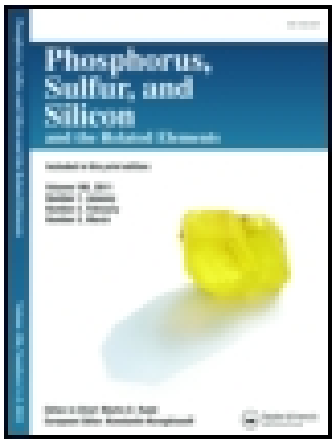

Phosphorus, Sulfur, and Silicon and the Related Elements

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PPN pyrophosphate: A New Reagent for the Preparation of Nucleoside Triphosphates

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PPN pyrophosphate: A New Reagent for the Preparation of Nucleoside Triphosphates

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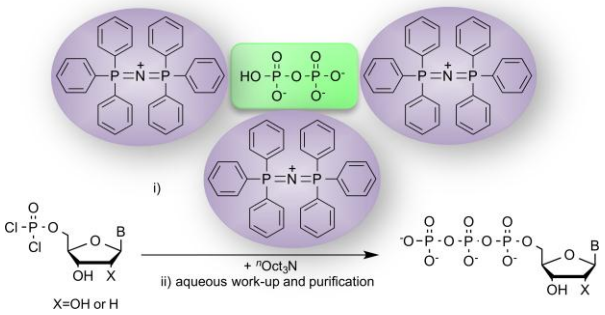
PPN PYROPHOSPHATE FOR NTP SYNTHESIS

Abstract

Tris{*bis*(triphenylphosphoranylidene) ammonium} (PPN) pyrophosphate was accessed via aqueous precipitation and desiccation. The reagent was investigated as a replacement for highly hygroscopic alkylammonium salts in Ludwig-Yoshikawa reactions for the preparation of nucleoside-5'-triphosphates.



Keywords

phosphorylation, triphosphate, nucleotide

INTRODUCTION

Nucleoside-5'-triphosphates (NTPs) provide the building blocks for polymerase enzymes and a multitude of other biological and biotechnological systems. Analogues are widely used for enzyme mechanism studies.[1] Over the last ~25-50 years, a small number of key methods have been adopted for the preparation of NTPs and other phosphoanhydrides,[2] although new methods are now becoming available.[3-14] Often, these approaches rely on tri- and tetralkylammonium salts as organic-solvent-soluble sources of nucleophilic phosphate**.** Unfortunately, these salts are extremely hygroscopic,[15-20] with deleterious consequences on the water-sensitive reactions that use them. PPN salts do not form heavily water-clathrated structures, and their preparation through aqueous precipitation has been established and applied synthetically.[21-22] Here we prepare for the first time, a PPN salt of pyrophosphate ion and investigate its utility as a source of nucleophilic pyrophosphate in the Ludwig modification of the Yoshikawa phosphorylation.[23]

RESULTS AND DISCUSSION

PPN salts can be prepared using PPNCl,[24] which is soluble in warm water, and the sodium salt of the desired anion,[25] where the PPN salt precipitates or crystallises on mixing the solutions. We explored stoichiometry and pH parameters in order to optimise the preparation of $PPN_3HP_2O_7$ using PPNCl and partially neutralised tetrasodium

pyrophosphate. A 3:1 PPN-pyrophosphate target stoichiometry was chosen in order to retain the nucleophilicity of the pyrophosphate trianion, while keeping pH low to avoid the formation of PPN.OH. A 2:1 ratio of PPN.Cl to sodium pyrophosphate (pH-adjusted to pH 5.0) afforded PPN pyrophosphate in 62% yield. ^{31}P NMR analysis revealed a PPN–pyrophosphate ratio of 3.2:1.

[Insert Scheme 1]

The material was desiccated, and its moisture content before and after exposure to the atmosphere was assessed visually and by thermogravimetric analysis (TGA) in comparison to tetraalkylammonium salts. Tris(tetrabutylammonium) hydrogen pyrophosphate became visibly ‘wet’ after exposure to the atmosphere (see Supplemental Materials), whereas PPN pyrophosphate appeared unchanged. TGA showed that the PPN salt contained only 1.2% water (approximately 1.2 molar equivalents) after desiccation, despite its isolation from aqueous solution, and this value rose to only 3.7% on exposure to the atmosphere for 22 h.

Preliminary experiments using PPN pyrophosphate explored the use of additional drying of PPN pyrophosphate using molecular sieves, the addition of collidine, the addition of tri-*n*-octylamine, reaction time, temperature and stoichiometry. The conclusions of these studies were that additional drying increased conversion level, as did the additions of collidine and tri-*n*-octylamine. Four ribonucleosides and thymidine, as a representative deoxyribonucleoside, were investigated as substrates (Scheme 2, Table 1).

[Insert Scheme 2]

[Insert Table 1]

Reactions were quenched with triethylammonium bicarbonate (TEAB) buffer followed by crude nucleotide isolation through extraction and selective precipitations with NaI/acetone. Rigorously controlled precipitation conditions served to reduce cTMP ion, which can be problematic during chromatography. ^{31}P NMR assessments of the crude nucleotides showed good conversion levels, with excess pyrophosphate and traces of cTMP and monophosphate being the P-containing impurities.

DEAE chromatography followed by cation exchange to sodium ions afforded each of the triphosphates **1-5** in yields of 21-48%.

CONCLUSIONS

PPN pyrophosphate is easily dried and displays limited moisture uptake from the atmosphere. It is a convenient and effective replacement for tri- and tetraalkyl ammonium pyrophosphates in (d)NTP preparation.

EXPERIMENTAL

Drying of solvent and reagents

Triethyl phosphate was vacuum distilled from CaH_2 and stored under N_2 with activated 3 Å molecular sieves. Trioctylamine (1 mL) was dried over KOH pellets overnight. PPN pyrophosphate (0.4 mmol, 2 eq.) was dissolved in dry MeCN (0.8 mL) and dried with

activated 3 Å molecular sieves under N_2 overnight. 2,4,6-trimethylpyridine was vacuum distilled from CaH_2 and phosphoryl chloride was distilled under dry conditions. Nucleosides (0.2 mmol, 1 eq.) was dried at 80 °C under vacuum over P_2O_5 overnight.

Preparation of PPN pyrophosphate (tris{(*bis*(triphenylphosphoranylidene)ammonium} pyrophosphate). Tetrasodium pyrophosphate decahydrate (0.79 g, 1.77 mmol) was dissolved in distilled water (25 mL) at 50 °C and the pH of the solution was adjusted to pH 5 with 1 M hydrochloric acid. This solution was added dropwise, with mixing, to a solution of *bis*(triphenylphosphoranylidene)ammonium chloride (2.00 g, 3.48 mmol) in distilled water (260 mL) at 50 °C. The mixture was transferred to six 50 mL centrifuge tubes, allowed to cool to room temperature and the tubes were centrifuged at 4,000 rpm for 20 min at 18 °C. The supernatant was removed and the solid residues were combined, recrystallized from distilled water (45 mL) at 80 °C, collected by centrifugation at 4,000 rpm for 20 min, dried, ground to a powder and further dried in a vacuum desiccator over P_2O_5 for 2 days to give PPN pyrophosphate as a white powder (1.45 g); mp 234-238 °C; ν_{max}/cm^{-1} 3371br (O-H), 1437s, 1248 (P=O), 1112s (P=O); δ_H (400.13 MHz, d_6-DMSO) 7.51-7.70 (m, phenyl H); δ_P (161.96 MHz, d_6-DMSO) –4.72 (2P, s, PPi), 20.8 (6.4P, s, PPh_3); δ_C (100.60 MHz, d_6-DMSO), 125.7 (J_{C-P} 54, 2.1 Hz), 128.7-128.3 (m), 130.9-130.6 (m), 132.7; ES^+ *m/z* 539 ($[PPN+H]^+$, 100%).

Phosphorylation Procedure

Dry triethyl phosphate (1 mL for uridine, cytidine and thymidine or 2.5 mL for guanosine and adenosine) was added to the nucleoside (0.2 mmol, 1 eq.) under an inert

atmosphere. The mixture was heated to 80 °C briefly to dissolve the nucleoside, if necessary, then stirred on an ice-salt bath at approx. –10 °C under an inert atmosphere. Separately, freshly distilled phosphoryl chloride (0.24 mmol, 1.5 eq.) and dry 2,4,6-trimethylpyridine (0.2 mmol, 1 eq.) were added drop-wise via syringe to the nucleoside solution, and the mixture was stirred at –10 °C for 90-120 min. PPN pyrophosphate solution in dry acetonitrile (0.4 mmol in 1 mL, 2 eq., with molecular sieves in stock solution) and dry n-trioctylamine (0.4 mmol, 2 eq.) were added to the nucleoside solution separately via syringe and the mixture was stirred at 0 °C for 2 h then RT for 20 h. Triethylammonium bicarbonate solution (5 mL, 0.1 M) was added to the solution, followed by chloroform (5 mL) and the mixture was stirred for 30 min. The aqueous layer was separated and washed with chloroform (2×20mL). The aqueous layer was transferred to a centrifuge tube, and the phosphorus-containing components were precipitated by addition of NaI (80 mg), followed by acetone (20 mL). The centrifuge tube was agitated for 30 min, cooled at 0 °C for 30 min and centrifuged at 4,000 rpm for 5 min at 4 °C. The supernatant was removed and the solid was re-suspended in TEAB buffer (3 mL, 0.1 M). NaI in acetone (5 mL, 0.1 M) was added dropwise to the mixture, while stirring at 0 °C. Stirring was maintained for 30 min at 0 °C, and the solid was collected by centrifugation (4,000 rpm, 5 min at 4 °C). The solid and supernatant were analysed by ^{31}P NMR spectroscopy to ensure the selective precipitation of NTP, where the majority of cTMP remained in the supernatant. If the supernatant retained NTP (by ^{31}P NMR spectroscopy), additional NaI/acetone was added, and an additional crop of

NTP precipitate was collected. The combined NTP precipitates were then dissolved in TEAB buffer, and then subjected to DEAE chromatography.

DEAE purification of NTPs

The crude NTP was dissolved in 2 mL of distilled water and was loaded onto a 50 mL bed-volume DEAE Sepharose Fast Flow column and nucleotide products were eluted using a 5-60% gradient of 1 M triethylammonium bicarbonate solution with a flow rate of 4.5 mL min^{-1}. Fractions corresponding to nucleoside triphosphate signals (at approximately 0.3 M buffer concentration) were collected and lyophilised to give the purified NTP, which was exchanged to the Na salt through NaI/acetone precipitation.

Supplementary Data

Time-lapse photography of PPN pyrophosphate and tetrabutylammonium salts on exposure to the atmosphere, crude ^{31}P NMR spectra, and ^{31}P, ^{1}H and ^{13}C NMR spectra of NTPs after chromatography and isolation as Na salts.

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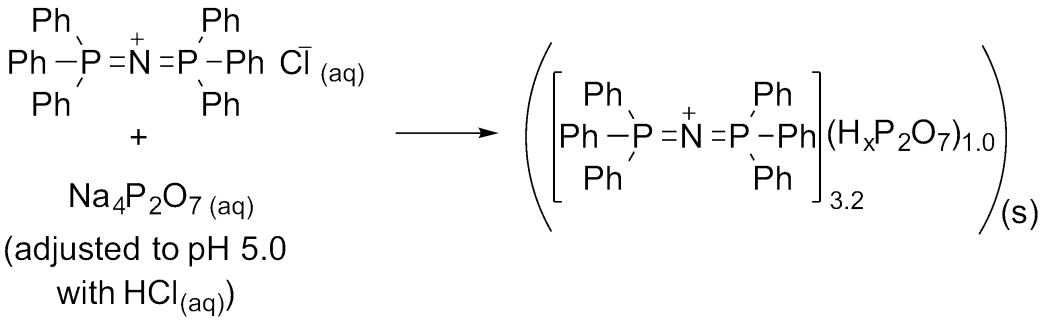
Scheme 1



Scheme 2

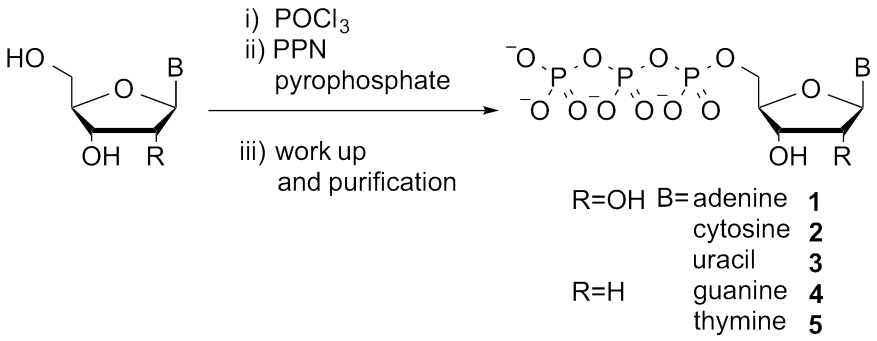


TABLE 1 Use of PPN pyrophosphate in (d)NTP syntheses

Entry	Nucleoside	Product	Estimated (^{31}P NMR) conversion to (d)NTP (%)	Yield of Na^+ salt (%)
1	adenosine	ATP **1**	76	30
2	cytidine	CTP **2**	66	48
3	uridine	UTP **3**	62	36
4	guanosine	GTP **4**	82	42
5	thymidine	TTP **5**	65	21