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SHORT COMMUNICATION

One-pot synthesis of nucleotides and conjugates in aqueous medium

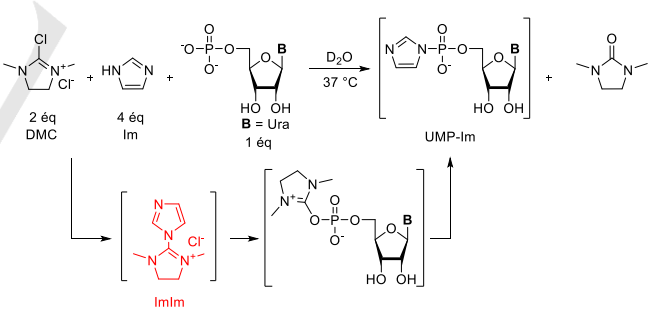
Anaïs Depaix,[a] Suzanne Peyrottes,[a] and Béatrice Roy*[a]

Abstract: Herein, we describe a one-pot synthesis, in a mixture of water-acetonitrile, of nucleoside 5'-polyphosphates and some derivatives starting from their corresponding nucleoside 5'-monophosphates. Phosphorimidazolide intermediates are formed in the presence of imidazole and 2-chloro-1,3-dimethylimidazolinium hexafluorophosphate. Under these mild conditions, nucleoside 5'-di- and 5'-triphosphates, dinucleoside 5',5'-polyphosphates as well as some nucleotide analogues modified either on the nucleoside or on the phosphate moieties, were obtained in moderate to high yields.

Introduction

Given the importance of nucleotides and their derivatives in biological processes, numerous methods have been developed to access to these compounds and their structural analogues.[1] Their scope ranges from mechanistic probes to versatile chemical tools for assay development and biomedical applications. As example, nucleoside 5'-triphosphates are the cornerstone of genome analysis and medical diagnostics.[2] Di(uridine)-tetraphosphate (Up_4U, diquafosol) is an agonist of the P2Y2 purinergic receptor, used to treat dry eye disease.[3] Dinucleotides also appear to be a promising new class of antiplatelet agents.[4] Moreover, a large number of nucleotide analogues and their conjugates have been synthesized and studied as inhibitors/promoters of therapeutically relevant enzymes.[5]

Methods for the chemical synthesis of these derivatives are based either on P(III) or P(V) chemistry.[1] Phosphoramidates, in general, and phosphorimidazolides in particular, have been extensively used as intermediates for pyrophosphate bond formation in anhydrous organic solvents.[6] This approach is based on the nucleophilic substitution of nucleoside 5'-monophosphates (NMPs) activated as phosphoramidate monoesters, by suitable inorganic phosphate salts. The products obtained are nucleoside 5'-diphosphates (NDPs),[7] nucleoside 5'-triphosphates (NTPs),[8] dinucleoside 5',5'-polyphosphates (DNPs),[9] and conjugates such as NDP-sugars[10] and CDP-choline.[11] Nucleoside phosphorimidazolide intermediates can be obtained by the reaction of NMPs with 1,1'-carbonyldiimidazole (CDI). These reactions are usually performed in anhydrous organic solvents, thus requiring the preparation of tri- or tetra-alkyl ammonium salts of both the NMP and the phosphate reagent. In 2012, Tanaka et al. have made a breakthrough in the chemical synthesis of NDP-sugars by performing the coupling of a sugar-1-phosphate with a nucleotide in D_2O.[12] The key reagent for the synthesis is 2-imidazolyl-1,3-dimethylimidazolinium chloride (ImIm), formed in situ (Scheme 1), by mixing 2-chloro-1,3-dimethylimidazolinium chloride (DMC) and imidazole (Im). ImIm can activate a NMP into its corresponding phosphoroimidazolide (NMP-Im), which is then converted to the desired sugar nucleotide by reaction with the appropriate sugar-1-phosphate salt. Interestingly, phosphorylated reagents are used as their commercially available and water-soluble sodium and potassium salts. However, only 6–10 % of NMP introduced in the reaction ends up in the final NDP-sugar due to the use of 4 equiv. of NMP for 1 equiv. of sugar-1-phosphate and low yields. Finally, this protocol allows to obtain only a few milligrams of the desired compounds. In 2014, the Fiedler research group reported a pyrophosphorylation method of peptides in water by reacting a peptide monophosphate with *O*-benzylphosphorimidazolide in the presence of zinc chloride. [13]



Scheme 1. Proposed activation mechanism of UMP to UMP-Im by ImIm. [12]

Our main objectives were to improve Tanaka's conditions[12] and to expand this approach to the synthesis of various families of nucleotides, while using H_2O instead of D_2O. Consequently, our protocol is cheap and suitable for large scale synthesis.

Results and Discussion

At the outset, activation of several NMPs (UMP, CMP, AMP) was performed following the reported conditions (NMP/DMC/Im 1/2/4 in D_2O at 40 °C).[12] The reaction progress was monitored by ^{1}H-decoupled ^{31}P NMR spectroscopy which gives two signals at approximately 2 and –8 ppm corresponding to NMP and NMP-Im, respectively. Activation was incomplete after 1 h and the

[a] Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université de Montpellier, ENSCM, Université de Montpellier, Campus Triolet, cc 1705, Place Eugène Bataillon, 34095 Montpellier cedex 5, France
E-mail: beatrice.roy@umontpellier.fr
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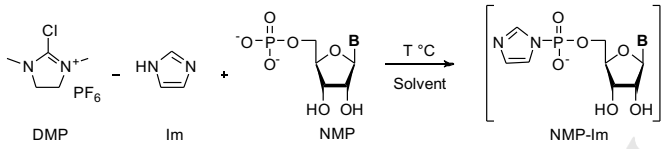
formation of symmetrical dinucleoside diphosphate Np_2N as a by-product was observed (singlet at around –11 ppm, see supplementary information). Indeed, the reported conversion of UMP to UMP-Im in D_2O was 70 %, while it was only 40–50 % in H_2O. This difference may be attributed to the reduced nucleophilicity of D_2O compared to H_2O in hydrolysis reaction.[12,14]

As 2-chloro-1,3-dimethylimidazolinium chloride is highly hygroscopic and therefore difficult to handle, we replaced it with its hexafluorophosphate salt (DMP, Scheme 2). This last was obtained by treatment of DMC with KPF_6 in acetonitrile.[15] The counter-ion exchange resulted in a lower solubility of the reagent in water, which was overcome by the addition of an organic co-solvent (CH_3CN or THF). In addition, we hypothesized that this co-solvent would also reduce the extent of NMP-Im hydrolysis, and therefore prevent dimer formation. Secondly, cost-effective D_2O was replaced by H_2O thus allowing to increase the reaction scale. Therefore, activation with UMP was optimized by varying the solvent mixtures, temperature, as well as the relative ratio of UMP/DMP/Im (Scheme 2 and supplementary information).



Scheme 2. Optimization settings of the activation conditions.

The activation step was almost complete after 1h when using 1 equiv. of UMP, 3 equiv. of DMP and 6 equiv. of Im in H_2O/CH_3CN (1/1, v:v) at 40 °C. As shown in Figure 1, the ^{31}P NMR spectrum of the crude reaction mixture shows a signal at –8 ppm (UMP-Im), and no dimer is observed. Under the same experimental conditions, the activation was also almost complete for CMP and AMP (see supplementary information).

With these optimized reaction conditions in hand, we then examined the scope of this reaction for the preparation of various natural nucleotides (NDPs, NTPs). For this purpose, a 1–3 equiv. of the suitable and commercially available phosphate salts were added to the NMP-Im and allowed to react for 2 to 17 h (Scheme 3). We found that 30 min activation was better than 1 h to minimize the formation of by-products (symmetrical dinucleoside diphosphates, NMP). Subsequently, addition of the phosphate reagent was done after 30 min.

Nucleoside 5'-di- and 5'-triphosphates were obtained by using 1 equiv. of NaH_2PO_4 and 3 equiv. of $Na_4P_2O_6$, respectively. Crude products were purified by ion-exchange chromatography followed, in some cases, by a size exclusion chromatography to remove the $HNEt_3.PF_6$ salts. Conversion ranged from 35 to 49 %, and the compounds were isolated in 22–40 % yields (Table 1, entries 1–6). For NTP synthesis, we also replaced tetrasodium pyrophosphate by PPN pyrophosphate.[16] However, the latter was poorly soluble in the reaction medium, resulting in lower yields (data not shown).

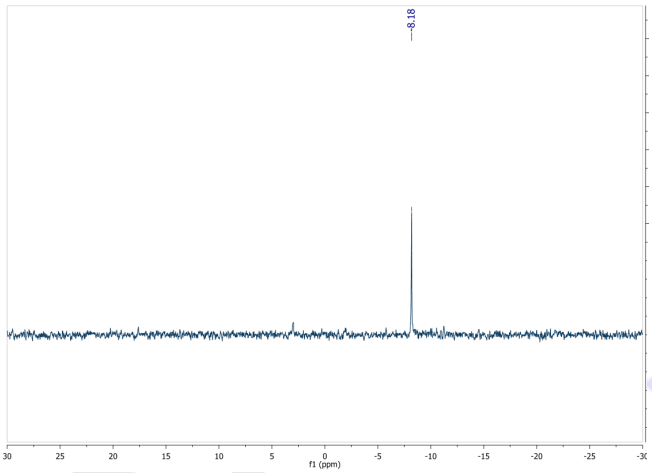


Figure 1. ^{31}P NMR (121 MHz, D_2O) after 1h of activation of UMP under the optimized conditions (UMP/DMP/Im 1/3/6 in H_2O/CH_3CN 1/1).

Then, we extended this approach to the synthesis of a nucleotide conjugate (CDP-choline) and various analogues modified either on the nucleoside or the phosphate moieties (Scheme 3). Thus, treatment of CDP-Im with choline phosphate (CholP) afforded CDP-choline in 34% isolated yield (Table 1, entry 7). Concerning analogues, two cases were considered: (i) synthesis of 5'-di- and 5'-triphosphates starting from a sugar-modified nucleoside 5'-monophosphate and (ii) synthesis of a β,γ-methylene NTP starting from a natural NMP.

As regards the nucleoside analogues (NAs), phosphorylation in cells is achieved by cellular or viral kinases.[5] The mechanism of action of antiviral or anticancer NAs involves the interaction of their 5'-phosphorylated derivatives with essential enzymes, such as DNA or RNA polymerases.[5] For example, 3'-azido-2',3'-deoxythymidine 5'-triphosphate (AZTTP) interact with HIV reverse transcriptase, leading to the inhibition of viral replication. Therefore, the availability of nucleotide analogues is required for biochemical and pharmacological studies. Using our optimized conditions, AZTMP was converted into AZT 5'-diphosphate (AZTDP) and 5'- triphosphate (AZTTP) in 49 % and 32 % yields, respectively (Scheme 3 and Table 1, entries 8–9). Secondly, we were interested in the synthesis of β,γ-methylene NTPs. Indeed, these non-hydrolysable compounds that are isosteric and isoelectronic with NTPs, are used to probe the mechanism of phosphoryl transfer in enzyme-catalysed processes[17] and receptors specificity.[18] Applying our procedure, UMP was converted into uridine 5'-β,γ-methylene triphosphate ($UDPCH_2P$) in 48 % yield using 1 equiv. of tetrasodium methylene diphosphonate as a reagent (Table 1, entry 10).

We also probed the ability of DMP/Im to activate inorganic phosphate or pyrophosphate. Indeed, Yanachkov at al. have reported the formation of P^1,P^2-diimidazolyl derivatives of pyrophosphate by treatment of the latter with CDI in DMF.[19] Under our optimized conditions (1 equiv. phosphate or pyrophosphate, 3 equiv. DMP, 6 equiv. Im), only minor products were observed by ^{31}P NMR monitoring (data not shown).

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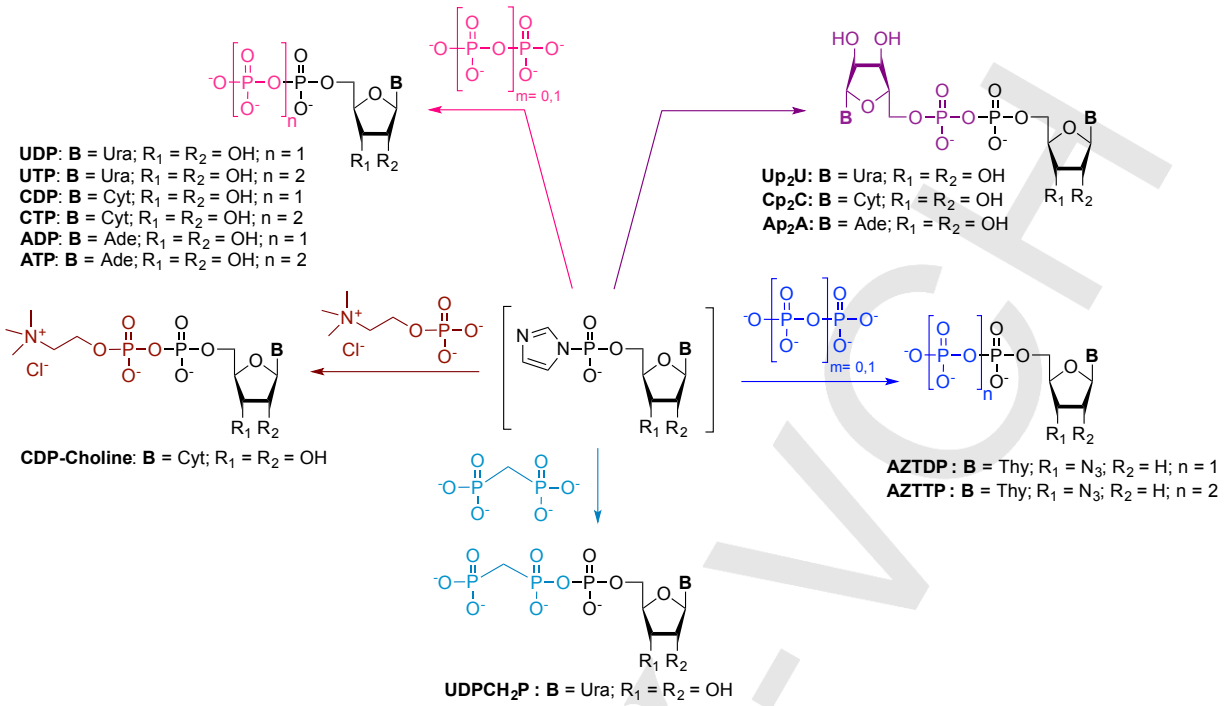

Scheme 3. Study of the scope of the reaction.

Finally, we took advantage of the formation of the dimers (Np_2N) as by-products in the activation step to isolate these compounds. Therefore, activation of several NMPs (UMP, CMP or AMP) was performed in one-pot without adding any other reagent than DMP and Im, until the ^{31}P NMR signal of NMP-Im disappeared. Conversion ranged from 31 to 70 % (Table 1, entries 11–13).

Compared to the existing methods in anhydrous media based on phosphorimidazolate intermediates,[1] our protocol has several advantages. While the first ones require technical expertise in handling under an inert atmosphere, the second does not require any particular skills to perform the chemical reactions and phosphorus reagents are used in their commercially available forms. Moreover, our protocol prevent carbonation of ribonucleotides usually observed with the use of CDI.[7] Our procedure applied for NDPs and NTPs gives yields in the same order of magnitude as anhydrous procedures[1, 7–9, 20–21] whereas better yields are reported in the literature for CDP-choline[11] and $UDPCH_2P$.[22] Last but not least, ~ 30–60 % of NMP introduced in the reaction ends up in the final product. This is a great advantage when it comes to the synthesis of nucleotide analogues starting from high-added value NMPs.

Conclusions

The attractive features of this one-pot strategy include absence of protecting groups on the starting material and convenient set-up (i.e. use of water, non-dry solvent and reagents, commercially available sodium or potassium salts). The experimental results demonstrated the applicability of the reported method for the synthesis of a variety of nucleotides. Finally, this protocol allows to synthesize hundreds of milligrams of the desired product.

Thus, our synthetic strategy provides a general and simple access to nucleotides, their conjugates as well as some of their analogues.

Entry	NMP[a]	Phosphorus reagent	Product	Yield [%]
1	UMP	NaH_2PO_4	UDP	47[b], 40[c]
2	UMP	$Na_4P_2O_7$	UTP	43[b],25[c]
3	CMP	NaH_2PO_4	CDP	42[b], 24[c]
4	CMP	$Na_4P_2O_7$	CTP	49[b], 24[c]
5	AMP	NaH_2PO_4	ADP	35[b], 27[c]
6	AMP	$Na_4P_2O_7$	ATP	43[b], 22[c]
7	CMP	CaCholP	CDP-Choline	34[c]
8	AZTMP	NaH_2PO_4	AZTDP	49[b]
9	AZTMP	$Na_4P_2O_7$	AZTTP	32[b]
10	UMP	$Na_4CH_2P_2O_6$	$UDPCH_2P$	48[b]
11	UMP	none	Up_2U	70[b], 68 [c]
12	CMP	none	Cp_2C	31[b]
13	AMP	none	Ap_2A	50[b], 39[c]

[a] Used as their commercially available disodium salts. [b] Conversion rate based on the corresponding NMP and calculated from signal integrations in the ^1H NMR or ^{31}P spectrum of the crude reaction mixture. [c] Isolated yields.

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Experimental Section

2-chloro-1,3-dimethylimidazolinium hexafluorophosphate (DMP) was synthesized according to Kitamura. [15] General procedure for nucleotide synthesis: NMP disodium salt (0.41 mmol) was placed in a small test tube with a stir bar, and dissolved in H_2O/CH_3CN 1/1 (1080 µL). The solution was heated to 40 °C and then DMP (1.23 mmol, 3 equiv.) and imidazole (2.45 mmol, 6 equiv.) were added. In the case of addition of a phosphorus reagent, it was added after 30 min, and the reaction was further stirred from 2 to 17 h. When suitable, additional solvent was added for a better solubility (supplementary information). At the end of the reaction, the crude reaction mixture was freeze-dried and purified by ion exchange chromatography on diethylaminoethyl (DEAE) Sephadex, using a gradient of triethylammonium hydrogen carbonate buffer (TEAB) at pH = 7.5. For some of them, the excess of hexafluorophosphate triethylammonium salts was removed by a supplementary size exclusion chromatography, performed on Bio-Gel P-2 Gel, fine (Bio-Rad laboratories) and using water as eluent.

Acknowledgements

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Keywords: nucleotides • phosphorylation • water chemistry •

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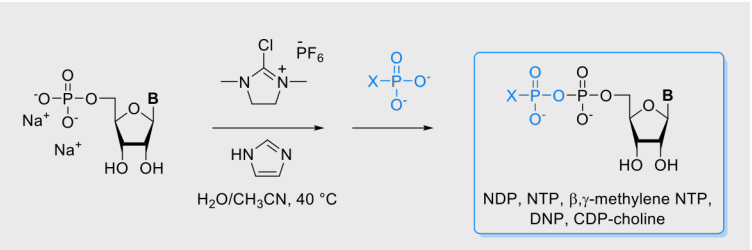

Key Topic: Nucleotide Synthesis

*Anaïs Depaix, Suzanne Peyrottes, Béatrice Roy**

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Title: One-pot synthesis of nucleotides and conjugates in aqueous medium

A two-step reaction sequence for the preparation of nucleoside 5'-polyphosphates in a mixture of water-acetonitrile was developed. This method also provides access to nucleotides analogues, modified either on the nucleoside or on the phosphate moieties.

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