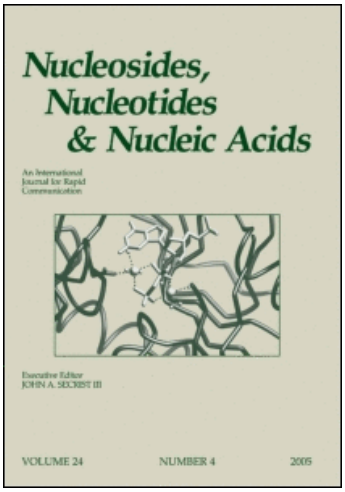

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 19 April 2010

To cite this Article Gillerman, Irina and Fischer, Bilha(2010) 'An Improved One-Pot Synthesis of Nucleoside 5'-Triphosphate Analogues', Nucleosides, Nucleotides and Nucleic Acids, 29: 3, 245 — 256
To link to this Article: DOI: 10.1080/15257771003709569
URL: http://dx.doi.org/10.1080/15257771003709569

Nucleosides, Nucleotides and Nucleic Acids, 29:245–256, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003709569

AN IMPROVED ONE-POT SYNTHESIS OF NUCLEOSIDE 5′-TRIPHOSPHATE ANALOGUES

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□ *Nucleoside 5′-triphosphate (NTP) analogues are valuable tools for biochemical and medicinal research. Therefore, a facile and efficient synthesis of NTP analogues is required. Here, we report on an improved nucleoside 5′-triphosphorylation procedure to obtain pure products after liquid chromotagrpahy (LC) separation with no need for high performance liquid chromatography (HPLC) purification. To improve the selectivity of the reaction we attempted the optimization of several parameters such as solvent, pyrophosphate nucleophilicity, time and temperature of the reaction. Eventually, the reaction was optimized by decreasing the temperature to −15° C and increasing the reaction time to 2 hours, based on monitoring time-dependent product distribution using ^{31}P NMR. Furthermore, the NTPs were obtained as pure products after LC separation, which was impossible in the original Ludwig procedure. Good yields were obtained for all studied natural and synthetic nucleosides.*

Keywords Nucleoside 5′-triphosphate analogues; nucleoside 5′-triphosphorylation; Ludwig procedure

INTRODUCTION

Nucleoside 5′-triphosphate (NTPs) are involved in many biological processes including purinergic signaling, neurotransmission, DNA replication, transcription, and translation. Correspondingly, synthetic analogues of NTPs have been widely used as inhibitors[1–3] and substrates of NTP-binding enzymes,[4,5] agonists,[6] and antagonists[7] of nucleotide receptors, as radioactive, fluorescent,[8–10] and other labeled NTP[11–13] probes. As a result, a number of synthetic methods have been developed for the preparation of NTPs.[14,15]

Commonly used methods for the synthesis of NTPs rely on the application of nucleoside 5′-monophosphates activated as phosphoramidate,[16,17] morpholidate,[18–20] or imidazolidate,[21,22] and involve a three-step reaction starting from the corresponding nucleoside. The nucleoside is

Received 27 January 2010; accepted 17 February 2010.
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5′-monophosphorylated followed by activation and the addition of pyrophosphate salt. In most cases, the final transformation to nucleoside-5′-triphosphate is slow, and the yields are moderate.

Likewise, the conversion of nucleoside 5′-*H*-phosphonate to NTP, involving a silyl-*H*-phosphonate converted to a reactive pyridinium phosphoramidate and treatment with $(NBu_4)_3HP_2O_7$, results in only moderate yields of NTP.[23]

Other methods for NTP synthesis involve the activation of the 5′-position of nucleoside with tosylate,[24,25] 8-quinolyl monophosphate, or 2,2,2-tribromoethyl morpholinochloridate and then reaction with tripolyphosphate.

The most widely used "one-pot, three-step" nucleoside 5′-triphosphate synthesis, developed by Ludwig[26] and others,[27] involves the generation of nucleoside dichlorophosphoridate[28] followed by reaction with bis-(tri-*n*-butylammonium) pyrophosphate and hydrolysis of the resulting cyclic intermediate. A related method involves treatment of dichlorophosphoridate with inorganic phosphate, instead of pyrophosphate.[29]

Both of these methods do not require the protection of the nucleoside and the reaction proceeds in one pot without isolating any intermediates. Yet, the second method applying inorganic phosphate, yields both nucleoside-5′-triphosphate and diphosphate analogues.

Although the Ludwig procedure[26] is currently the most common for the preparation of many nucleotide analogues, the yields are often low because of the formation of various byproducts.[7,30] Furthermore, these byproducts complicate the isolation of the desired NTP product. Ludwig reported 86% yield of the adenosine 5′-triphosphorylation reaction.[26] Yet, the purity of the product was judged in that report by thin layer chromatography (TLC) only. In Ludwig's procedure[26] the only reported byproduct was adenosine 3′-triphosphate that was formed in less than 0.5% yield.

However, byproducts obtained in this reaction can be detected only by more contemporary analytical methods such as ^{31}P NMR and mass spectrometry (MS). These byproducts were identified by Knoblauch[7,30] for a uridine nucleoside analogue as the corresponding 2′-monophosphate; 3′-monophosphate; 5′-monophosphate; 3′-monophosphate; 5′–monophosphate; 2′,3′-cyclo-monophosphate; 2′,3′-cyclophosphate-5′-triphosphate; and trimetaphosphate.

Most of the above-mentioned methods for nucleoside 5′-triphosphorylation, suffer limitations such as several synthetic steps, moderate yields, and the formation of various byproducts, which complicate the isolation of the NTP product. The limitations of the currently available synthetic methods and especially the difficulty to purify the product from similar byproducts prompted us to optimize the nucleoside-5′-triphosphorylation reaction.

We selected the Ludwig 5′-phosphorylation procedure for optimization as it is a one pot reaction, with no need of isolating the reaction intermediates, and of protection of the 2′- and 3′-ribose hydroxyl groups. To optimize the reaction procedure and decrease by-products formation we modified several parameters such as solvent, time, temperature, and pyrophosphate salt. Here, we report on an improved nucleoside 5′-phosphorylation method. By decreasing reaction temperature and prolonging the second step of the reaction, we obtained pure NTP product in a good yield after liquid chromatography (LC) separation with no further need for high performance liquid chromatography (HPLC) purification.

RESULTS AND DISCUSSION

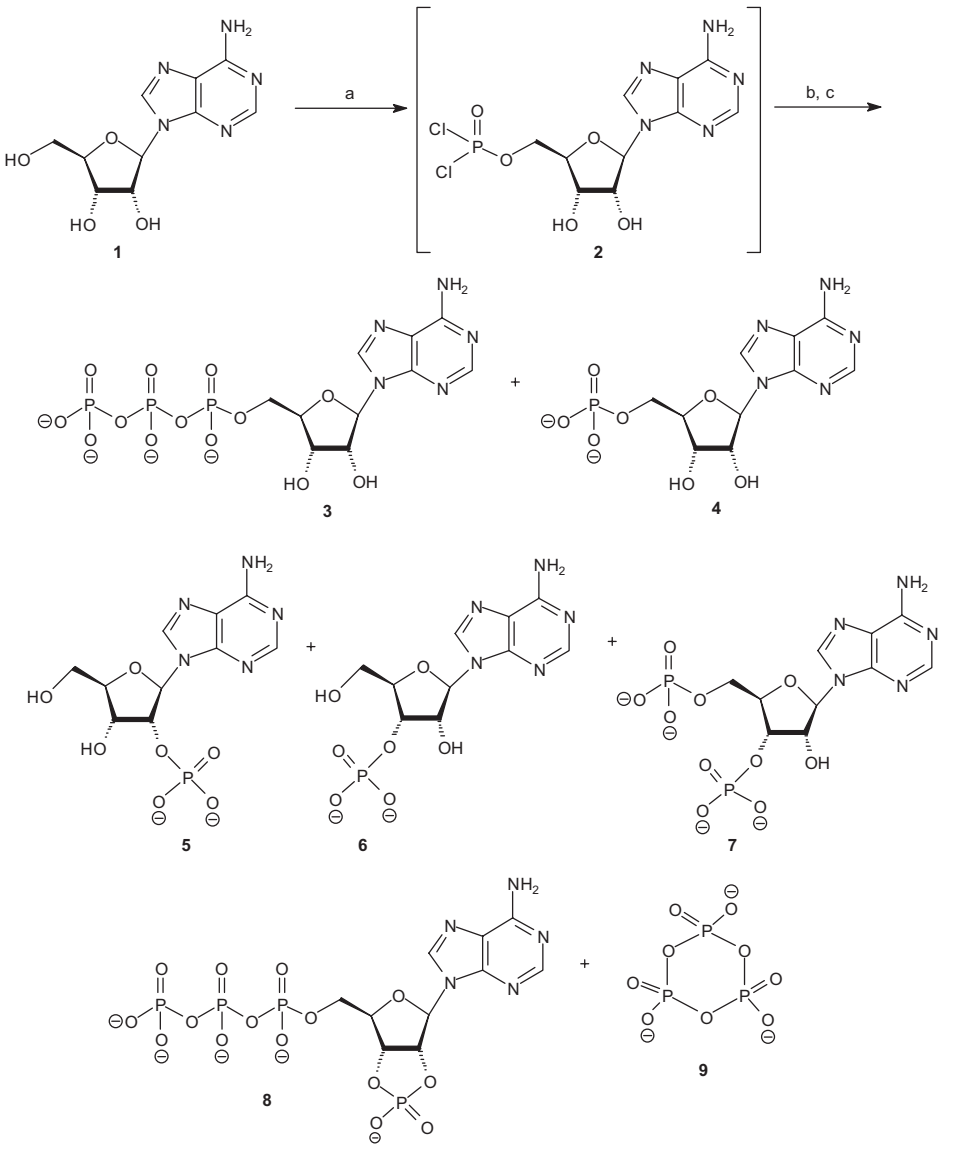
When we repeated Ludwig's one-pot triphosphorylation procedure, using adenosine, **1,** as the starting material, we identified several major byproducts, **4–9**, in addition to the product ATP, **3** (Scheme 1).

^{31}P NMR spectrum of compound **8** shows four signals three of which correspond to the 5′-triphosphate group: −10 (Pγ), −11 (Pα), and −23 (Pβ) ppm and a fourth signal at +20 ppm, integrating as one phosphorus atom, representing the 2′,3′-cyclic phosphate.[7]

^{31}P NMR spectrum of trimetaphosphate, **9**, shows one signal for all three phosphorus atoms at −20 ppm, while that of 5′- or 2′- or 3′-monophosphate, **4–6**, shows a signal at 3 to −5 ppm. Nucleoside-5′-monophosphate, **4**, is the major byproduct (ca. 60% yield), due to hydrolysis of nucleoside 5′-dichlorophosphoridate which did not react further with the pyrophosphate salt. Yet, byproduct **4** does not complicate the isolation of nucleoside-5′-triphosphate, **3**, as do byproducts, **8**, **9**, due to their similar charge to that of **3**.

Byproducts **8** and **9** are formed in the second step of the triphosphorylation reaction (Scheme 1) when dimethylformamide (DMF) is added to the reaction mixture, as DMF reacts with $POCl_3$ remaining from the first step, to produce Vilsmeier reagent[31] (Schemes 2 and 3). Intermediate, **10**, is more reactive than phosphoryl oxychloride and reacts with the nucleosides 2′ and 3′ hydroxyl groups, to form nucleosides 2′,3′-cyclic phosphate, **8**. Byproduct, **9**, is formed when Vilsmeier reagent, **10**, undergoes a nucleophilic attack by pyrophosphate followed by hydrolysis (Scheme 3).

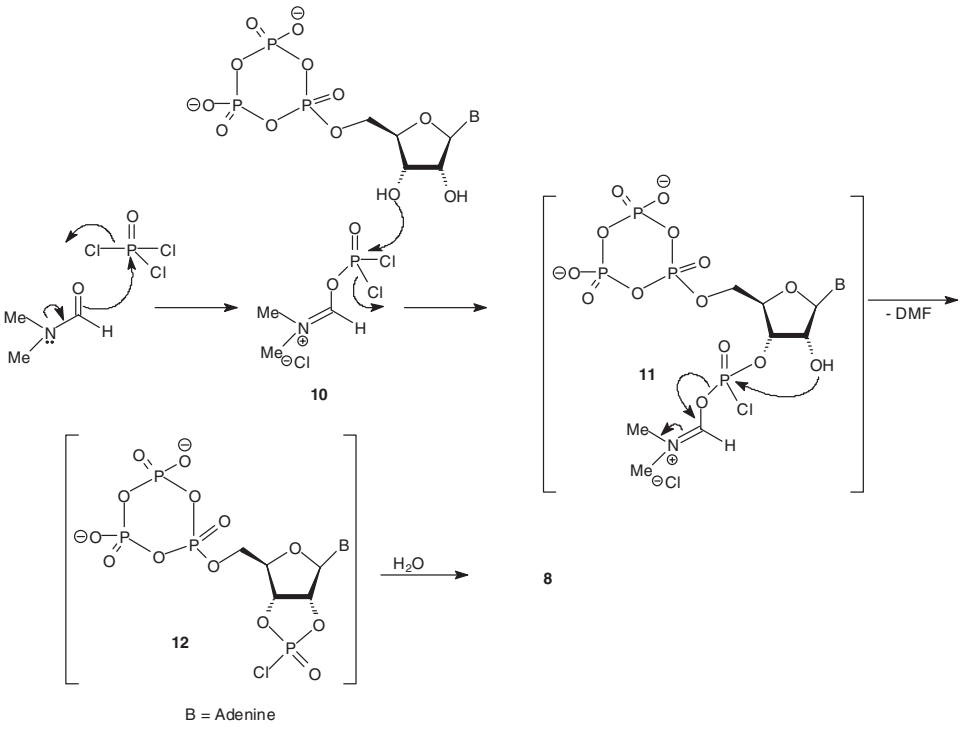
In an attempt to reduce or even eliminate the formation of byproducts **8** and **9,** we tried to optimize the following parameters of the 5′-triphosphorylation reaction: solvent, nucleophilicity of the pyrophosphate reagent, temperature, and time.



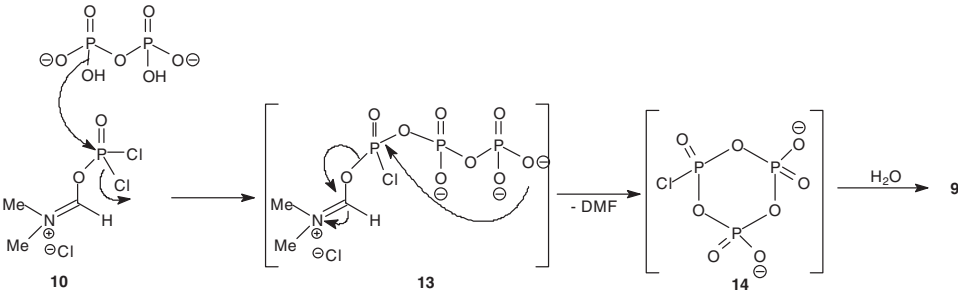
SCHEME 1 Reagents and conditions: a. $(MeO)_3PO$, proton sponge, $POCl_3$, 2 hours, 0°C; b. $(HNBu_3)_2H_2P_2O_7$, NBu_3/DMF, 1 minute, 0°C; c. TEAB, RT, 45 minutes.

Selection of Solvent

The nature of the solvent has an important role in the 5′-monophosphorylation reaction. It should dissolve all of the components of the reaction: unprotected nucleoside, phosphorylation reagent and base, and it should be dry, aprotic, and inert to the reagents. Yet, unprotected nucleoside is not soluble in common organic solvents. Polar solvents such



SCHEME 2 Formation of adenosine 5′-triphosphate, 2′, 3′-cyclic phosphate, **8**.



SCHEME 3 Formation of trimetaphosphate, **9**, from pyrophosphate and Vilsmeier reagent.

as DMF or dimethyl sulfoxide (DMSO) are also not useful as they react with the phosphorylating reagent, phosphoryl oxychloride.

Recently, 5′-monophosphorylation in acetonitrile was reported,[32] however our attempts to dissolve both adenosine and phosphoryl oxychloride in acetonitrile have failed. Other aprotic solvents such as: dioxane and triethyl amine did not dissolve the reaction mixture either.

Previously, we reported that reaction of adenosine with $PSCl_3$ is accelerated in pyridine[33] (15 minutes) as compared to the corresponding reaction in triethyl phosphate (12 hours),[34] due to activation of $PSCl_3$ by pyridine.

Here, we performed the reaction by treating adenosine with $POCl_3$ in pyridine at 0°C, for 45 minutes, followed by the addition of pyrophosphate in DMF and hydrolysis. The crude reaction mixture was analyzed by mass spectrometry. A 9-phosphate-membered cycle and pyridinium polyphosphate were identified as products indicating that most of the pyrophosphate salt was polymerized and only a minute amount of adenosine triphosphate (ATP) was formed.

Trimethylphosphate was suggested to accelerate monophosphorylation of nucleosides,[28,35] and interactions of the trimethylphosphate with $POCl_3$ were proposed to form an active intermediate.[36,37] Therefore, the solvent of choice remained trimethyl phosphate.

Attempts to Improve the Nucleophilicity of Pyrophosphate

The commonly used 5′-triphosphorylation protocol involves the addition of 7 equivalents of pyrophosphate tri-n-butylammonium salt dissolved in DMF to the dichlorophosphoridate intermediate, **2**. We found that despite the large excess of pyrophosphate commonly used for the preparation of NTP only ca. 50% of the adenosine 5′-dichlorophosphoridate, **2**, is converted to triphosphate, **3**. This observation indicates that either the pyrophosphate is not a strong nucleophile, or that the reaction time (1 minute)[26] is not long enough. To test this hypothesis on the limited nucleophilicity of pyrophosphate we prepared a solution of $H_2P_2O_7(Bu_4N)_2$ salt in DMF. Indeed, with the more hindered counter-ion of pyrophosphate the major product was AMP rather than ATP (AMP:ATP ratio was 2:1) as judged by ^{31}P NMR.

Yet, attempts to improve the pyrophosphate nucleophilicity by preparing "naked" pyrophosphate from $P_2O_7H_2K_2$ and 18–6 crown ether, were not successful, as the mixture of pyrophosphate potassium salt and crown ether 18–6 was not soluble in DMF. Attempts to improve the solubility of the pyrophosphate potassium salt in DMF led us to the preparation of the mixed potassium, tetra butyl ammonium salts $(P_2O_7)K_2(Bu_4N)_2$ or $(P_2O_7)K(Bu_4N)_3$. Yet, the mixture of these salts and crown-ether 18–6 did not dissolve in DMF either.

Optimization of the Time and Temperature of the Reaction

According to Ludwig's 5′-triphosphorylation protocol, the second step of the reaction (step b, Scheme 1) is performed for 1 minute at 0°C. The low temperature of the reaction allows it to be more selective towards phosphorylation at 5′ versus 2′ and 3′ positions, and the short reaction time is supposed to decrease byproduct formation. Yet, as mentioned above, a variety of byproducts are still formed. Thus, to minimize byproduct formation we lowered the reaction temperature and increased the reaction time. We

TABLE 1 Reaction conditions and distribution of several byproducts as determined by ^{31}P NMR

Entry	Temp (°C)	Time (minutes)	4	8	9	3
1	0	4	2.83	0.185	0.65	1
2	−10	4	3.02	0.11	0.25	1
3	0	10	3.39	0.165	0.45	1
4[a]	−15	10	1.94	0.016	0.32	1
5	−15	120	0.71	0	0	1

[a]Two equivalents of $POCl_3$ were added instead of 1.2 equivalents.

first performed the 5′-phosphorylation of adenosine with $POCl_3$ (1.2 equivalents) and proton sponge (3 equivalents) at −10°C for 2 hours (first step) followed by the addition of $(H_2P_2O_7(Bu_3NH)_2)$ and mixing at 0°C for 4 minutes (second step). The reaction mixture was analyzed by ^{31}P NMR. Indeed, the amount of byproducts **8** and **9** (Table 1, entry 2) was decreased, but the ratio AMP:ATP remained about the same, as compared to entry 1 (regular Ludwig's reaction conditions). We concluded that a low temperature at the second step of the reaction is required to decrease by-products formation.

When we further decreased the temperature (−15°C) and increased reaction time (10 minutes) of the second step, maintaining temperature below −10°C at the pyrophosphate addition (entry 4) by-product formation further decreased and AMP:ATP ratio was about 2:1. Namely, maintaining a low temperature of both steps of the reaction is required for decreasing byproduct formation. We concluded that the optimum reaction temperature is −15°C for both steps of the reaction.

To optimize the reaction time of the second step of the reaction, we monitored the time-dependent distribution of the by-products using ^{31}P NMR. The reaction was performed at −15°C, NMR samples were taken from the reaction at 10, 60, 120, and 180 minutes, and analyzed by NMR. Thus, ^{31}P NMR spectrum of the 5′-triphosphorylation of adenosine showed after 6 minutes the AMP:ATP ratio of 1.7:1. Moreover, no trimetaphosphate, **9,** and 2′,3′-cyclic-AMP-5′-triphosphate, **8**, by-products were observed, unlike the products obtained under the Ludwig conditions (Figure 1A). An optimal ratio of AMP:ATP 0.71:1 was observed after 2 hours and no additional by-products were detected (Figure 1B). Thus, the optimal reaction time and temperature for the second step of 5′-triphosphorylation of adenosine, 2 hours and −15°C, resulted in 51% yield of ATP.

Optimization of the Reaction Conditions for U, G, and C Nucleosides

These optimized reaction conditions were applied also to other nucleosides besides adenosine and the reactions were monitored by ^{31}P

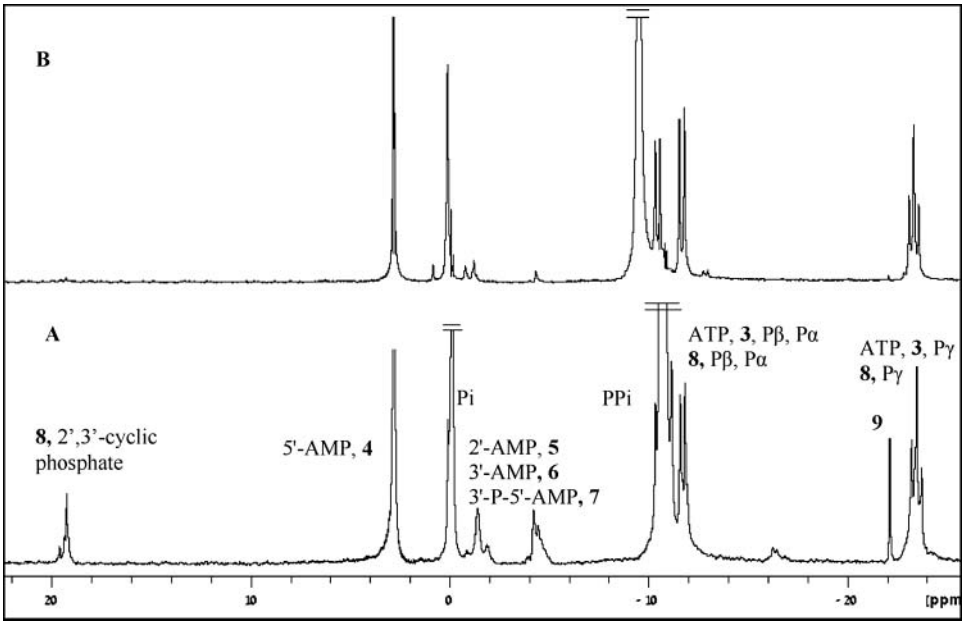


FIGURE 1 A) ^{31}P NMR of crude mixture obtained with the original Ludwig's 5′-triphosphorylation conditions; B) ^{31}P NMR of crude mixture obtained with the improved 5′-triphosphorylation conditions.

NMR. Indeed, for uridine the UMP:UTP ratio after 2 hours for the second step at −15°C was 0.8:1 (isolated yield 55%), while for guanosine GMP:GTP ratio was 0.62:1 (isolated yield 54%). However, the reaction with cytosine resulted in a complex products mixture. The cytosine exocyclic amine reacts with $POCl_3$ and produced additional byproducts, such as N^4-monophosphate.[38,39] Namely, the 5′-triphosphorylation of cytosine requires N^4-protected cytosine, for example, 4-dimethylamidine N-protected cytosine.[40] 5′-Triphosphorylation of cytidine provided a protected CMP:CTP ratio of 0.55:1 at −15°C for 1 hour (isolated yield 61%).

Utilization of the New Reaction Conditions for Synthetic Nucleosides

When applying Ludwig's conditions to natural nucleosides, the product was possible to isolate by HPLC only; however, applying these conditions to synthetic nucleosides such as N^6-methylamino- and N^6, N^6 -dimethylamino- adenosine resulted in a very complicated mixture nearly impossible to isolate by HPLC. Thus, applying our optimized conditions to 5′-phosphorylation of N^6- and N^6, N^6-dimethylamino-adenosine analogues resulted in obtaining pure products in 74% and 73% yield, respectively, after a facile LC separation.

Conclusion

We propose here conditions for an efficient one-pot conversion of various nucleosides to the corresponding 5′-triphosphate analogues. Furthermore, our conditions inhibit completely the formation of by-products **8** and **9,** which not only reduce the reaction yield but make the ion-exchange LC purification of the final product almost impossible due to their number of charges which is similar to that of the product. Under our conditions the only by-product is NMP, which is easily separated from the NTP product. Thus, by reducing the temperature to −15°C instead of 0°C and by prolonging the reaction time after pyrophosphate salt addition to 2 hours, instead of 2 minutes, we were able to obtain nucleoside-5′-triphosphate analogues in good yields after a facile LC separation (51–74% yields) with no further need of HPLC purification. This finding is not trivial as most of the byproducts formed in the original Ludwig procedure make it impossible to obtain a pure product after LC separation. The current method is compatible with all natural nucleosides and is also applicable to synthetic nucleoside derivatives.

EXPERIMENTAL

General

Crude reaction mixtures and pure products were analyzed by ^{31}P NMR using Bruker AC-200 and DPX-600 spectrometers. ^{31}P NMR spectra was recorded in D_2O (pD 8), using 85% H_3PO_4 as an external reference. Nucleotides were also analyzed by mass spectrometry using negative fast atom bombardment (FAB) and were desorbed from a glycerol matrix at low resolution. Phosphoryl oxychloride was freshly distilled. Trimethyl phosphate was dried over molecular sieves 4Å. Nucleoside and proton sponge were dried overnight in a vacuum oven. All reactions were performed in flame-dried, argon-flushed, two-neck flasks sealed with rubber septa, and phosphoryl oxychloride was introduced with a syringe. Primary purification of the nucleotides was achieved on an LC (Isco UA-6, Lincoln, NE, USA) system using a column of Sephadex DEAE-A25, swollen in 1 M $NaHCO_3$ at 4°C for 1 day. The resin was washed with deionized water before use. The LC separation was monitored by ultraviolet (UV) detection at 280 nm. Ammonium bicarbonate gradients of 0–0.2 M (300 mL water and 300 mL NH_4HCO_3) followed by a gradient of 0.2–0.4 M (300 mL of each 0.2 and 0.4 NH_4HCO_3) were applied. The purity of the nucleotides was evaluated on HPLC (Hitachi, Schaumberg, IL, USA) system equipped with UV detector with fixed wavelength (263 nm). An analytical reverse-phase column (Gemini 5u, C-18, 110A, 150 × 4.60 mm, 5 μm, Phenomenex, Torrance, CA, USA) was used in two different solvent systems. 1) A: CH_3CN and B: triethylammonium acetate (TEAA) pH 7. An

isocratic elution was applied (5:95), flow rate 1 mL/min; 2) linear gradient of phosphate buffer/CH_3CN 95:5 to 90:10, flow rate 1 mL/min.

5′-Triphosphorylation Reaction—A Typical Procedure

Nucleoside (0.37 mmol) was dissolved in trimethyl phosphate by slightly heating the turbid solution. The clear solution was then cooled to −15°C and dry proton sponge was added (287 mg, 3 equiv.). The solution was stirred for 20 minutes. Phosphoryl oxychloride was added dropwise (40 μL, 0.44 mmol, 1.2 equiv.). After 2 hours at −15°C a mixture of Bu_3N (0.3 mL) and 1 M $H_2P_2O_7(Bu_3NH)_2$ in DMF (2.6 mL, 2.6 mmol, 7 equiv.) was added. After 2 hours at −15°C, a solution of 1 M TEAB (pH 8) was added (15 mL), and the clear solution (pH 7) was stirred at room temperature for 45 minutes and freeze-dried. The semisolid obtained, dissolved in a minimal volume of water, was separated at room temperature on a Sephadex DEAE-A25 column as described in the General section above. The relevant fractions were pooled and repeatedly freeze-dried to yield a white powder. The nucleoside-5′-triphosphates were obtained in >95% purity, as judged by HPLC (as described in General), without any further need for separation on HPLC.

Yields of NTP analogues obtained this way include the following. ATP:103.3 mg, 0.185 mmol; 51%, starting from adenosine; UTP: 107 mg, 0.2 mmol; 55%, starting from uridine; GTP: 120 mg, 0.21 mmol; 54%, starting from guanosine; 2-dimethylamidine-CTP 133 mg, 0.226 mmol; 61%, starting from 2-dimethylamidine-cytidine. N^6-Methylamino ATP was obtained in 74% yield. N^6, N^6 -Dimethylamino ATP was obtained in 73% yield. Spectroscopic data for the above NTP analogues are consistent with literature data.[41,42]

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