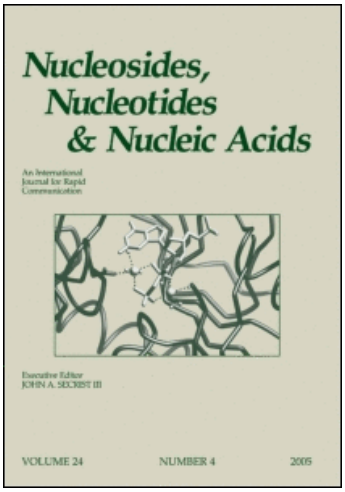

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AN ALTERNATIVE ROUTE FOR PREPARATION OF α-METHYLPHOSPHONYL-β,γ-DIPHOSPHATES OF THYMIDINE DERIVATIVES

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Abstract. An alternative "one pot" synthesis of α-methylphosphonyl-β,γ-diphosphates of thymidine and 3'-azidothymidine is proposed. p-Toluenesulphonic acid was used as desililating agent for triphosphate analogues.

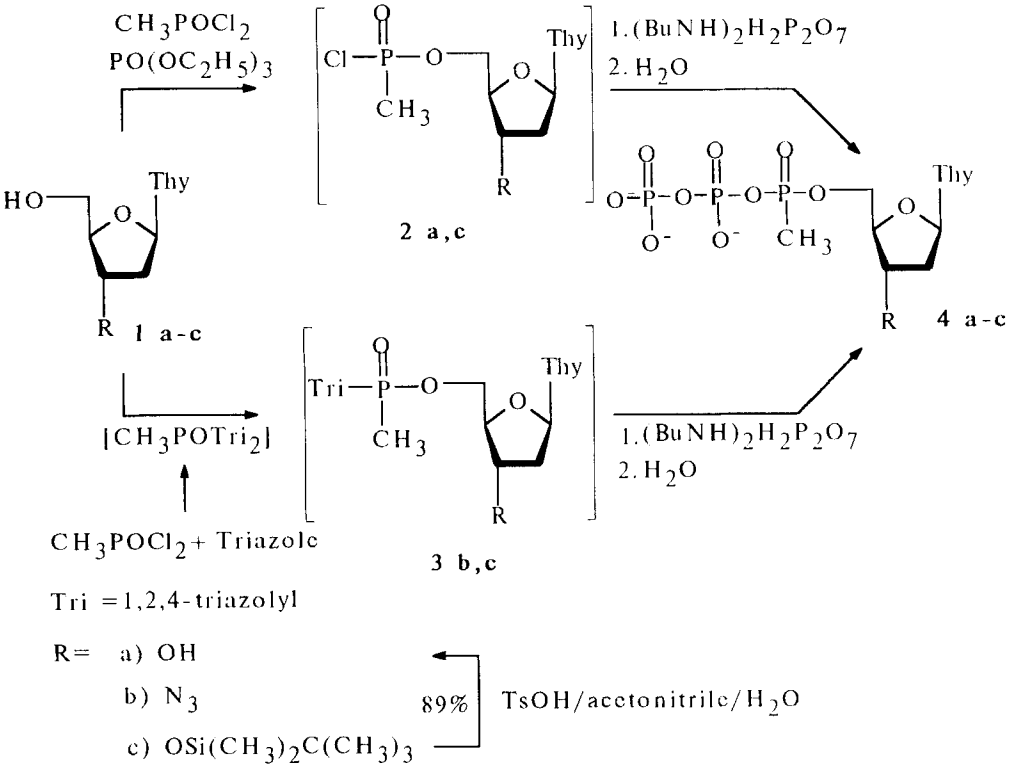
Thymidine 5'-α-methylphosphonyl-β,γ-diphosphate **4a** has been recently shown to be a substrate for terminal deoxynucleotidyltransferase[1], human placenta DNA polymerase α , reverse transcriptase of avian myeloblastosis and human immunodeficiency viruses[2]. During DNA biosynthesis catalyzed by these enzymes the thymidine 5'-methylphosphonyl residue is incorporated into a growing DNA chain, forming DNA fragments with non-ionic phosphodiester groups. The lability of α-phosphonyl-β,γ-diphosphate derivatives makes necessary to repeat its synthesis for long term biological experiments. The two step synthesis of **4a** described in [2] including preparation of thymidine 5'-methylphosphonate, its activation by N,N'-carbonyldiimidazole and condensation with tributylammonium pyrophosphate is rather time consuming and requires several chromatographic separations. In this work we propose an alternative route for synthesis of **4a** as well as 3'-azido-3'-deoxythymidine 5'-α-methylphosphonyl-β,γ-diphosphate **4b**.

We started from the methods used for preparation of nucleoside 5'-triphosphates [3] but methylphosphonic dichloride was taken instead of phosphorus oxychloride. The interaction of thymidine **1a** or 3'-azido-3'-deoxythymidine **1b** with CH_3POCl_2 in triethylphosphate was not as intensive as similar reactions of nucleosides with $POCl_3$ and was complete only in 10-15 h at 4^0C resulting in the formation of monophosphate derivatives **2a,b**.

Addition of tri-*n*-butylammonium pyrophosphate in DMF resulted in the transformation of monophosphate analogues **2a,b** into their triphosphate derivatives **4a,b**, respectively (Scheme). About 30% of **2a,b** were not involved in the reaction during the first 40 min. Further prolongation of the reaction does not result in any shift of equilibrium and does not increase the yield of **4a,b**. The nucleotides **4a,b** were isolated by ion-exchange chromatography using a linear gradient of NH_4HCO_3 and freeze-dried. It can be noticed that α-phosphonyl-β,γ-diphosphate derivatives are unstable under basic conditions. That is why some amount of nucleoside 5'-methylphosphonates formed during the freeze-drying. To purify the triphosphate analogues **4a,b** we used low pressure reversed phase chromatography in water. Two main peaks were collected. The first one contained nucleotide **4a** or **4b** and the second - corresponding thymidine or 3'-azido-3'-deoxythymidine 5'-methylphosphonates. The solution of **4a,b** was frozen, stored at -20°C and used as a stock solution for biochemical experiments. The reaction yield was estimated by measuring the optical density of **4a,b** solutions and taking the extinction coefficient of thymidine derivatives to be constant and equal to 9800. The yield of the reaction was 26%.

To reduce the reaction time and increase the yield of reaction products methylphosphonic dichloride was replaced with the appropriate triazolide as it was done before for preparation of nucleoside triphosphates [4] and α-thiotriphosphates [5]. Application of methylphosphonic *bis*(1,2,4-triazolide) requires protection of the thymidine 3'-OH group. Using acyl protection turned out to be impossible because α-phosphonyl-β,γ-diphosphate analogues were destroyed completely under alkaline conditions during the unblocking procedure, whereas inclusion of a dimethoxytrityl group prevented phosphorylation. For this reason we used 3'-O-*tert*-butyldimethylsilyl thymidine **1c** as an original compound, synthesized according to [6]. Nucleosides **1b** and **1c** were phosphorylated by triazolide in acetonitrile in 1-2 h, the reaction of **3b,c** with pyrophosphate was carried out over 40 minutes. Compounds **4b,c** were isolated as described above.

Our attempts to remove the protecting group from the triphosphate **4c** using tetrabutylammonium fluoride resulted in destruction of compound **4c**, evidently due to the high basicity of the fluorine ion. The most appropriate desilylating reagent was p-toluenesulfonic acid in aqueous acetonitrile. After deblocking **4a** was isolated by the low pressure reversed phase chromatography.



Scheme

It follows from the NMR spectra that triphosphates **4a-c** were obtained as a mixture of R and S diastereomers in equal amounts, which could not be separated by chromatographic methods in our conditions.

It is necessary to mention that DNA-polymerase catalyzed reactions require some specific criteria to substrate purity. The main point is the absence of triphosphates by-products. Small amounts of monophosphate derivatives, which can be formed under the storage of the solution play no role, as they are not recognized by DNA-polymerases. The absence of triphosphate by-products was proved by TLC, HPLC and FAB-mass analysis. In figure 1 the results of the ion-exchange HPLC analysis of compound **4a** after DEAE and reversed phase chromatography purifications are shown. The similar results were obtained for compounds **4b,c** (fig.2). The presented pictures allow us to conclude that the prepared nucleoside triphosphate analogues **4a-c** do not contain any triphosphate admixtures.

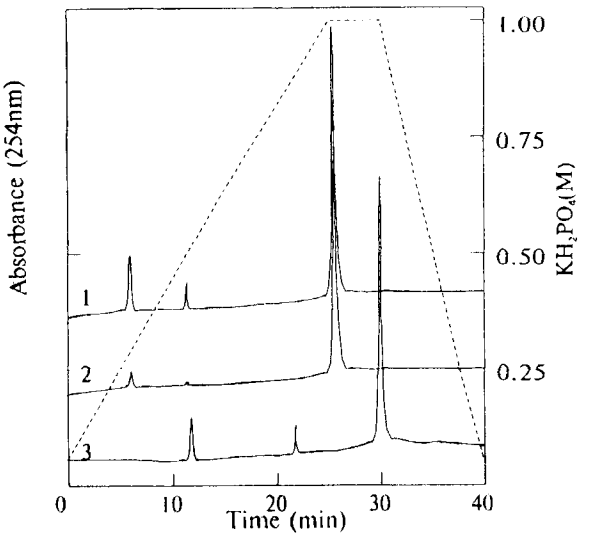


Fig.1 HPLC analysis of compound **4a** after DEAE (1) and reversed phase (2) chromatography purifications and TTP sample (3) carried out on the Hypersil APS column (5 μ, 4.6x250 mm); elution with KH_2PO_4 buffer (pH 5.5); flow rate 0.8 ml/min; detected by U.V. at 254 nm.;retention time for **4a** was 25.2 min and 30 min for TTP.

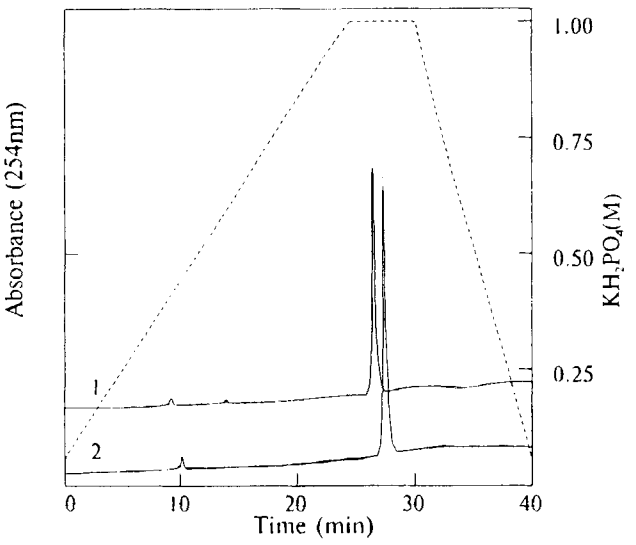


Fig.2 HPLC analysis of compounds **4b** (1) and **4c** (2) carried out on the Hypersil APS column (5 μ, 4.6x250 mm); elution conditions identical to those presented in fig. 1; retention time for **4b** was 26.7 min and 28 min for **4c**.

Experimental Section

Thin layer chromatography was carried out on the Kieselgel 60 F_{254} plates (Merck) in the systems: 2-propanol-25% NH_4OH-H_2O (7:1:2, v/v) (A) and dioxan-25% NH_4OH-H_2O (6:1:4,v/v) (B). HPLC was carried out on the columns Spherisorb C-18, 8 μ, 4x150 mm (C) and Hypersil APS, 5 μ, 4.6x250 mm (D) at the flow rate 0.8 ml/min. Elution was done with a linear gradient of acetonitrile in 0.1 M TEAB (pH 7.0) from 0 to 30% during 30 min, and with a linear gradient of KH_2PO_4 (pH 5.5) from 0.05 to 1.0 M during 25 min and 1.0 M during 5 min, respectively. For column chromatography DEAE-Toyopearl 650 M from Toyosoda (Japan) and siliconized silica gel LiChroprep RP-8 (40-63 μ) from Merck were used.

The ^{1}H-NMR spectra were registered with a Bruker Spectrospin spectrometer at 250 MHz and ^{31}P-NMR spectra at 101.26 MHz. FAB mass spectra were determined with Kratos MS 50 TC mass spectrometer.

Reaction of Nucleosides (1a and 1b) with methylphosphonic dichloride; General Procedure :

Nucleoside **1a** or **1b** (0.2 mmole) was dissolved in 2 ml of triethyl phosphate, methylphosphonic dichloride (40 μl, 0.3 mmole) was added and the mixture was kept overnight at 4°C. 0.5 M Solution of *bis*(tri-*n*-butyl ammonium) pyrophosphate (1.2 ml) was added to the mixture and 40 min later it was diluted with water up to 150 ml and put onto a DEAE (HCO_3^-) column (2.5x25 cm). The column was washed with 300ml of water and elution was carried out with linear gradient of NH_4HCO_3 from 0 to 0.4 M, pH 7.0, total volume 600 ml. Fractions eluted at 0.33-0.38 M, were freeze-dried. The residue was repurified by low pressure reversed phase chromatography on the column (2x20cm) with LiChroprep RP-8 in water. The first peak contained thymidine 5'-O-α-methylphosphonyl-β,γ-diphosphate (**4a**) (the yield was 27%) identical to that prepared earlier [2] or 3'-azido-3'-deoxythymidine 5'-O-α-methylphosphonyl-β,γ-diphosphate (**4b**).

*3'-azido-3'-deoxythymidine 5'-O-α-methylphosphonyl-β,γ-diphosphate (**4b**)*

The yield was 25%.

R_f 0.15 (A), 0.22 (B). Retention time was 18.5 min (C), 26.7 min (D).

M.S.: m/e=506 (M^+ +1), 523 (M^+ +1 +NH_3).

^{1}H-N.M.R. (D_2O/*tert*$BuOH_{int}$): δ = 7.60 (m, 1H, H-6); 6.30 (dd, 1H, $J_{1',2'a}$=2.4 Hz, $J_{1',2'b}$=6.5 Hz, H-1'); 4.60 (m, 1H, H-3'); 4.28 (m, 1H, H-4');

4.11-4.08 (m, 2H, $J_{5'a,5'b}$=12 Hz, H-5'a,b); 2.60-2.50 (m, 2H, $J_{2'a,2'b}$=11.4 Hz, H-2'a,b); 1.95 (m, 3H, C$\underline{H}_3$); 1.84 and 1.83 ppm (2 d, 3H, $J_{P,H}$=18.1 Hz, R and S diastereomers).

^{31}P-N.M.R. (D_2O/85%H_3PO_{4ext}): δ = 26.9 (d, $J_{\alpha,\beta}$ =20.5 Hz, P-α); -7.5 (br.s, P-γ); -26.2 ppm (t, $J_{\beta,\gamma}$=20.5 Hz).

Reaction of Nucleosides (1b and 1c) with methylphosphonic *bis*(1,2,4-triazolide); General Procedure:

Triazole (76 mg, 1.1 mmol) and triethylamine (81 μl, 1.1 mmole) were dissolved in acetonitrile (1.5 ml). Then methylphosphonic dichloride (70 μl, 0.5 mmol) was added and the mixture was kept for 40 min at room tempreture. The reaction mixture was centrifuged and supernatant containing methylphosphonic *bis*(1,2,4-triazolide) was added to the nucleoside **1b** or **1c** (0.2 mmole), dried by coevaporation with pyridine (3x1ml). Monitoring of the reaction was done by TLC in the system chloroform-methanol (9:1, v/v), following the disappearance of original nucleoside and formation of a substance characterized by a zero mobility. After 1.5 hour 0.5 M solution of *bis*(tri-*n*-butylammonium) pyrophosphate (1.2 ml) in DMF was added and the mixture was kept for 40 min, then the reaction mixture was adjusted to 150 ml with water and it was applied to the DEAE (HCO_3^-) column. Further isolation of the product was as in the above method. So we obtained the 3'-azido-3'-deoxythymidine 5'-O-α-methyl-phosphonyl-β,γ-diphosphate (**4b**) identical to that obtained by the above-described technique (the yield was 32%) or 3'-O-*tert*-butyldimethylsilyl-thymidine 5'-O-α -methylphosphonyl-β,γ- diphosphate (**4c**).

3'-O-tert-butyldimethylsilyl-thymidine 5'-O-α -methylphosphonyl-β,γ-diphosphate ***4c***.

The yield was 25%.

R_f 0.26 (A), 0.54 (B). Retention time 26.3 min (C) and 28.0 min (D).

M.S.: m/e=595 (M^++1), 612 (M^++1+NH_3).

^{1}H-N.M.R. (D_2O/*tert*$BuOH_{int}$): δ = 7.58 (m, 1H, H-6); 6.35 (dd, 1H, $J_{1',2'a}$=6.9 Hz, $J_{1',2'b}$= 13.7 Hz, H-1'); 4.86 (m, 1H, H-3'); 4.44-4.28 (m, 2H, $J_{5'a,4'}$=$J_{5'b,4'}$= 3.5 Hz, $J_{5'a,5'b}$= 11.6 Hz); 4.22 (m, 1H, H-4'); 2.41 (m, 2H, H-2'a,b); 1.95 (m, 3H, C$\underline{H}_3$); 1.83 (d, 3H, $J_{P,H}$= 17.1 Hz, C$\underline{H}_3$P); 0.98 (s, 9H, (C$\underline{H}_3$)$_3$CSi); 0.23 ppm (s, 6H, C$\underline{H}_3$Si).

^{31}P-N.M.R. (D_2O/85%H_3PO_{4ext}): δ = 26.2 and 26.1 (2 d, $J_{\alpha,\beta}$=21.2 Hz and 19.0 Hz, P-α, R and S diastereomers); -8.9 and -9.1 (2 br.s, P-γ, R and S diastereo-mers); -22.8 ppm (t, $J_{\beta,\alpha}=J_{\beta,\gamma}$=20.1 Hz, P-β).

Deblocking procedure for 4c

To the nucleotide **4c**, which was obtained in the experiment described above in 1 ml of water 0.5 M solution of p-toluenesulfonic acid in acetonitrile was added up to pH 1.5. After 2 hours 1 ml of pyridine was added to the reaction mixture, the solvent was evaporated and coevaporated with 2 ml of pyridine. The residue was dissolved in water (1 ml) and put onto LiChroprep RP-8 column (1.5x25 cm) and eluted with water. So we obtained **4a**, identical to that obtained before, with the yield of 89% relative to **4c**.

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REFERENCES

1. Higuchi, H., Endo, T. and Kaji, A. *Biochemistry*, 1990, **29**, 8747.
2. Victorova, L., Dyatkina, N., Mozzherin, D., Atrazhev, A., Krayevsky, A. and Kukhanova, M. *Nucleic Acids Res.*, 1992, **20**, 783.
3. Ludwig, J. *Acta Biochim. et Boiphis. Acad. Sci. Hung.*, 1981, **16**, 131.
4. Dyatkina, N., Atrazheva, E., Alexandrova, L., Krayevsky, A. and von Janta-Lipinski, M. *Bioorg. Chem. (russian)*, 1988, **14**, 815.
5. Dyatkina, N. and Arzumanov, A. In *Biophosphates and Their Analogues-Synthesis, Structure, Metabolism and Activity*; K.S.Bruzik and W.J.Stec Eds., Elsevier Science Publishers B.V.; Amsterdam, 1987; pp 395-399.
6. Codington, J.F., Fecher, R. and Fox, J.J. *J. Org. Chem.*, 1962, **27**, 163.

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