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NOVEL SYNTHESIS OF NUCLEOSIDE 5'-POLYPHOSPHATES

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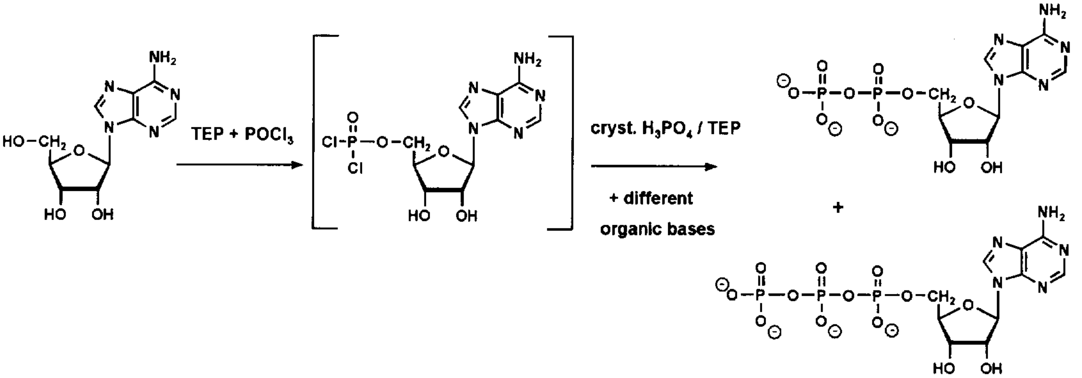
Abstract. We report a novel synthetic method to prepare nucleoside 5'-di- and triphosphates simultaneously. Their preparative separation and the possibilities to influence the product ratio were investigated. Preliminary results of the triphosphates to act as phosphate donors for the nucleoside diphosphate kinase (EC 2.7.4.6) are presented. Copyright © 1996 Elsevier Science Ltd

Since ribo- and deoxy-ribonucleoside 5'-triphosphates are involved in a large number of biochemical processes, various methods for their synthesis have been developed. In general, the procedures are based on the substitution of a leaving group of the nucleoside 5'-phosphate, namely chloro-[1,2], morpholino-[3] and imidazole-groups[4]. More recently, the enzymatic synthesis of nucleoside triphosphates was reported[5,6]. In contrast the synthesis of nucleotide diphosphates usually follow a different procedure where a tosyl group in the 5'-position is displaced by pyrophosphate[7]. Mishra and Broom[8] reported the synthesis of a triphosphate by substituting chloride of an activated nucleoside monophosphate by tributylammonium phosphate when they intended to generate diphosphates.

In order to synthesize base-modified nucleoside di- and triphosphates and to test their potential as phosphate donor for the enzyme nucleoside diphosphate kinase (NDP-kinase), we investigated the possibility to generate a mixture of di- and triphosphates in a one-pot reaction as depicted below.



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In a first attempt, unprotected adenosine was dissolved in triethylphosphate (TEP) and phosphorylated with 2 equivalents of $POCl_3$[9]. In a second phosphorylation step a 2M solution of crystalline phosphoric acid in TEP was added in different amounts (up to 10 molar equivalents). However, this procedure did not generate ATP and only minor amounts of ADP (Fig. 1). To increase the activation for a nucleophilic attack by H_3PO_4 in TEP we investigated the influence of organic bases. Catalytic amounts of N,N-diisopropylethylamine (DIPEA, 0.5 molar equivalents) yielded significant amounts of ADP and ATP. Five equivalents DIPEA shifted the ATP/ADP ratio in favor of ATP (Fig. 1), as was verified by analytical HPLC. Higher amounts of DIPEA had no further effect. Other organic bases (5 equivalents) led to different product compositions (Fig. 1). In particular 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) favoured the diphosphate formation. Variation of the amount of crystaline H_3PO_4 showed 5 equivalents H_3PO_4 to be optimal for the reaction (data not shown). The ratio of ATP/ADP increased with the loss of nucleophilicity of the organic base, giving the following ranking: Imidazole < DBU < tri-n-Butylamine < DIPEA.

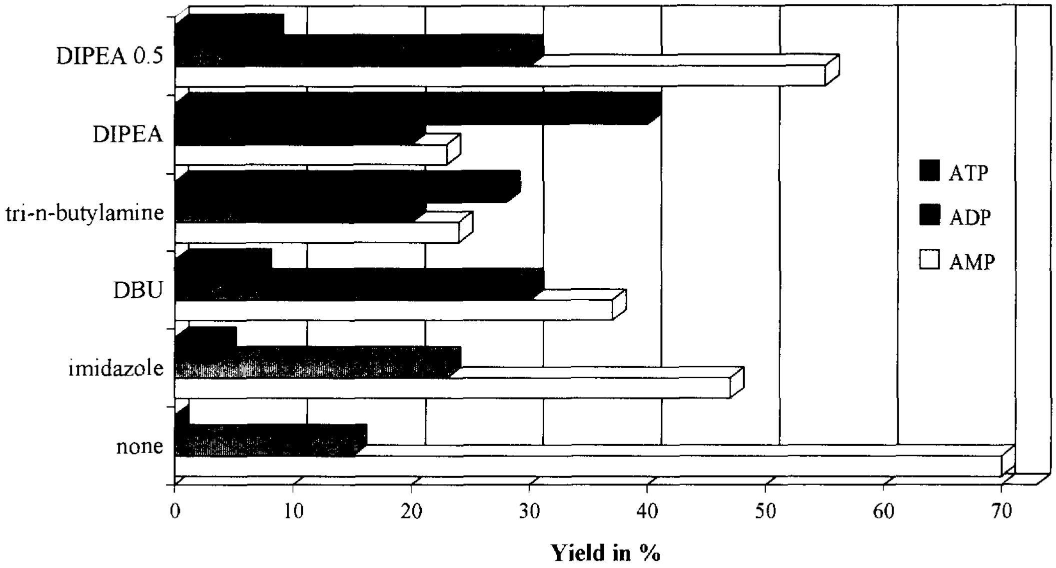
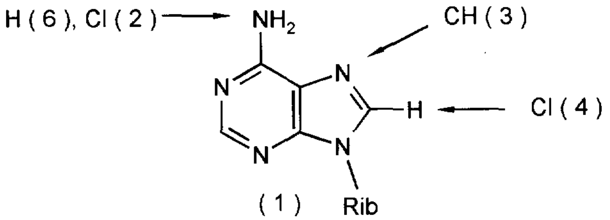
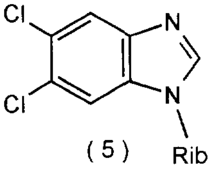


Figure 1: Product composition yielded in the presence of various organic bases. Bases are denoted on the ordinate and were used in 5 mol equivalents or 0.5 mol equivalents as is indicated for DIPEA.

The nucleoside di- and triphosphates were successfully separated on an analytical anion-exchange HPLC-column (Whatman Partisil SAX-10; 4.6×250 mm), by isocratic elution with 250 mM sodium phosphate buffer at pH 6. Preparative purification of the nucleoside di- and triphosphates was performed on an anion-exchange column (Whatman Partisil SAX-10; 22×250 mm) under similar conditions. Fractions were controlled for purity and freeze-dried. The overall yield of di- and triphosphates was 60% with DIPEA and this procedure was applied to other nucleosides with the following structures:



(1)



(5)

The table below shows the yields, as determined by HPLC, of nucleoside mono-, di- and triphosphates synthesised by the method described for ATP (total nucleotide yield about 80%).

nucleoside	monophosphate yield in %	diphosphate yield in %	triphosphate yield in %
adenosine (1)	22	20	40
6-Cl-purine riboside (2)	24	22	35
tubercidine (3)	22	17	22
8-Cl-adenosine (4)	37	31	15
5,6-DCl-BIR[10] (5)	12	10	65
nebularine (6)	8	31	57

Nucleotides were freed from inorganic phosphate by the following procedure: the freeze-dried fractions were dissolved in 10 mL of 1M triethyl ammonium formate (TEAF), pH 6.5. The mixture was applied to a glass column (30×200 mm) filled with LiChroprep® RP-18 (25-40µm, 100g). Elution (4mL/min) was performed with 25 mM TEAF, pH 6.5, under slight pressure (1.25 bar). Inorganic phosphate eluted while the respective nucleotide stuck to the column. The nucleotides were detected by UV absorption and inorganic phosphate was monitored by a commercially available blue color test (Aquamerck® 14661). TEAF and the nucleotides did not crossreact with this assay. When the eluate showed no more blue color reaction, the nucleotides were eluted with water. The pooled fractions were freeze-dried and analysed by HPLC and UV-spectroscopy. ^{1}H- and ^{31}P-NMR and FAB-MS-data were identical with previously published data[11] or are stated below. Isolated yield were about 75% to 85% of the percentage given in the table above. Purities were higher than 95%.

The properties of the synthesised triphosphates to substitute ATP in biochemical processes were tested with NDP-kinase from *Dictyostelium discoideum*. This enzyme catalyses the reaction ATP + NDP → ADP + NTP. We measured the activity of the nucleotides to act as a phosphate donor in an assay similar to that discribed by Bourdais et al.[12] with a standard concentration of 0.5 mM nucleoside triphosphate and 0.1 mM ^{14}C-ADP as phosphate acceptor. The following table shows the relative rates of the synthesised nucleoside triphosphates compared to ATP:

compound [13]	ATP	6-Cl-PuTP	TuTP	8-Cl-ATP[14]	5,6-DClBITP[15]	NeTP
NDP-kinase activity in % relative to ATP	100	110	155	140	145	130

The values above indicate that all modified nucleotides were better substrates as phosphate donors than ATP. Since this data are relative rates measured for a single concentration of the respective phosphate donor, we obtained no information about K_M- or V_{max}-values. Triphosphates modified in position 1 or 2 used in similar experiments were poorer substrates compared to ATP (data not shown). This preliminary results give first hints for the structural requirements of the phosphate donor, but further investigations will be needed.

References and notes:

1. Ludwig, J. *Acta Biochim. Biophys. Acad. Sci. Hung.*, **1981**, *16*, 131-133.
2. Kovacs, T.; Otvos, L. *Tetrahedron Lett.*, **1988**, *29*, 4225-4228.
3. Moffat, J. G. *Can. J. Chem.*, **1964**, *42*, 599-604.
4. Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.*, **1965**, *87*, 1785-1788.
5. Simon, E.S.; Grabowski S.; Whitesides G.M. *J. Org. Chem.*, **1990**, *55*, 1834-1841.
6. Schobert, B. *Anal. Biochem.*, **1995**, *226*, 288-292.
7. Davisson, V. J.; Davis, D. R.; Dixit, V. M.; Poulter, C. D. *J. Org. Chem.*, **1987**, *52*, 1794-1801.
8. Mishra, N. C.; Broom, A.D. *J. Chem. Soc., Chem. Commun.*, **1991**, 1276-1277.
9. Yoshikawa, M.; Kato, T.; Takenishi, T. *Bull. Chem. Soc. Jpn.*, **1969**, *42*, 3505-3508.
10. 5,6-DClBIR = 5,6-dichlorobenzimidazole-β-D-ribofuranoside.
11. Scheit, K. H. *Nucleotide analogs, synthesis and biological functions*; John Wiley & Sons, New York 1980 and references cited therein.
12. Bourdais, J.; Biondi R.; Safrati, S.; Guerreiro, C.; Lascu, I.; Veron, M. *J. Biol. Chem.* **1996**, *271*, 7887-7890.
13. adenosine-5′-triphosphate (ATP); 6-chloropurine-β-D-ribofuranosyl-5′-triphosphate (6-Cl-PuTP); tubercidine-β-D-ribofuranosyl-5′-triphosphate (TuTP); 8-chloro-adenosine-5′-triphosphate (8-Cl-ATP); 5,6-dichloro-benzimidazole-β-D-ribofuranosyl-5′-triphosphate (5,6-DCl-BITP), and nebularine-5′-triphosphate (NeTP) = purine-β-D-ribofuranosyl-5′-triphosphate, respectively.
14. 8-Cl-ATP: NMR spectra in D_2O (ppm rel. internal TMS and external 85% H_3PO_4 resp.): ^1H δ 8.28 (s, 1H, H-2), 6.11 (d, 1H, J = 7.2 Hz, H-1′), 5.21 (dd, 1H, J = 7.5 Hz, J = 6.1 Hz, H-2′), 4.63 (dd, 1H, J = 6.1 Hz , J = 4.3 Hz, H-3′), 4.28 (m, 1H, H-4′), 4.21 (m, 2H, H-5′); ^{31}P δ -10.3, -10.9, -22.7; FAB-MS in glycerine: negative mode m/z 540 [M-H], 506 [M-Cl], 460 [M-H-PO_3H], 257 [$P_3O_{10}H_4$], positive mode m/z 542 [M+H]; UV-spectra: λ_{max} = 262 nm.
15. 5,6-DCl-BITP: NMR spectra in D_2O (ppm rel. internal TMS and external 85% H_3PO_4 resp.): ^1H δ 8.54 (s, 1H, H-2), 7.92 (s, 1H, H-4), 7.88 (s,1H, H-7), 5.99 (d, 1H, J = 6.5 Hz, H-1′), 4.67 (dd, 1H, J = 6.5 Hz, J = 5.6 Hz, H-2′), 4.51 (dd, 1H, J = 5.6 Hz, J = 3.4 Hz, H-3′), 4.35 (m, 1H, H-4′), 4.26 (m, 1H, H-5′), 4.16 (m, 1H, H-5′); ^{31}P δ -10.1 (d, J = 19.4 Hz), -10.9 (d, J = 24 Hz), -22.6 (dd, J = 19.4 Hz, J = 24 Hz); FAB-MS in glycerine: negative mode m/z 557 [M-H], 523 [M-Cl], 257 [$P_3O_{10}H_4$], positive mode m/z 559 [M+H]; UV-spectra: λ_{max} = 253, 286, 294 nm.

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