were collected: H_2O × 7, 5% aq MeOH × 7, 10% aq MeOH × 6. The fractions were analyzed by ESI-MS and the pure product was present from fraction 4 of the H_2O washes to fraction 3 of 10% MeOH washes, which were pooled and concentrated. The residue was re-dissolved in H_2O (1.5 mL) and lyophilized in a microfuge tube to yield **4** (5.18 mg, 9.93 μmol, 39%). ESI-MS; $[M - H^+]^-$: expected: 520.26 found: 520.0; selected NMR data supporting the structural assignment of 4: ^1H NMR (250 MHz, D_2O) δ 5.45 (H_1, dd, $J_{1,2}$ = 3.2,$J_{1,P}$ = 7.2 Hz), 2.04 (s, $NCOCH_3$), 0.82 (t, decane-CH_3); ^{13}C: δ 94.5 (C_1): ^{31}P: δ −10.7 (P_2, P-O-decane), −13.2 (P_1, GlcNAc-O-P); $^1H/^{31}P$ HMBC: 5.45/−13.2 (GlcNAc-H_1/P_2), 3.94/−13.2 (GlcNAc-H_2/P_2), 3.89/−10.7 (decane-CH_2/P_2).
16. Bacterial cultures of *Escherichia coli* VW187 (O7:K1) were grown and used as the enzyme source in Gal-transferase assays as described.[9,10] Enzyme products using acceptor **3** or newly synthesized acceptor **4** were isolated by C18 Sep-Pak columns. Mammalian β4-Gal-transferase I (lactose synthase, Sigma) was assayed as described.[17]
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