Synthetic Methods

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A Simple Synthesis of Sugar Nucleoside Diphosphates by Chemical Coupling in Water**

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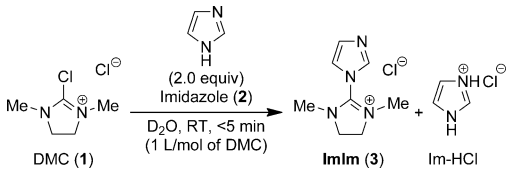
The use of glycosyltransferases in chemoenzymatic oligosaccharide synthesis is attractive, provided that the enzymes are available, since it eliminates the tedious multistep protection-deprotection and chemical glycosylation procedure that characterizes classic chemical synthesis.[1,2] The sugar nucleoside diphosphates (sugar-NDPs) that most glycosyltransferases require as glycosyl donors (e.g. UDP-Glc, UDP-Gal, ADP-Glc etc.) can be prepared either chemically or through enzymatic methods including recycling systems. The enzymatic preparation of sugar-NDPs has the advantages of being simple and compatible with glycosyltransferase reactions but requires access to the necessary enzymes. In contrast, chemical synthesis is more complex but is especially powerful for the preparation of analogues that cannot easily be prepared using enzymes owing to a low tolerance of the enzyme for substrate modifications.

Chemical methods for the synthesis of sugar-NDPs have recently been comprehensively reviewed and eloquently discussed.[3] The most commonly used procedures involve the formation of the pyrophosphate linkage by coupling of an activated nucleoside-5′-monophosphate (NMP, typically a morpholidate or imidazolide) with the sugar-1-phosphate; in these reactions the addition of catalysts like nitrogen-containing heterocycles can effect substantial improvements in both rate and yield.[4,5] Newer methods include the "*cyclo*Sal" phosphotriester coupling[6] and mixed phosphite–phosphate formation followed by oxidation.[7] Direct coupling of anomerically activated monosaccharides with nucleoside diphosphates, such as uridine 5′-diphosphate, represents a useful alternative approach (reviewed in reference [3]). These chemical procedures are normally performed in anhydrous organic solvents and usually employ per-*O*-acetylated sugars and phosphates, such as their tetraalkylammonium salts, that are soluble in organic solvents. The syntheses are notoriously difficult to reproduce from laboratory to laboratory, especially when researchers have little previous experience with chemical sugar nucleotide synthesis.

We report herein a simple method for the chemical synthesis of sugar-NDPs in a one-pot reaction in water. The procedure involves only the sequential addition of commercially available compounds, and the crude product solution can be used directly as a glycosyl donor source in glycosyltransferase-mediated oligosaccharide synthesis.

We found that the reaction of 2-chloro-1,3-dimethylimidazolinium chloride (DMC, **1**)[8,9] with imidazole (**2**) in D_2O at room temperature gave 2-imidazolyl-1,3-dimethylimidazolinium chloride (ImIm, **3**), within five minutes, along with imidazole hydrochloride (Im-HCl; Scheme 1). The formation of **3** was confirmed by ^{1}H NMR spectroscopy and ESI–MS.[10] We then examined whether **3** would convert phosphate groups into reactive phosphorimidazolide groups in aqueous solution. Phosphorimidazolides have been extensively used for pyrophosphate bond formation in anhydrous organic solvents.[3]



Scheme 1. Reaction of DMC (**1**) with imidazole (**2**) gives ImIm (**3**) as a reactive intermediate.

Uridine monophosphate (UMP) disodium salt (**4**), DMC (**1**), and imidazole (**2**) were dissolved in D_2O at 37 °C, and the reaction was monitored by ^{1}H NMR spectroscopy (Figure 1).

The signal corresponding to H-5 of uracil in **4** ($\delta =$ 8.01 ppm) decreased and simultaneously a peak assigned to H-5 of UMP-imidazolide (UMP-Im, **5**; $\delta = 7.62$ ppm) increased for up to one hour where the maximum conversion to **5** was approximately 70 %. The reaction was also monitored using ^{31}P NMR spectroscopy, where the intensity of the characteristic[5,11] signal for UMP-imidazolide **5** (UMP-Im, $\delta = -8.2$ ppm) was observed. ESI–MS on the crude reaction mixture further confirmed the formation of UMP-Im (m/z 373.3; see the Supporting Information). An identical mass spectrum was obtained if DMC and imidazole were first reacted for five minutes and subsequently UMP was added.

Reaction times longer than one hour led to hydrolysis of **5** and subsequent increased dimerization (**4** + **5**) to give UMP-dimer (**7**; Figure 1). If the reaction was allowed to proceed for 16 h, only hydrolysis product (**5**→**4**) and dimer **7** were seen: at that stage the reactive species **3** and **5** were fully hydrolyzed, thereby rendering the solution chemically benign.

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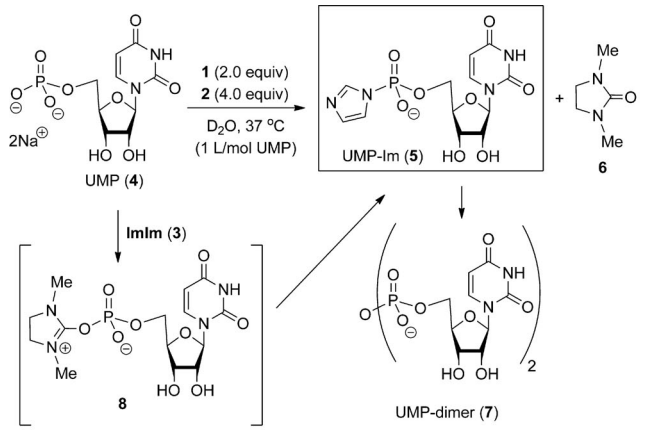


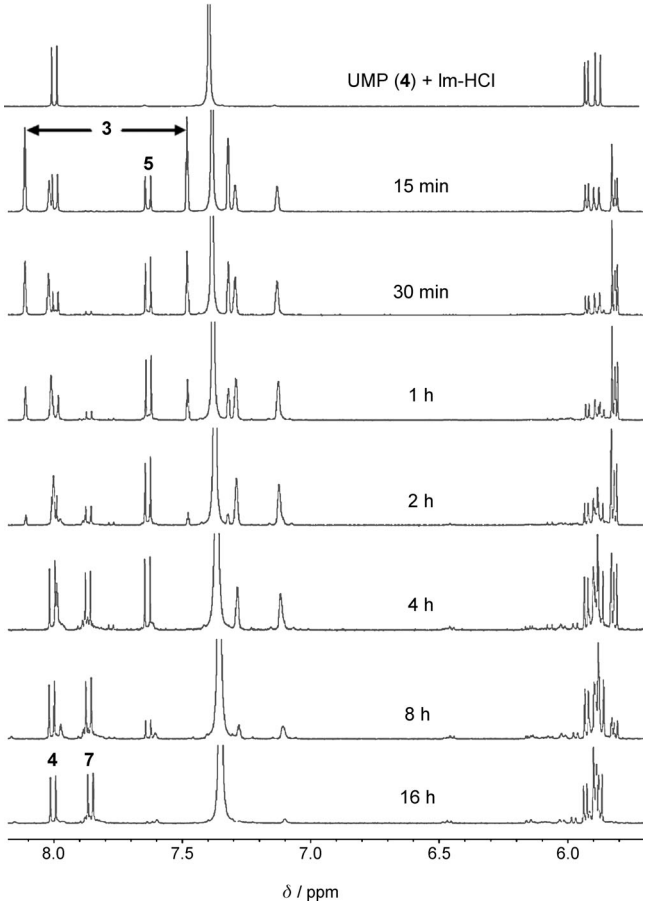


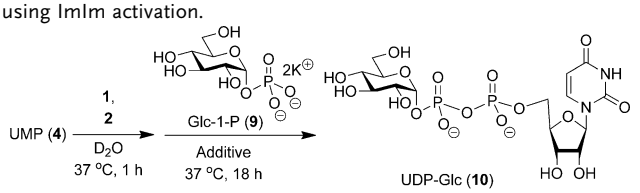
Figure 1. Activation of UMP (**4**) in D_2O with ImIm monitored by ^1H NMR spectroscopy.

A plausible reaction mechanism is presented in Figure 1, where the rapidly formed ImIm activates the phosphate via an intermediate isourea that is then captured by imidazole to produce UMP-Im and urea **6**. In an independent experiment where DMC alone was incubated with UMP for 24 h at 37 °C, the only products observed were urea **6** (40%) and UMP-dimer (<5%) along with 60% of unreacted DMC and more than 95% unreacted UMP (see the Supporting Information).

The reactions described above were optimized in D_2O, because its use facilitates reaction monitoring by ^1H NMR spectroscopy. However, activation of **4** was also examined in H_2O, where the yield of UMP-Im (**5**) was consistently lower, only 60–70% of that obtained in D_2O. Both **3** and **5** were hydrolyzed much faster in H_2O than in D_2O (verified by kinetic NMR monitoring, see the Supporting Information); this result is attributed to the known solvent isotope effect of water in hydrolysis reactions.[12] The use of D_2O is therefore advantageous in preserving a high concentration of the active species (**3** and **5**) in aqueous solution.

We next examined conditions for coupling the in situ formed UMP-Im (**5**) with glucose-1-phosphate (Glc-1-P, **9**) to give UDP-glucose (UDP-Glc, **10**) as the target sugar-NDP. The products were quantitated by ^{31}P NMR spectroscopy by using the beneficial D_2O as solvent (Table 1). The activation step to produce **5** (**1** + **2** + **4**) was run for one hour (at which time the majority of the ImIm (**3**) was consumed) before adding Glc-1-P (dipotassium salt) **9**. It is important to minimize the activation of Glc-1-P by unhydrolyzed ImIm, because this activation leads to the formation of glucose-1,2-cyclic-phosphate. The rate of formation of UMP-Im (**5**) is, however, dependent on the concentration of ImIm, so a compromise in reaction time and corresponding yield must be made to provide the highest possible concentration of reactive **5** for coupling to Glc-1-P.

Table 1: Optimization of reaction conditions for UDP-Glc synthesis using ImIm activation.



Entry	D_2O [L mol^{-1} of UMP]	Ratio (**4**/**1**/**2**/**9**)	Additive	Yield [%][a]
1	1.0	1:2:4:1	none	20
2	1.0	1:2:4:1	TEAC	23
3	1.0	1:2:4:1	[15]crown-5	21
4	1.0	2:4:8:1	none	34
5	0.5	2:4:8:1	none	43
6	0.5	4:8:16:1	none	57
7	0.5	4:8:16:1	none	43[b]

[a] Yields of **10** based on Glc-1-P and calculated from the corresponding signal integrations in the ^{31}P NMR spectrum of the crude mixture.
[b] Yield of isolated **10**, which was purified by ion-exchange chromatography on DEAE-Sephacel after treatment with alkaline phosphatase for 24 h at 30 °C; the yield is based on Glc-1-P and was determined by UV spectrosopy at λ_{max} 260 nm.

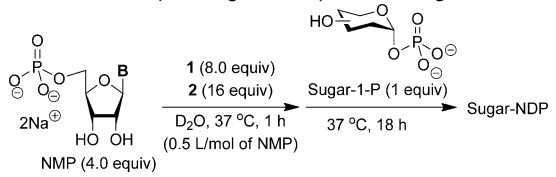
Coupling reactions were examined under a variety of conditions at 37 °C for 18 h (Table 1). Addition of tetraethylammonium chloride (TEAC) or [15]-crown-5 was not beneficial (Table 1, entries 1–3). Furthermore, use of dimethyl sulfoxide (DMSO) as a co-solvent led to a decrease in coupling yield (data not shown). An increase in the concentration of **5** would be predicted to improve the efficiency of coupling with **9**. Indeed, higher yields were obtained when the amounts of **1**, **2**, and **4** were increased and the volume of D_2O

decreased (Table 1, entries 4–6). The highest yield of crude product **10** was 57%, estimated by NMR spectroscopy (Table 1, entry 6). To facilitate isolation of **10**, we used alkaline phosphatase to cleave the phosphate group in unreacted **4** and then purified the product by ion exchange chromatography on DEAE-Sephacel leading to isolation of **10** in 43% yield (Table 1, entry 7).

The scope of the reaction with respect to different NMPs and sugar-1-phosphates was briefly explored as summarized in Table 2. The optimized conditions[13] led to the formation of UDP-Gal in a reasonable yield (59%). Although adenosine monophosphate (AMP) possesses a free amino group available for potential reaction, ADP-Glc was successfully obtained in a 41% yield. However, guanosine monophosphate (GMP) has limited solubility in water and required a larger volume of D_2O (1 L/mol of GMP) resulting in a diminished yield. To activate GMP efficiently, an increase in both the amount of **1** and **2** and in the time for the activation step resulted in the formation of GDP-Man in a yield close to 50% (23% isolated).

One objective of this work was to provide a method for the sugar-NDP preparation that could be performed without any skill in organic synthesis, thus eliminating the need for sugar protection and anhydrous organic solvents while employing only commercially available reagents. However, as alluded to above, the second objective was that the final sugar-NDP be present in a chemically benign solution that could directly serve as the source of donor in a glycosyltransferase-catalyzed synthesis of oligosaccharides.

Table 2: Substrate scope in sugar-NDP synthesis using ImIm activation.

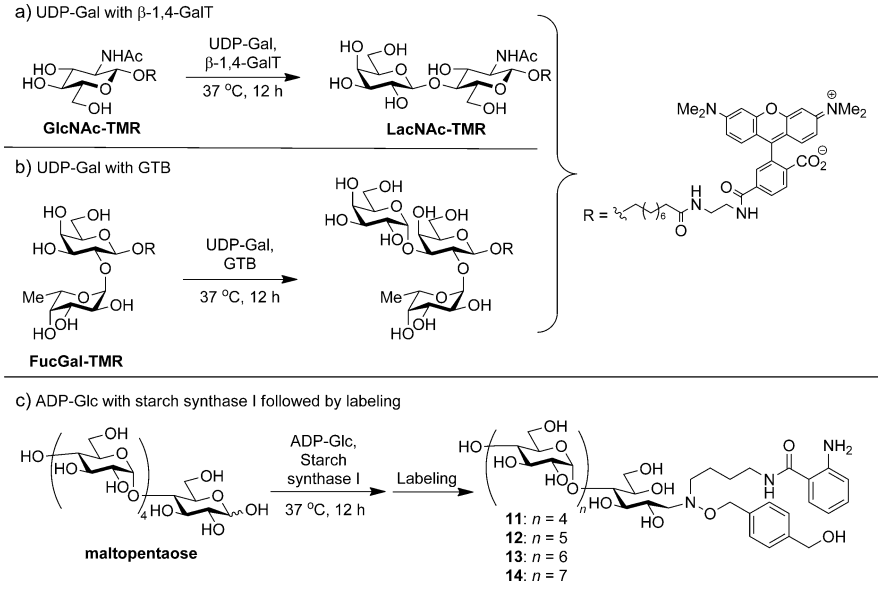


Entry	NMP	Sugar-1-P	NDP-Sugar	Yield [%][b,c]
1	UMP (**4**)	Gal-1-P	UDP-Gal	59 (35)
2	AMP	Glc-1-P	ADP-Glc	41 (32)
3[a]	GMP	Man-1-P	GDP-Man	< 50 (23)

[a] DMC (16 equiv), imidazole (32 equiv), and D_2O (1 L mol^{-1} of GMP) were used and the activation step was performed for 2 h. [b] Yields of the coupling product were based on sugar-1-P and calculated from the corresponding signal integrations in the ^{31}P NMR spectrum of the crude mixture. [c] In parentheses, the yields of isolated coupling products are given, which were purified by ion-exchange chromatography on DEAE-Sephacel after alkaline phosphatase treatment for 24 h at 30°C; yields were based on sugar-1-P and determined by UV spectroscopy at λ_{max} 260 nm (uracil and adenine) and λ_{max} 252 nm (guanine). In the scheme, **B** stands for the nucleobases U, A, or G.

To this end, the unprocessed reaction mixtures containing UDP-Gal (Table 2, entry 1) and ADP-Glc (Table 2, entry 2), were evaluated as donors for two galactosyltransferases and one glucosyltransferase, respectively (Scheme 2). The mixture containing crude UDP-Gal synthesized by the present method was added to either bovine β-1,4-galactosyltransferase[14] (an inverting galactosyltransferase) or human-blood-group-B galactosyltransferase[15] (a retaining galactosyltransferase, GTB) in the presence of their known glycosyl acceptors GlcNAc-TMR[16] and FucGal-TMR,[17] respectively. After incubation for 12 h, both galactosyltransferase incubations resulted in complete conversion of the acceptor to the galactosylated product when monitored by MALDI-TOF MS and capillary electrophoresis (see the Supporting Information). When compared to using pure commercial UDP-Gal, the synthetic mixture gave a 2–4 times slower reaction in the initial rate (see the Supporting Information). This result was attributed to inhibition of the enzymes by both UMP and UMP-dimer (**7**), which had inhibition constants (K_i) in the same range as the Michaelis–Menten constant (K_m) for UDP-Gal.[18] However, the synthetic UDP-Gal mixture remained completely effective for milligram-scale product synthesis in an overnight incubation.

The crude product mixture containing synthetic ADP-Glc (Table 2, entry 2) was incubated with recombinant barley starch synthase I in the presence of maltopentaose as an acceptor (Scheme 2c). After the incubation, the resultant mixture was labeled with a fluorescent tag at the reducing end.[19] Ultra-performance liquid chromatography (UPLC)–MS analysis revealed that 1, 2, or 3 glucose residues had been transferred to the acceptor, thereby giving maltooligosaccharides **12**–**14** as products. In this case, inhibition of the enzyme was not observed: in fact the reaction proceeded more rapidly than with commercial ADP-Glc (see the Supporting Information).



Scheme 2. Enzymatic reactions: a) UDP-Gal with bovine β-1,4-galactosyltransferase (β-1,4-GalT); b) UDP-Gal with GTB; c) ADP-Glc with barley starch synthase I followed by fluorescent labeling.

In conclusion, the new ImIm reagent formed in situ can activate nucleoside 5′-monophosphates in D_2O to give a reactive phosphorimidazolide intermediate. Coupling of this intermediate with sugar-1-phosphates in D_2O produced NDP-sugars in useful yields based on the sugar-1-phosphate as the limiting reagent. All reactions occur in one pot, and the procedure involves only the timed sequential addition of commercial chemicals. Furthermore, all chemically reactive species are hydrolyzed by the end of the coupling reaction, thus the crude NDP-sugar solutions can be used directly without any purification for preparative oligosaccharide synthesis by glycosyltransferases.

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