Ball-Milling to Access Dinucleoside Polyphosphates and Analogues

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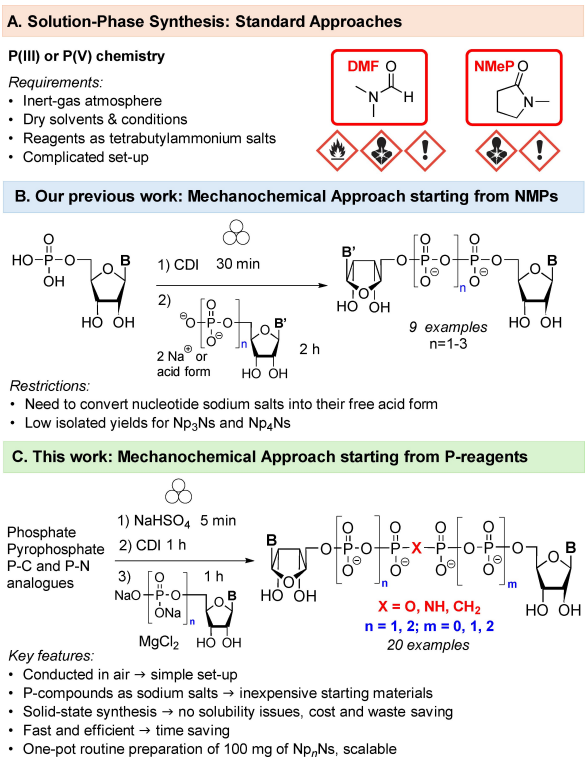
Abstract: Dinucleoside 5',5'-polyphosphates (Np_nNs) play essential roles in various biological processes. Access to these high value-added compounds, while avoiding the many drawbacks of solution phase synthesis, is a challenge that has been overcome thanks to mechanochemistry. A straightforward solid-state synthesis based on activation of phosphate or pyrophosphate sodium salts to their diimidazolides, followed by coupling with commercially available nucleotides, under air conditions, gave symmetrical Np_nNs. The scope ranged from Np_3Ns to highly challenging Np_6Ns. The methodology has also been implemented using medronic acid and imidodiphosphate to obtain Np_nNs analogues, some of which are unprecedented.

Keywords: dinucleotides; bisphosphonates; phosphorus; biomolecules; mechanochemistry

Linear dinucleoside 5',5'-polyphosphates (Np_nNs, where n represents the number of phosphates and N the nucleosides)[1] are endogenous substances that play important intra- and extracellular roles in biology,[2–5] with remarkable actions on vascular tone regulation,[6,7] platelet aggregation,[8,9] and ocular physiology.[10] Therefore, these compounds and their analogues are highly relevant in the medical field. For example, diquafosol tetrasodium (Up_4U) is a potent and selective agonist for the P2Y2 receptor, used for the treatment of dry eye syndrome.[11,12] To date, the most widely used strategies to access linear dinucleotides are solution-phase syntheses based on P(III) or P(V) chemistry (Scheme 1A).[1] They usually involve the activation of a 5'-nucleotide followed by coupling with a second nucleotide or inorganic pyrophosphate, affording dinucleotides containing up to five bridging phosphate groups,[13–23] and we have previously implemented this approach by mechanochemistry (Scheme 1B).[24] Alternatively, the synthesis of dinucleotides using pyrophosphate or cyclotriphosphate as starting materials has been developed by Wright[25] and Taylor,[26] respectively. More recently, Jessen reported several phosphoramidite strategies to prepare efficiently a large range of phosphorylated derivatives, including some dinucleotides.[27–29] Herein we report a rapid, inexpensive, and convenient solid-state synthesis of symmetrical Np_nNs ($n = 3$–6), as well as P–C and P–N analogues, that avoids most of the drawbacks of the current methods, using polyphosphates and nucleotides as their sodium salts (Scheme 1C). In addition, the protocol developed does not require anhydrous conditions, allowing the entire procedure to be carried out in air. Importantly, this work describes the first mechanochemical synthesis of long chain Np_nNs and analogues, including some hitherto unknown compounds.

Although some reported methods are efficient, they require the use of dry, polar aprotic solvents, mainly *N*,*N*-dimethylformamide (DMF) and *N*-methyl-2-pyrro-



Scheme 1. Strategies to access dinucleotides. (**A**) Drawbacks of solution-phase synthesis. (**B**) Our mechanochemical approach starting from nucleoside 5'-monophosphates (NMPs) reported in 2019.[24] (**C**) The new mechanochemical synthesis allows to prepare dinucleotides with phosphate chains up to 6 as well as P–C and P–N analogues.

lidone, which are to be restricted by the European Chemicals Agency (ECHA) due to their hazardous nature,[30] and need to be performed under inert atmosphere.[31] In addition, synthesis of dinucleotides in solution requires the use of pre-dried nucleotides and inorganic (poly)phosphates obtained beforehand as their tris or tetra-*n*-butylammonium counterions for solubility in organic medium. Finally, yields decrease significantly as the length of the polyphosphate chain increases.[1]

Mechanochemistry has recently gained importance as an emerging solvent-free activation technique, not only in organic or (metallo)organic synthesis, but also in polymer science or materials chemistry.[32] For example, we and others reported the solvent-free mechanochemical synthesis of dinucleotides (Scheme 1B).[24,33] We were also able to prepare longer chain derivatives, albeit at low isolated yields, e.g. 40% for Up_3U[24] and 11% for Up_4U (unpublished data).

Our first goal was to activate pyrophosphoric acid or its sodium salts with 1,1'-carbonyldiimidazole (CDI) under ball-milling conditions. In this regard, Wright's group reported the quantitative activation of tetrakis-tributylammonium pyrophosphate with CDI in dry DMF under anhydrous conditions, to form quantitatively the corresponding diimidazolides.[25] In the present work, reactions were performed in air, using a vibratory ball-mill (vbm), at a frequency of 30 Hz in a 15 mL Teflon milling jar and a single 10 mm diameter ball made of stainless steel. Acetonitrile (MeCN) was added as a liquid assistant for grinding (LAG).[24] Extensive optimization studies are described in the Supporting Information and summarized in the $^{31}P\{^{1}H\}$ NMR tracing experiments shown in Figure 1.

Initial studies were conducted with pyrophosphoric acid, which was rapidly abandoned due to its high hygroscopicity and its instability under mechanochemical conditions. Milling of sodium pyrophosphates (0.158 mmol $Na_2H_2P_2O_7$ or $Na_4P_2O_7$) with 4 equiv. of CDI for 1 h led to the monoactivated pyrophosphate and its diimidazolide as minor products (Figure 1B). Short-time ball milling of pyrophosphates in the presence of an acidic additive, i.e. sodium hydrogen sulfate,[34] was found crucial for the outcome of the activation step. The best conversion was obtained by milling $Na_2H_2P_2O_7$ with 2 equiv. $NaHSO_4$ for 5 min, before adding CDI (Figure 1C). By contrast, performing the same reaction in one step was inefficient, giving mainly monoactivation (Figure 1D). Then, the optimization of the coupling step was performed using the disodium salt of P^1,P^2-Di(1-imidazolyl)pyrophosphate[25] and uridine 5'-monophosphate (UMP) sodium salt. Several parameters were screened such as the number of equivalents of UMP and $NaHSO_4$, the presence of divalent cations (e.g., Zn^{2+}, Mg^{2+}), and time (see the Supporting Information). Indeed, divalent cations have been shown to facilitate anhydride bond formation either in solution-phase[35] or in solid-phase[33a] synthesis. The metal ion is thought to perform two functions, activating the electrophilic P(V) center and templating the incoming phosphate nucleophile and the P(V) electrophilic center.[35] Gratifyingly, we could identify experimental conditions (3 equiv. UMP, 2 equiv. $NaHSO_4$, 1 equiv. $MgCl_2$) to convert efficiently the diimidazolide into Up_4U (see the Supporting Information). Finally, we performed the one-pot mechanosynthesis of Up_4U by successively implementing the three optimized steps. We were pleased to obtain an almost complete disappearance of the activated pyrophosphate intermediate (Figure 1E). Indeed, the ^{31}P NMR spectrum shows signals of the major product Up_4U, the remaining UMP, and Up_2U as minor side-product. Reducing the number of equivalents of CDI from 4 to 3 limited the formation of Up_2U and improved atom economy (Figure 1F), affording Up_4U in 69% isolated yield after purification by RP-18 chromatography, followed by ion exchange on Dowex-Na^+. Finally, to confirm the importance of the first milling with $NaHSO_4$ before activation with CDI, the reaction was performed in only two steps, with the use of $NaHSO_4$ maintained only in the last step. Accordingly, no Up_4U was observed under these conditions (Figure 1G). The optimal conditions were successfully

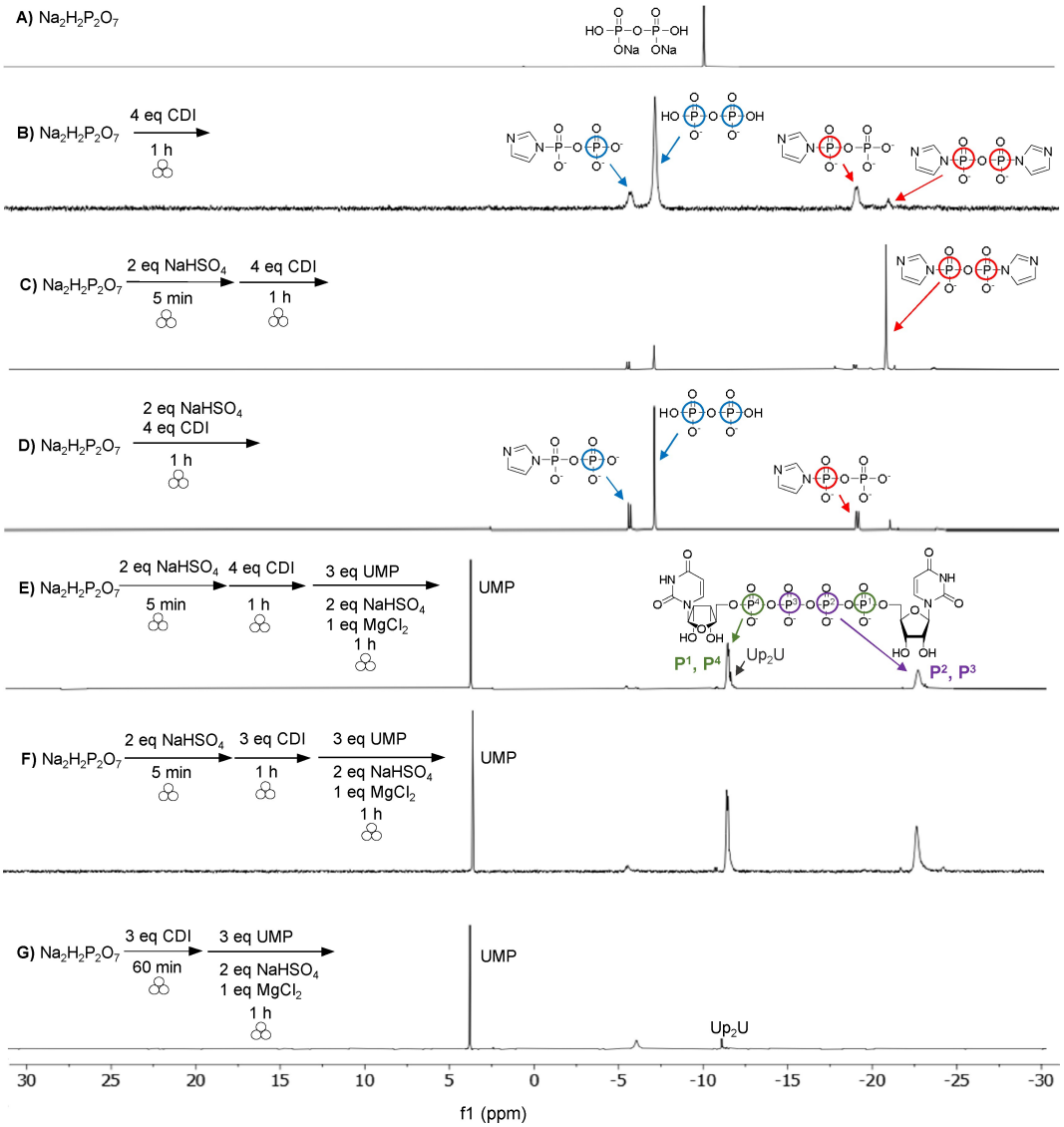
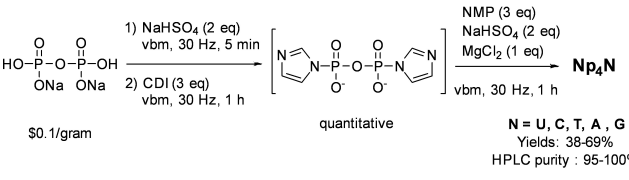


Figure 1. ^{31}P {^1H} NMR study (D_2O, 162 MHz) monitoring the optimization of Up_4U's mechanosynthesis. All reactions use 0.158 mmol (35 mg) of $Na_2H_2P_2O_7$ as starting material. In experiments B to G, MeCN (0.3–0.95 μL.mg^{-1}) was added as liquid additive (for more details, see the Supporting Information). A) $Na_2H_2P_2O_7$. B) After ball milling of $Na_2H_2P_2O_7$ with 4 equiv. CDI, 1 h. C) 2 equiv. $NaHSO_4$, 5 min then addition of 4 equiv. CDI, 1 h. D) 2 equiv. $NaHSO_4$ and 4 equiv. CDI, 1 h. E) 2 equiv. $NaHSO_4$, 5 min then addition of 4 equiv. CDI, 1 h and finally 3 equiv. uridine 5'-monophosphate (UMP), 1 equiv. $MgCl_2$, 2 equiv. $NaHSO_4$, 1 h. F) 2 equiv. $NaHSO_4$, 5 min then addition of 3 equiv. CDI, 1 h and finally 3 equiv. UMP, 1 equiv. $MgCl_2$, 2 equiv. $NaHSO_4$, 1 h. G) 3 equiv. CDI, 1 h and then 3 equiv. UMP, 1 equiv. $MgCl_2$, 2 equiv. $NaHSO_4$, 1 h.

applied to several pyrimidine (T,C) or purine (A,G) nucleoside 5'-monophosphates, to give rise to the desired Np_4Ns in modest to good yields and high purity (Scheme 2).[36] Even though obtaining 100 mg of these high value-added Np_nNs is already highly convenient in the field of nucleotides, scaling up the reaction for Up_4U using larger milling jars was possible and made it easy to prepare hundreds of milligrams of this therapeutically important P2Y2 receptor agonist (see the Supporting Information).[11,12]



Scheme 2. Optimized conditions for the synthesis of Np_4N. All reactions were carried out with 35 mg of $Na_2H_2P_2O_7$. The synthesis of Gp_4G required 4 equiv. GMP and 3 equiv. $NaHSO_4$. MeCN (0.3–0.95 μL.mg^{-1}) was added as liquid additive (for more details, see the Supporting Information).

We then proceeded to investigate the substrate scope of this methodology for more challenging dinucleotides. In this regard, only a few Np_6N have been reported in the literature.[1] For example, Up_6U has only been described once, albeit as a minor side product in the course of the synthesis of Up_5U.[18] The one-pot three steps approach described in Scheme 2 was implemented by replacing UMP with uridine 5'-diphosphate (UDP) sodium salt to afford Up_6U in 54% isolated yield (Figure 2A).

We then applied the procedure to modified Np_nNs where the central bridging oxygen atom is replaced by a methylene (CH_2) or an imido (NH) group. Indeed, methylenebisphosphonate and imidobisphosphonate isosteres of pyrophosphate are widely used as tools in various binding and enzymatic activity studies.[37–41] Moreover, with the exception of $Up_2CH_2p_2U$, access to these derivatives has been rarely studied. We started with the P–C analogue of pyrophosphate, which is commercialized as medronic acid (Table 1, entries 1–5). As anticipated, the acidification step was not required anymore. In addition, only 2.5 equiv. of CDI and a 30 min milling time were found necessary to reach a full conversion to the diimidazolide (Figure 2B–C). After this one-pot activation procedure, several purine and pyrimidine NMPs were added in the presence of $MgCl_2$ leading to the desired β,γ-methylenedinucleoside tetraphosphates in 35–69% yields (Table 1, entries 1–4).

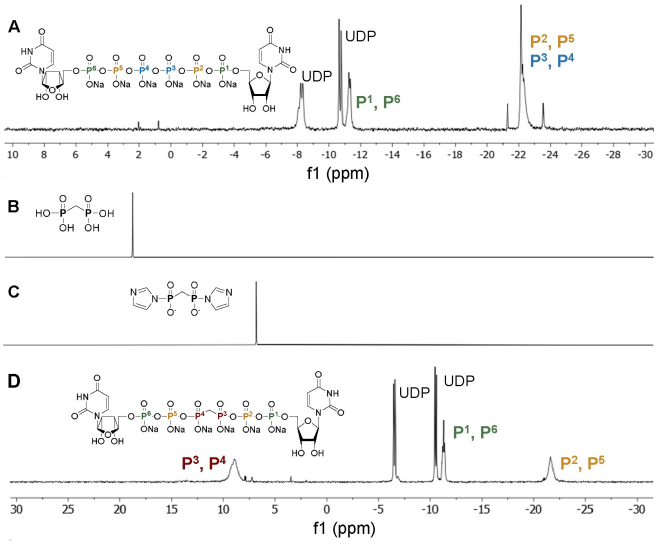
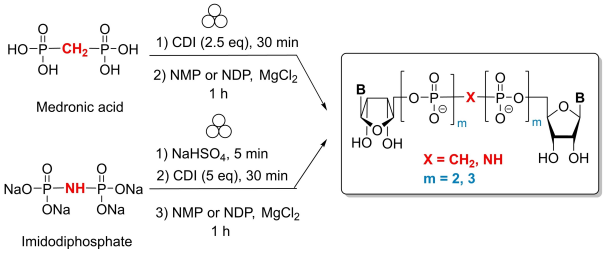


Figure 2. ^{31}P $\{^1H\}$ NMR spectra (D_2O, 162 MHz). A) Crude reaction mixture obtained when applying the one-pot three steps strategy reported in Scheme 1 with UDP instead of NMPs. B) Medronic acid. C) Crude reaction mixture after activation of 35 mg of medronic acid, 2.5 equiv. CDI, vbm, 30 Hz, 30 min. D) Crude reaction mixture obtained when applying the one-pot two-steps strategy reported in Table 1 entry 5. MeCN (0.3–0.95 $\mu L.mg^{-1}$) was added as liquid additive (for more details, see the Supporting Information).

Table 1. Scope of the mechanochemical synthesis of dinucleotide analogues.

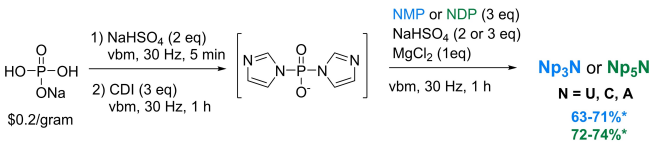


Entry	Starting material	Nucleotide[b]	Product	Yield (%)[d]
1	Medronic acid[a]	UMP	$Up_2CH_2p_2U$	55
2		CMP	$Cp_2CH_2p_2C$	55
3		AMP	$Ap_2CH_2p_2A$	69
4		GMP[c]	$Gp_2CH_2p_2G$	35
5		UDP	$Up_3CH_2p_3U$[e]	19
6		UMP	Up_2NHp_2U[e]	51
7	Imidodiphosphate[a]	AMP	Ap_2NHp_2A[f]	39
8		CMP	Cp_2NHp_2C[e]	43

[a] The reaction was performed starting either from 35 mg of medronic acid or 35 mg of imidodiphosphate.
[b] Nucleotides were used as sodium salts.
[c] In the second step, 4 equiv. GMP and 3 equiv. $NaHSO_4$ were used.
[d] Yield of isolated product.
[e] Unprecedented compound.
[f] Reported in the literature using a chemoenzymatic procedure.[39]

The same procedure applied to UDP gave rise to the original compound $Up_3CH_2p_3U$ (Table 1, entry 5 and Figure 2D). In the case of P–N derivatives, we started from the commercially available imidodiphosphate tetrasodium salt. A first acidification step with $NaHSO_4$ and a larger excess of CDI were required to optimize the one-pot synthesis, giving rise to Np_2NHp_2N in 39–51% yields (Table 1, entries 6–8). As far as we know, this is the first reported chemical synthesis of these imido derivatives.

Finally, we investigated the mechanochemical synthesis of dinucleotides with an odd number of phosphate groups, namely Np_3Ns and Np_5Ns. For the latter, the few reported methods require dry DMF, strictly anhydrous conditions and reagents as TBA salts.[1] These drawbacks could be circumvented with our mechanochemical approach. Indeed, the one-pot three steps strategy was developed starting from phosphate instead of pyrophosphate (see the Supporting Information). A screening of phosphoric acid, sodium phosphate and disodium phosphate led to the selection of sodium phosphate as the best starting material for activation. Dinucleoside 5',5'-tri-, and pentaphosphates were obtained with good conversion rates (Scheme 3). To the best of our knowledge, this is the first example of



Scheme 3. One-pot three steps synthesis of dinucleosides tri- and pentaphosphates starting from sodium phosphate. *Conversions were determined by integration of the corresponding signals in the ^{31}P {^{1}H} NMR spectra for Np_3N and Np_5N. MeCN (0.3–0.95 µL.mg^{-1}) was added as liquid additive (For more details, see the Supporting Information).

activation of orthophosphate to prepare symmetrical dinucleotides with an odd number of phosphate groups.

In conclusion, we have developed an environmentally friendly procedure to prepare dinucleotides and analogues that offers significant advantages over current methods. Indeed, unlike most solution-phase methods, it can be performed in air, with P-compounds as sodium salts, in the absence of dry DMF or *N*-methyl-2-pyrrolidone, resulting in a simple set-up. This is also a huge advantage in terms of cost and time, as it allows to avoid the preparation of reagents as TBA salts and their drying (high vacuum, co-evaporation with toluene), the use of dry solvents and inert gas line techniques. Long chain Np_nNs are high value-added compounds due to their complicated synthesis and isolation. In this study, dinucleoside tetraphosphates were obtained in good yields starting from inexpensive sodium pyrophosphate and nucleoside 5'-monophosphates. The scope was expanded to longer chain Np_nNs and analogues, including new compounds, which would facilitate studies of their biochemical, medical, and biotechnological applications. This ball-mill approach which enables the routine synthesis of dinucleotides on a 100 mg scale, could be easily scaled-up using larger milling jars.

Experimental Section

General Procedure for the Synthesis of Dinucleoside 5',5'-Tetraphosphates

A 15 mL Teflon milling jar was charged with pyrophosphate disodium salt (0. 035 g, 0.158 mmol, 1.0 equiv.), $NaHSO_4$ (0.038 g, 0.315 mmol, 2.0 equiv.), MeCN (22 µL, 0.3 µL.mg^{-1}) and a 10 mm stainless ball. After milling at a frequency of 30 Hz for 5 minutes, CDI (0.076 g, 0.473 mmol, 3.0 equiv.) and MeCN (46 µL, 0.6 µL.mg^{-1}) were added, followed by grinding for 1 h at 30 Hz. Then, $MgCl_2$ (0.015 g, 0.158 mmol, 1.0 equiv.), $NaHSO_4$ (0.038 g, 0.315 mmol, 2.0 equiv.), NMP disodium salt (0.473 mmol, 3.0 equiv.) and MeCN (0.95 µL.mg^{-1}) were added. The reaction vessel was vibrated at 30 Hz for 1 h. The reaction mixture was solubilized in deionized water and analyzed by ^{31}P NMR. The compounds were purified by reversed-phase chromatography (Redi*Sep* Gold® C18Aq column) or by ion-exchange chromatography (DEAE Sephadex® A-25 chloride resin). Subsequent cation exchange with a DOWEX 50W-X8 (Na^+ form) followed by freeze drying afforded Np_4Ns as their sodium salts.

CAUTION: It should be noted that CO_2 is released during activation with CDI, leading to a slight increase in pressure in the closed vessel.

Acknowledgements

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