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A new chemical method of synthesis of modified nucleoside [^{32}P]phosphates

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A simple method of chemical phosphorylation for modified nucleosides with [^{32}P]orthophosphoric acid in the presence of BrCN is described. The yields of 5′-[^{32}P]nucleoside monophosphates achieved are 50–65% at nominal specific radioactivities of ca. 1000 Ci/mmol. The mechanism of phosphorylation in the presence of heterocyclic amines is studied.

Keywords: modified nucleosides; [^{32}P]orthophosphoric acid; phosphorylation; synthesis of [^{32}P]labeled nucleotides

Introduction

Phosphorus-bearing compounds are widely spread and are involved in numerous important cellular processes. Isotope-labeled nucleoside 5′-triphosphates have been used for many years as tools for studying various biochemical processes, particularly, of nucleic acids. The interest in such compounds has increased recently due to the discovery of antiviral activity of a number of modified nucleosides. The active forms of most of these compounds are the corresponding 5′-triphosphates. The use of ^{32}P or ^{33}P labeling enables the studies of both intracellular transformations and metabolic pathways of these compounds to be performed with high sensitivity. Today, labeled phosphorylated nucleosides are mainly obtained using enzymatic procedures, for example, the method of Johnson and Walseth for 5′-[^{32}P]phosphates of all natural nucleosides.[1] The method is based on 5′-phosphorylation of nucleoside 3′-phosphates with [γ-^{32}P]ATP in the presence of phage T4 polynucleotide kinase (PNK T4) followed by removal of the 3′-phosphate group with nuclease P_1. PNK T4 can phosphorylate all natural nucleoside 3′-phosphates. However, if 3′-phosphorylation is impossible due to specific features of the nucleoside structure, this approach is inapplicable.

While enzymatic methods are highly selective and provide high product yields, they cannot be used for the introduction of phosphate groups into nucleosides modified at the sugar residue. Therefore, development of effective phosphorylation procedures for modified nucleosides is an important radiochemical task.

Phosphorylation of nucleosides with phosphorus oxychloride is the most effective chemical method of preparation of nonlabeled nucleoside phosphates. The product yields are high and insensitive to the nucleoside structure.[2] However, when applied to radioactive synthesis there were insurmountable problems. In particular, the direct preparation of high specific activity phosphorylating agent by irradiation of $POCl_3$ in an (n, γ) reaction is impossible. The synthesis of $^{32}POCl_3$ based on [^{32}P]orthophosphoric acid (a readily available source of ^{32}P) and nonlabeled phosphorus pentachloride resulted in considerable isotope dilution of the product.[3] Additionally, low yields and the high volatility of the radioactive agent militate against $^{32}POCl_3$ as a phosphorylating agent for the synthesis of labeled nucleotides. Various carbodiimides are used for activation of phosphorus components; but under the conditions of radioactive synthesis, where low concentrations of the radioactive component are common, the reaction rates are so low that satisfactory yields can only be achieved after 24 h. Numerous radiolysis products accumulated in the reaction mixture during this period hampering isolation of the target product. In addition, the radioactive component is only partially consumed. Another published procedure involves activation of phosphate groups with 4-dimethylaminopyridine (DMAP) or *N*-methylimidazole (MeIm) by formation of the corresponding phosphoramidate.[4] These compounds are highly reactive, but their preparation is based on the use of an oxidative–reductive pair Py_2S–Ph_3P and is characterized by low reaction rates and formation of numerous side products.[4]

Comparing the efficacy of coupling agents (carbodiimides, 2,4,6-triisopropylbenzenesulfonyl chloride, trichloroacetonitrile and BrCN) in the reaction of 5′-phosphorylation of 3′-azido-3′-deoxythymidine (AZT) with $H_3^{32}PO_4$, we showed that (in this series) BrCN was most effective.[5] It is noteworthy that BrCN had been used earlier for oligonucleotide synthesis.[6]

The aim of the current study was to evaluate the generality of the method and to determine its limits for phosphorylation of unnatural nucleosides and their analogues. We were also interested in the selectivity of phosphorylation of primary hydroxyl groups relative to amino groups in nucleic bases. In addition, we wanted to confirm that thia groups are unreactive

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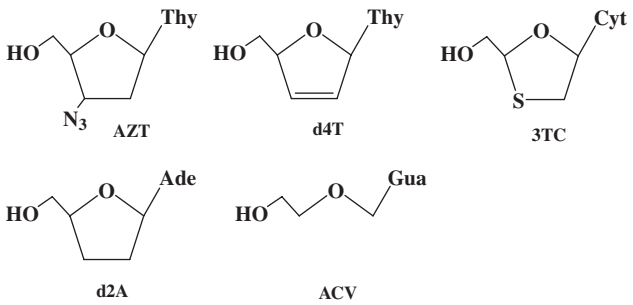
to the given conditions and that there no adverse interactions between BrCN and unsaturated bonds.

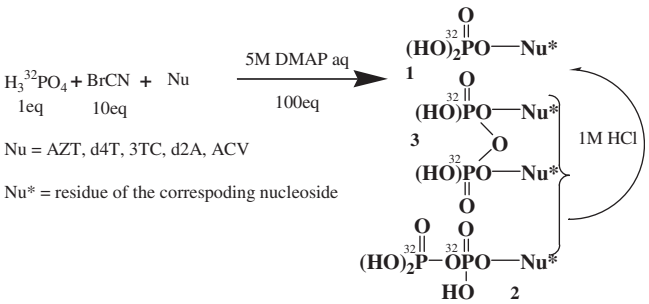
We studied BrCN-mediated phosphorylation of several nucleoside-based antiviral agents, whose monophosphates are inaccessible by enzymatic or biochemical methods. These are AZT, 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), 2′,3′-dideoxyadenosine (d2A), 2′-deoxy-3′-thiacytidine (3TC) and acyclovir (ACV). We also studied the mechanism of this reaction in the presence of heterocyclic amines.

Results and discussion

The target phosphates were synthesized under the conditions used for the synthesis of AZT[5] with a small modification: DMAP rather than pyridine was used as a base (Scheme 1). The ratios of components relative to 1 eq of phosphoric acid was Nu:BrCN:DMAP = 10:100:100; the reaction time was 1–2 h (for ACV, 24 h). Nucleoside diphosphates **2** and bis(nucleoside) diphosphates **3** were isolated as side products. Hydrolysis of these compounds with 1 M HCl increased the yields of resulting nucleoside 5′-[^{32}P]phosphates **1** by 5–10 %. Total yields of **1** of 50–65% were achieved, with ACV monophosphate as an exception. The low solubility of ACV in aqueous–organic mixtures prevents a high concentration in the reaction mixture, which results in a low reaction rate. In addition, ACV is partially degraded in the presence of BrCN. Our attempts to increase ACV solubility by decreasing the amount of water resulted in negligible yields of target monophosphates. The addition of dimethylformamide (DMF) while retaining the H_3PO_4:H_2O ratio led to significant ACV degradation. The yield of ACV monophosphate did not exceed 10% even after 24 h.



Structure 1



Scheme 1

We also studied the selectivity of phosphorylation of hydroxyl groups in nucleosides bearing amino groups. The UV spectra of phosphorylation products of 3TC and d2A recorded at varying pH showed that the amino groups remained intact. Therefore, NH_2- groups need not be protected in these reactions. Moreover mass spectrometry and NMR data also demonstrated that the 3TC thia group was unmodified. We also showed that no addition of BrCN to the d4T double bond occurred.

Using natural thymidine, uridine and adenosine we studied the selectivity of (2′)3′/5′-phosphorylation. The reaction was performed under the conditions similar to the synthesis of compound **1**, but with a 20-fold excess of the nucleoside component. Phosphorylation parameters are given in Table 1. Clearly, phosphorylation of thymidine and 2′-deoxyuridine was unselective, the product ratio merely reflecting the number of primary and secondary hydroxyl groups. Similar results were obtained for adenosine, in which the ratio of primary:secondary hydroxyl groups is 2:1.

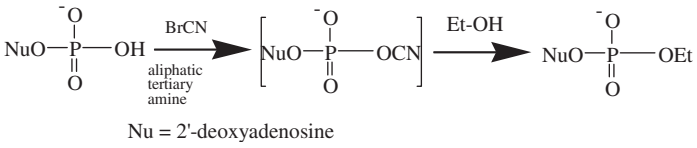
In a previous study[7] it was shown that a tertiary amine is a necessary component in the phosphorylation reaction activated by BrCN. The mechanism of activation of the phosphorus component and the formation of the intermediate cyanate[6] are shown in Scheme 2. The authors indicated that the cyanate is highly reactive and is rapidly hydrolyzed by water giving symmetrical bisnucleoside diphosphates of type **3** in anhydrous conditions.

In our previous study we observed no significant changes in either reaction kinetics or in phosphate yields.[5] Advantages of pyridine over other tertiary amines were increased solubility of nucleotides in aqueous–pyridinium systems and the higher stability of pyridine in the presence of BrCN. In this work we studied the effects of amines on the reaction rate and the extent of phosphorylation using AZT as a model substrate. The amines of varying pK_a given in Table 2 were used.

Table 1.

	Ratio of phosphorylation products (%)		
Nucleoside	5′-Phosphate	3′-Phosphate	3′,5′-Diphosphate
Thymidine	53	44	3
2′-Deoxyuridine	50	45	5
Adenosine	39	60[a]	1[b]

[a]A mixture of 2′- and 3′-phosphates.
[b]A mixture of 2′,5′- and 3′,5′- diphosphates.



Scheme 2

Table 2.

	Triethylamine	Py	MeIm	DMAP
pK_a	10.78	5.17	6.95	9.70

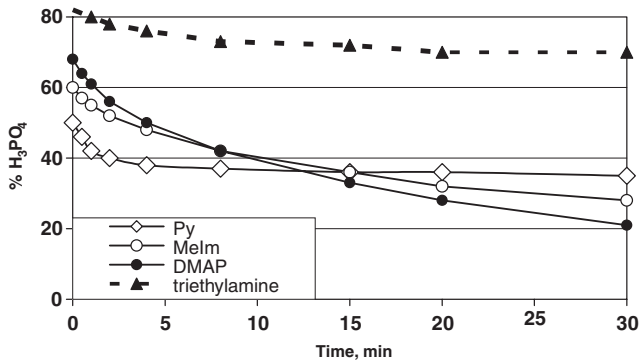
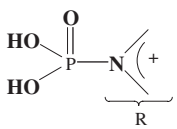


Figure 1. Dynamics of consumption of H_3PO_4 in the reaction of AZT phosphorylation (detection by radioactivity). The data are obtained by autoradiographic scanning of a TLC plate.



4a R = DMAP
4b R = MIm
4c R = Py

Structure 2

Kinetic analysis of curves of the phosphorylation reaction shows that with aromatic amines the higher the pK_a value, the higher the conversion of phosphoric acid and the slower the reaction. With triethylamine, conversion of phosphoric acid is the poorest, although triethylamine has the highest pK_a of the amines studied.

In the presence of DMAP or MeIm, phosphoric acid is consumed even after 10–15 min (Figure 1), which does not agree with low stability of both BrCN and of intermediate cyanate in aqueous solutions. The half-life of BrCN in 0.25 M aqueous solution of *N*-morpholinoethanesulfonate (pH > 7.5) is less than 1 min.[6] Since the reaction rate was lower in the presence of heterocyclic amines than in the case of aliphatic tertiary amines, we were able to analyze the composition of the intermediate reaction products in the presence of DMAP or MeIm. Using reverse phase high performance liquid chromatography (HPLC), we detected a hydrophobic compound bearing ^{32}P in both reactions at early time points (1–2 min). The amount of this intermediate varied with time. Its retention factor on thin layer chromatography (TLC) was slightly lower than that of the corresponding phosphate. The ^{1}H NMR spectrum contained no new resonances in addition to those of the starting aromatic amine. In the ^{31}P NMR spectrum, resonances at −8.1 ppm for a DMAP derivative and −9.9 ppm for a MeIm derivative were detected, consistent with for phosphoroamidates of type **4**. Authentic compounds **4a** and **4b** were prepared and characterized as previously described,[4] and were identical to the compounds isolated from the phosphorylation reaction and the published data. We were unable to isolate and characterize intermediate **4c** as it dimerizes under anhydrous conditions and is highly moisture-sensitive and rapidly hydrolyzed. This behavior of phosphoroamidate **4c** agrees with the published data.[7]

We propose the mechanism of nucleoside coupling with phosphoric acid in the presence of BrCN and a heterocyclic amine as shown in Scheme 3.

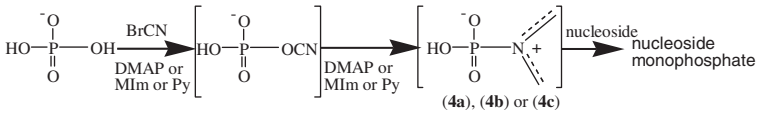
Thus, if the solubility of compounds is very low in organic solvents, the major reaction product is a phosphoroamidate of type **4** (as was observed in the case of ACV) (Table 2). An increase in the reaction time has negligible affect on the phosphate yield, since the water contained in the reaction mixture hydrolyzes phosphoroamidate **4** prior to its interaction with the nucleoside (Table 3).

It is noteworthy that the use of BrCN in phosphorylation reactions enables further esterification of the resulting phosphates without their isolation provided that the second alcoholic component is able to be added in approximately a 3–5-fold excess in the reaction mixture. This approach was used for the two-step synthesis of AZT 5′-(cholinium [^{32}P]phosphate) from $H_3^{32}PO_4$ and choline followed by addition of excess AZT.[8]

To summarize, we have developed an effective chemical method of synthesis of the phosphorylated forms of modified nucleosides using $H_3^{32}PO_4$ as a phosphorus donor and BrCN as an activating agent. The approach uses aqueous solutions, which is especially important for nucleosides with low solubility in anhydrous organic solvents. It was found that nucleic base amino groups are unaffected in the course of such a phosphorylation and, hence, these groups need not be protected. [^{32}P]phosphorylation of thia- and double bond-containing nucleosides is also possible with this method.

Experimental

Five molar BrCN in acetonitrile, triethylamine and H_3PO_4 were obtained from Merck; DMAP, MeIm and pyridine from Fluka; [^{32}P]orthophosphoric acid (8000 Ci/mmol) was from the Institute of Reactor Materials (Zarechny, Sverdlovskaya obl.); AZT and 3TC were a kind gift of AZT Association (Moscow, Russia).



Scheme 3

Table 3.

Nucleoside	Ratio of phosphorylation products (%)					Yield of 5′-phosphate (%)[a]
	5′-Phosphate (**1**)	5′-Diphosphate (**2**)	Bisnucleoside diphosphate (**3**)	(**4a**)	P_i	
AZT	55	3	14	11	10	62
d4T	58	4	10	12	9	65
3TC	53	3	5	9	24	59
d2A	46	1	11	18	20	50
ACV[b]	7	<1%	<1%	82	~10	7
ACV[c]	9	<1%	<1%	<1%	~90	9

[a]After treatment of the reaction mixture with 1 M HCl.
[b]Reaction time 4 h.
[c]Reaction time 24 h.

UV spectra were recorded on a Shimadzu UV-1201 spectrophotometer (Japan). ^{1}H NMR and ^{31}P NMR spectra (with proton–phosphorus decoupling, 85% H_3PO_4 as an external standard) were recorded on a Bruker AMX III-400 spectrometer with working frequencies 400 and 162 MHz, respectively. HPLC was performed on a Gilson chromatograph (France) supplied with UV detector type 115 and flow radioactivity detector type 170, on a Lichrosorb RP-18 (5 μm) column (4 × 150 mm) eluted in a ion-pair mode; flow rate 0.5 ml/min; solution A: 50 mM triethyl ammonium bicarbonate; solution B: 75% ethanol; flow rate 0.5 ml/min. The following gradient was used: 0–5 min (0% B), 5–10 min (0→20% B), 10–30 min (20→30% B). TLC was carried out on Kieselgel 60 F254 plates (Merck, Germany) in the following systems: chloroform–methanol 9:1 (system A) and dioxane–iPrOH–water–25% aqueous NH_3 3:3:3:1 (system A). Radioactive products were detected using Instant Imager electronic autoradiography (Packard Instrument Company, USA). The reaction mixtures were quantified using the above instrument and the supplied programs. UV and NMR spectra were recorded using the products obtained in the reactions with indicator quantities of $H_3^{32}PO_4$ (molar activity 0.1 Ci/mmol). Mass spectra were recorded on a COMPACT MALDI-4 mass spectrometer (Kratos Analytical) using the products stored at −80°C until their molar activity decreased to ~0.1 mCi/mmol.

AZT-5′-[^{32}P]phosphate

A solution of 10 mCi $H_3^{32}PO_4$ in 0.05 M HCl (20–50 μl against the specific activity) was added to a solution (10 μl) of 1 mM H_3PO_4; the solvents were removed in vacuum and the residue was dissolved in 50% aqueous pyridine (15 μl). A solution of 2 M AZT (20 μmol) in 5 M DMAP (10 μl) was added, cooled to 0°C and a solution of 5 M BrCN in acetonitrile (2 μl) was added. After 30 min the solvent was removed under vacuum, the residue was coevaporated twice with water (50 μl × 2), dissolved in water (50 μl) and purified by HPLC to give 62% of the product with nominal specific activity 1000 Ci/mmol.

d4T-5′-[^{32}P]phosphate was obtained similarly to *AZT 5′-[^{32}P]phosphate* in a yield of 65%, nominal specific activity 1000 Ci/mmol.

d2A-5′-[^{32}P]phosphate was obtained similarly to *AZT 5′-[^{32}P]phosphate* by interaction of 0.5 Ì d2A (5 μmol) solution (10 μl) in 5 M DMAP. The yield 57%; nominal specific activity 1000 Ci/mmol.

3TC-5′-[^{32}P]phosphate was obtained similarly to *AZT 5′-[^{32}P]phosphate* by interaction of 0.5 Ì d2A (5 μmol) solution (10 μl) in 5 M DMAP. The yield 59%; nominal specific activity 1000 Ci/mmol.

Phosphamides (4a) and (4b)

Phosphamides (**4**) were obtained by the reaction of DMAP or MeIm and orthophosphoric acid in DMF in the presence of excesses of dipyridyl disulfide and triphenylphosphine as described in Reference 4. The yield of (**4a**) was 66%, and (**4b**) 51% relative to orthophosphoric acid.

Comparative dynamics of AZT phosphorylation in the presence of DMAP, MeIm, Py or Et_3N

A solution of 0.1 mCi $H_3^{32}PO_4$ in 0.05 M HCl (2–5 μl against the specific activity) was added to a solution (10 μl) of 1 mM H_3PO_4; the solvents were removed in vacuum and the residue was dissolved in 50% aqueous pyridine (20 μl). The solvents were removed in vacuum and the residue was dissolved in 5 M DMAP, MeIm, Py or Et_3N. A solution of 2 M AZT (20 μmol) in 5 M solution of the corresponding amine (10 μl) was added, cooled to 0°C and a solution of 5 M BrCN in acetonitrile (2 μl) was added. Aliquots (1 μl) were loaded on TLC plates and chromatographed in system B.

Acknowledgement

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