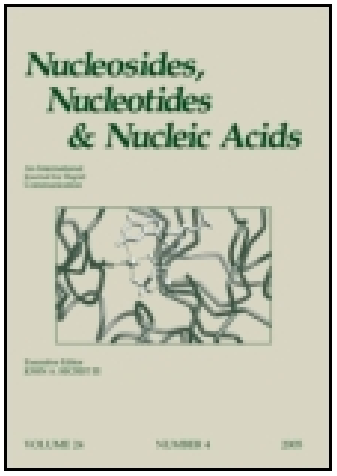

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Synthesis of 3′-Azido-3′-Deoxythymidine-Terminated Oligonucleotide

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SYNTHESIS OF 3'-AZIDO-3'-DEOXYTHYMIDINE-TERMINATED OLIGONUCLEOTIDE

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ABSTRACT. A method of completely chemical synthesis of 3'-azido-3'-deoxythymidine-terminated oligonucleotides *via* 5'-H-phosphonate of AZT is described.

3'-Azido-3'deoxythymidine (AZT) is a nucleoside analogue which inhibits replication of the human immunodeficiency virus 1 (HIV-1), and is widely used in AIDS therapy. AZT likely inhibits reverse transcriptase of HIV-1 by being incorporated into the primer during replication, which results in formation of a dead-end template-primer complex. The therapeutic use of AZT is limited by the ability of HIV-1 to acquire resistance through mutations in the reverse transcriptase gene[1]. Combining AZT with the specific targeting properties of antigene oligonucleotides[2] may increase the therapeutic value of this compound and provide a new tool for the study of reverse transcriptase action mechanism.

Here we describe improved method of synthesis of AZT-terminated oligonucleotides. Existing methods of synthesis of such oligonucleotides include enzymatic attachment of AZT *via* its 5'-triphosphate by terminal transferase, followed by product isolation, which is a laborious and time-consuming process[3,4]. Our method avoids this step by using solid-phase chemical synthesis of oligonucleotides which includes 3'-azido-3'-deoxythymidine at the terminal stage. The synthesis is accomplished in 5'→3' direction starting from 3'-protected 2'-deoxynucleoside-5'-hydrogenphosphonates with pivaloylchloride as coupling reagent.

The preparation of 5'-H-phosphonates of nucleosides was performed in several stages shown in Fig. 1. At the first stage 3'-dimethoxytrityl derivatives of 2'-deoxynucleosides

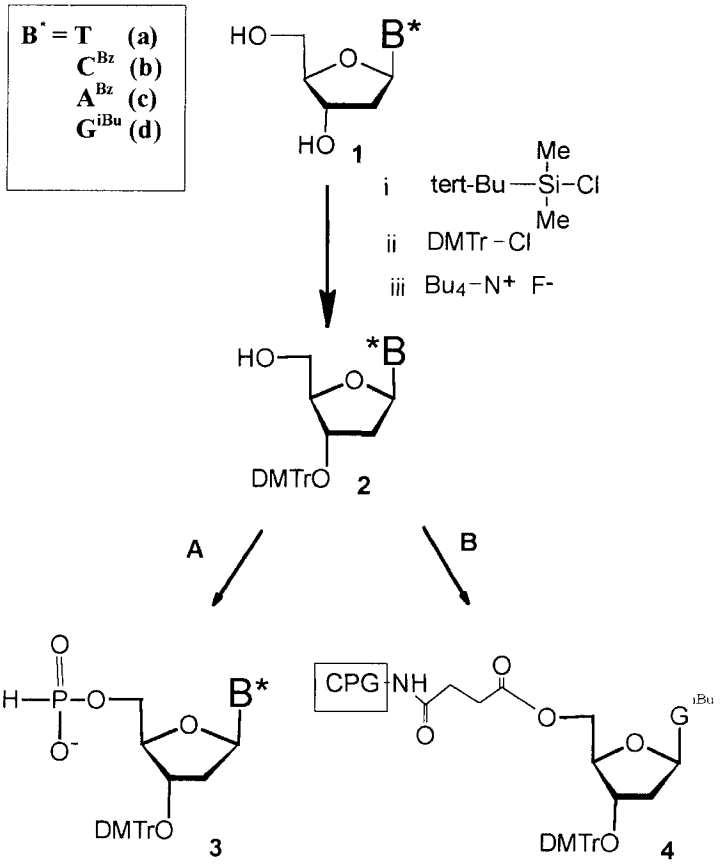

FIG. 1

(**2a-d**) were obtained. 5'-Hydroxy groups of nucleosides were blocked with *tert.*-butyldimethylsilyl protection using interaction with *tert.*-butyldimethylsilylchloride in pyridine (step i). Then, without isolation of product, protection of 3'-hydroxy groups was carried out using 4,4'-dimethoxytritylchloride (step ii). Elimination of 5'-*tert.*-butyldimethylsilyl protective group was performed by treatment of tetrabutylammonium fluoride in tetrahydrofuran without isolation of 5',3'-blocked derivatives (step iii). Completion of all reactions was determined qualitatively until starting compound disappears on TLC. 3'-Dimethoxytrityl derivatives of nucleosides were isolated using column chromatography on aluminium oxide with the linear gradient of methanol

concentration in dichloroethane in presence of TEA. Purity of products was in the range 92-98% as determined by analytical reverse phase HPLC.

At the next stage 5'-H-phosphonates of 3'-blocked nucleosides (**3a-d**) were synthesised (Fig. 1, path A). 3'-(4,4'-Dimethoxytrityl)nucleosides (**2a-d**) were treated with the mixture PCl_3 and 1,2,4-triazol in methylene chloride in presence of N-methyl morpholine. The structure of newly synthesised compounds was confirmed by ^{1}H-NMR (see Tabl. 1) and ^{31}P-NMR spectroscopy data. There were characteristic coupling constants of H-P (620 Hz) both at ^{31}P-NMR spectra without proton decoupling (approx. 4.8 ppm) and at ^{1}H-NMR spectra (approx. 6.7 ppm).

3'-(4,4'-Dimethoxytrityl)-N2-iso-butiryl-2'-deoxyguanosine served also as initial compound for preparation of CPG-support (**4**) (Fig. 1, path B). It was treated with succinic anhydride and then activated with *p*-nitrophenol. Control pore glass (CPG) with free amino groups was worked up by prepared compound, unmodified amino groups were capped by acetic ahhydride[5].

Hydrogenphosphonate of AZT has been synthesised utilising tris-(1,1,1,3,3,3-hexafluoro-2-propyl)-phosphite[6] (Fig. 2). In this case the product (**5**) was obtained with almost quantitative yield after being isolated using fast chromatography on the silica gel with chloroform-methanol (9:1) as eluent, which contained 2.5% TEA.

Because we are planning to study the inhibitory activity of 3'-AZT-terminated oligonucleotides in the elongation reactions using phage M13 DNA (+)-strand template, the following sequence corresponding to M13 single-strand primer was selected for solid phase synthesis: 5'-d[GTAAAACGACGGCCAG-AzT]-3'.

The synthesis of oligonucleotide was carried out on the synthesiser «ASM 102U» (Novosibirsk, Russia) using H-phosphonate scheme of condensation in 5'→3' direction. 5'-H-Phosphonate of AZT was attached at the last step of oligonucleotide synthesis.

Deblocked 3'-AZT-terminated oligonucleotide was isolated by high performance ion-exchange chromatography on DEAE-Separon (Fig. 3) followed by reverse phase chromatography. Its structure was confirmed by analysis of snake venom phosphodiesterase and bacterial alkaline phosphatase digest products[7].

Thus method of synthesis of 3'-terminated oligonucleotides described here allow to use the possibilities of synthetic approach for preparation of new class of antigene oligonucleotides bearing biologically active azide group.

TABLE 1. Chemical shifts in ^{1}H-NMR spectra ($CDCl_3$, δ, ppm)*.

1'-CH	2'-CH_2	3'-CH	4'-CH	5'-CH_2	T **2a,3a**	C **2b,3b**	A **2c,3c**	G **2d,3d**	H-P **3a-d**
6.1t	2.4dd	4.4m	3.8m	4.1m	1.8c (5-CH_3) 7.6s (6-CH)	6.1d (5-CH) 7.6d (6-CH) 7.8m (Bz)	8.1s (2-CH) 8.6s (8-CH) 7.4m (Bz)	1.2m (2(CH_3) iBu) 3.1k (CH- iBu) 5.1s (1-NH) 7.6s (8-CH)	6.71d (J = 620 Hz)

* Signals of DMTr group in **2a-d, 3a-d**: 3.7 m (6H, -OCH_3); 6.8 m, 7.2 m (13H).

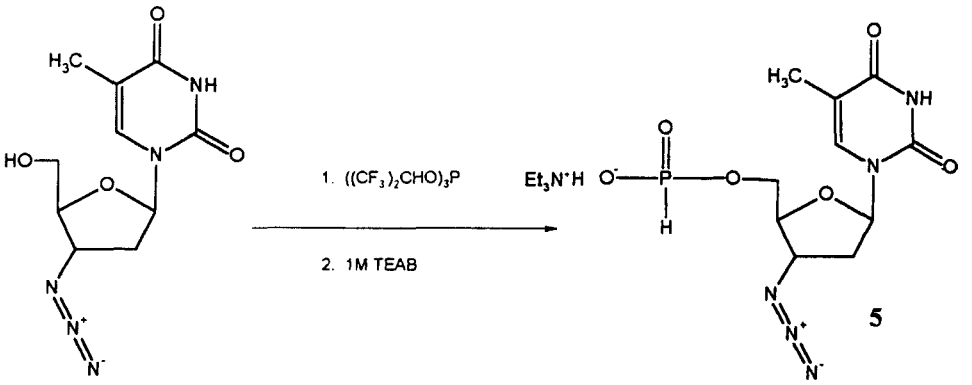

FIG. 2.

EXPERIMENTAL

NMR spectra were recorded on Bruker MSL 200 spectrometer for ^{1}H and ^{31}P nuclei (200.13, 81.01 MHz) respectively. Tetramethylsilane and chloroform, both as internal standard, and 85% H_3PO_4 as external standard were used. ^{31}P-NMR spectra were recorded with and without proton decoupling. IR spectrum was recorded on Specord 75 IR spectrophotometer. Column chromatography was performed on silica gel (0.06-0.20 mm) (Merck) and aluminium oxide, neutral, Brockmann II (Reanal). TLC was carried out on

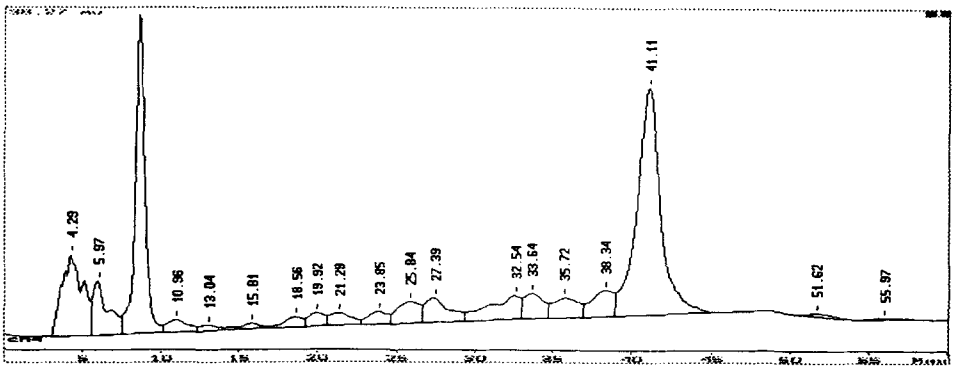

FIG. 3. Ion-exchange HPLC of oligonucleotide 5'-d[GTAAAACGACGGCCAG-AzT]-3'.

Kieselgel 60 F 254 (Merck) with detection by UV light using system $CHCl_3$-MeOH-TEA, 89:10:1. Ion-exchange HPLC was carried out on Beckman System Gold with column DEAE-Separon Bio, 250x4 mm. HPLC analysis of enzymatic degradation products was performed on Beckman System Gold using column Serva ODS, 15x4.6 mm. Initial nucleosides were thymidine, N4-benzoyl-2'-deoxycytidine, N6-benzoyl-2'-deoxyadenosine, N2-iso-butiryl-2'-deoxyguanosine (Olainfarm, Latvia). AZT was kindly delivered by «Association AZT» (Russia). Tris-(1,1,1,3,3,3-hexafluoren-2-propyl)-phosphite was prepared according C.Reese[6]. Analytical HPLC of synthesised 3'-(4,4'-dimethoxytrityl)-2'-deoxynucleosides was performed on chromatograph Knauer with column Separon SGX RPS, C_{18}, 7 mm, 250x8 mm, using the linear gradient of acetonitrile concentration in 0.1 M triethylammonium acetate, pH 7.5, from 50 to 100 % in 25 min at 2 ml/min.

3'-(4,4'-Dimethoxytrityl)-2'-deoxynucleoside (2a-d)

Initial 2'-deoxynucleoside (10 mmole) was dried by evaporating with dry pyridine (2x20 ml), dissolved in dry pyridine (15 ml) and *tert.*-butyldimethylsilylchloride (11 mmole) was added to this solution. The mixture was stirred at 20°C until the reaction was completed according to TLC. Then solution of 4,4'-dimethoxytritylchloride (15 mmole) in dry pyridine (15 ml) was added and the reaction mixture was stirred at 20°C. The reaction was monitored by TLC. After completion of the reaction absolute methanol (25 ml) was added, and after 30 min the mixture was evaporated in vacuo to dryness. The residue was dissolved in chloroform (150 ml), the organic layer was washed with 5% aqueous solution

of sodium bicarbonate (3x100 ml), dried with Na_2SO_4, evaporated to dryness and re-evaporated with toluene. The residue was dissolved in dry tetrahydrofuran (3 ml) and 1M tetrabutylammonium fluoride in tetrahydrofuran (7 ml) was added to the solution. The mixture was kept at 20°C until the reaction was completed according to TLC. After usual work up the product was isolated by column chromatography on aluminium oxide using the linear gradient of methanol from 0 to 10% in dichloroethane in 2 h with 1% TEA.

Yield, %: **2a** 56; **2b** 55; **2c** 50; **2d** 48. Analytical HPLC, retention times (min): **2a**, 10.61 (98%); **2b**, 13.79 (95%); **2c**, 13.55 (97%); **2d**, 11.71 (92%).

5'-Hydrogenphosphonate 3'-(4,4'-dimethoxytrityl)-2'-deoxynucleoside, triethylammonium salt (3a-d)

PCl_3 (25 mmole) was added to a solution of 1,2,4-triazole (80 mmole) and N-methyl morpholine (240 mmole) in methylene chloride (160 ml). After 30 min of stirring the mixture was cooled to 0°C, then respective 3'-(4,4'-dimethoxytrityl)-2'-deoxynucleoside (**2a-d**) (5 mmole) in methylene chloride (50 ml) was added dropwise over 30 min. After completion of the reaction according to TLC. 1M triethylammonium bicarbonate (TEAB) buffer (150 ml) was added and the mixture was stirred for another 30 min. The aqueous layer was extracted by methylene chloride (2x100 ml). The combined organic layers were dried with Na_2SO_4 and evaporated to dryness, re-evaporated with toluene. The residue was chromatographed on a column with silica gel using the linear gradient of methanol from 0 to 10% in dichloroethane in 1 h with 2% TEA.

Yield, %: **3a** 84; **3b** 64; **3c** 58; **3d** 56; R_F: **3a** 0.30; **3b** 0.25; **3c** 0.35; **3d** 0.17.

5'-hydrogenphosphonate 3'-azido-3'-deoxythymidine, triethylammonium salt (5)

3'-Azido-3'-deoxythymidine (0.62 g, 2.31 mmole) was dissolved in dry pyridine (16 ml) and tris-(1,1,1,3,3,3-hexafluoren-2-propionyl)-phosphite (1.38 ml, 4,62 mmole) was added to the solution. After 10 min of stirring a mixture of 1 M TEAB and TEA (50:1, v/v, 10 ml) was added. Then methanol (50 ml) was added, and the mixture was evaporated to dryness. The product was isolated by column chromatography on silica gel using as eluent the step gradient of methanol in methylene chloride with 2 % TEA (5-15 %). Yield 0,91g (91 %); R_F (system A); IR, n: 2100 cm^{-1} (N_3); ^{1}H-NMR ($CDCl_3$), δ, ppm: 1.28 m (9H, 3(CH_3) TEA); 1.89 s (3H, 5-CH_3); 2.35 dd (2H, 2'-CH_2); 3.02 m (6H, 3(CH_2) TEA); 3.65

m (1H, 4'-CH); 3.92-4.10 m (2H, 5'-CH_2); 4.38 m (1H, 3'-CH); 6.22 t (1H, 1'-CH); 6.90 d (1H, J_{H-P} = 620 Hz, H-P); 7.65 s (1H, 6-CH); ^{31}P-NMR, ($CDCl_3$), δ: 3,51 ppm.

DNA synthesis

Oligonucleotide synthesis was performed on the Biosan DNA synthesiser model «ASM-102U».

Protokol of oligonucleotide synthesis:

Acetonitrile wash	100 ml
Deprotection with 3% $CHCl_2COOH$ in CH_2Cl_2	1 min
Acetonitrile wash	100 ml
Coupling (4x25 ml 0,1 M PivCl + 0,05 M H-P-compound)	~1 min

After completion of all cycles of the synthesis, oxidation was performed by iodine (0.1 M in a mixture THF-Py-H_2O, 8:1:1) over 20 min. The obtained sequence was deprotected and cleaved from the solid support by treatment with concentrated ammonia for 8 h at 50°C. The obtained oligonucleotide was purified on DEAE-Separon Bio column; buffer A, 12.5% acetonitrile; buffer B, 1 M ammonium acetate in 12.5% acetonitrile; gradient, 20-80 % B in 1 h.

Enzymatic degradation of oligonucleotide

The oligonucleotide (0.2 OD) solution in 100 ml of the following buffer (50 mM Tris-HCl, pH 8.6; 50 mM sodium chloride; 7 mM $MgCl_2$) was digested by 1.2 U of snake venom phosphodiesterase at 37°C for 18 h. This digestion was followed by the treatment of 1.2 U of bacterial alkaline phosphatase at 37°C for 6 h. Aliquots of this solution were analysed by HPLC on a column Serva ODS using the linear gradient of acetonitrile (2.5-50 % in 50 min) in 0.1 M triethylammonium acetate. Analysis showed that ratio of unmodified nucleosides over AZT corresponds to theoretically calculated one.

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