SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING 4-N-(ω-AMINOHEXYL)-5-METHYLDEOXYCYTIDINE*

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Oligodeoxyribonucleotides containing 4-N-(ω-aminohexyl)-5-methyldeoxycytidine have been synthesized. It has been shown that a method of preparation based on the use of a monomeric component containing 4-N-(ω-aminohexyl)-5-methyldeoxycytidine is more reliable and promising than transamination at the level of an oligonucleotide containing 5-methyl-4-triazolyl-6-deoxythymidine. A structure of the monomeric component of oligonucleotide synthesis bearing a hexylamine grouping with trifluoroacetyl protection in the aliphatic amino group has been put forward. The structure of the modified deoxyribonucleotides obtained was confirmed not only by the method of chemical modification, degradation, and exhaustive enzymatic hydrolysis but also by obtaining derivatives at the aliphatic amino group with fluorescein isothiocyanate.

At the present time, methods are being developed for the synthesis of oligodeoxyribonucleotides to which biologically active molecules of d-biotin and fluorescent markers can be covalently attached. Such compounds are being used successfully for the nonradioactive diagnosis of genetic diseases, for the analysis of the primary structure of DNA, for the inhibition of the translation of mRNA, and for solving a number of other questions of modern molecular biology [1]. One of the promising methods of obtaining such compounds is the introduction into oligodeoxyribonucleotides of a single aliphatic amino group that is capable of reacting with electrophilic groupings in the molecules of proteins and of nonradioactive markers. Two approaches to the chemical synthesis of oligodeoxyribonucleotides bearing an aliphatic amino group exist: 1) a monomeric synthon contains the desired functional groups [2]; 2) a monomeric synthon contains a functional group which gives the desired compound when an oligonucleotide that has been synthesized from it is treated with an appropriate amino [3].

In the present paper we describe the preparation of oligodeoxyribonucleotides (I)-(IV) (Table 1) containing 4-N-(ω-aminomethyl)-5-methyl-2'-deoxycytidine. Both approaches to the synthesis of such compounds were tried out. For the first of the approaches we proposed the structure of a monomeric component of the synthesis bearing a hexylamine "stalk" with

TABLE 1. Synthesis of Oligodeoxyribonucleotides Containing 4-N-(ω-aminohexyl)-5-methyldeoxycytidine d(X)

Oligodeoxyribonucleotide	Yield with respect to the mono- or dimethoxytrityl group, %		Amount after isolation, OU_{260}
	on the first nucleotide	per stage	
5′ 3′			
I. d(ACCACCGCGTX)	70	97	25
II. d(AGAGCCGCCACCCTCX)	63	97	40
III. d(GTAAAACGACGGCCAGX)	22	91	8
IV. d(GGGCGCATTATAGGAX)	63	97	16

*Abbreviations. Tri) 1,2,4-triazole; MMTr) monomethoxytrityl; iPr) isopropyl; Py) pyridine; DMS) dimethyl sulfate; DEPC) diethyl pyrocarbonate.

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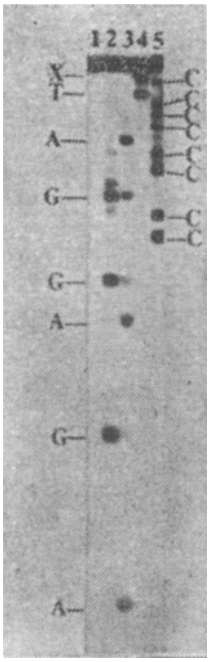
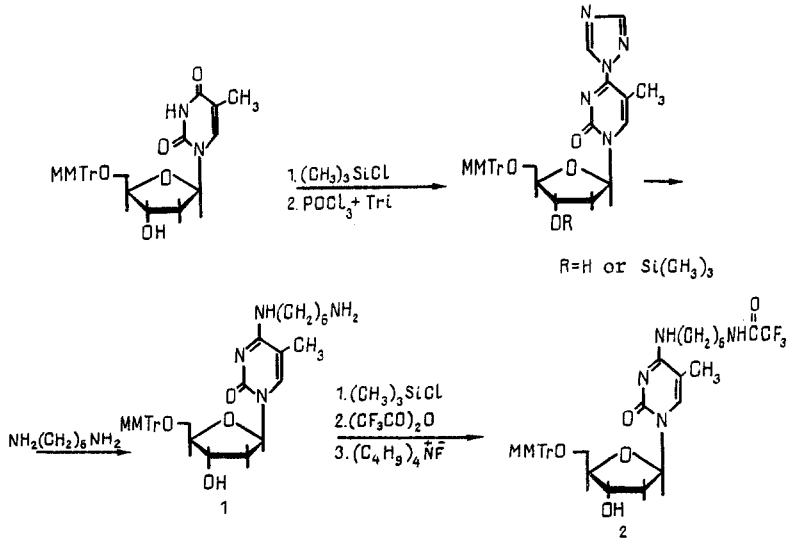


Fig. 1. Analysis by the method of chemical modification and degradation of the nucleotide sequence of compound (II): 1) initial oligodeoxyribonucleotide; cleavage; 2) at the G units (DMS) 3) the A units (DEPC); 4) at the T units ($KMnO_4$); 5) at the C units (NH_2OH). Electrophoresis in 20% polyacrylamide gel containing 7 M urea.

trifluoroacetyl protection at the aliphatic amino group (Scheme 1, compound 2). This protective group proved to be stable during oligonucleotide synthesis and was hydrolyzed under the conditions of the deblocking of the desired ribonucleotide. The structure of the compound (2) obtained was confirmed by PMR spectroscopy. The inclusion of (2) in an oligonucleotide chain was achieved by standard procedures [4]. The oligodeoxyribonucleotides containing 4-N-(ω-aminohexyl)-5-methyldeoxycytidine were obtained by the phosphoramidite method on a Viktoriya-4 M automatic synthesizer.

Scheme 1



The primary structures of the oligodeoxyribonucleotides (I-IV) (Table 1) were confirmed by the method of chemical modification and degradation [5, 6]. It must be mentioned that 4-N-(ω-aminohexyl)-5-methyldeoxycytidine, in contrast to deoxycytidine, does not interact with hydroxylamine but it does take part in a reaction with potassium permanganate, by analogy with deoxythymidine (Fig. 1). To determine the nucleotide composition of the oligodeoxyribonucleotide synthesized, and also to confirm the presence of the modified unit in them, compounds (I-IV) were hydrolyzed with a mixture of phosphomonoesterase (PME) and phosphordiesterase (PDE) from snake venom. The hydrolysates obtained were analyzed with the aid of reversed-phase HPLC under specially selected conditions ensuring the effective separation of the nucleotides (Fig. 2).

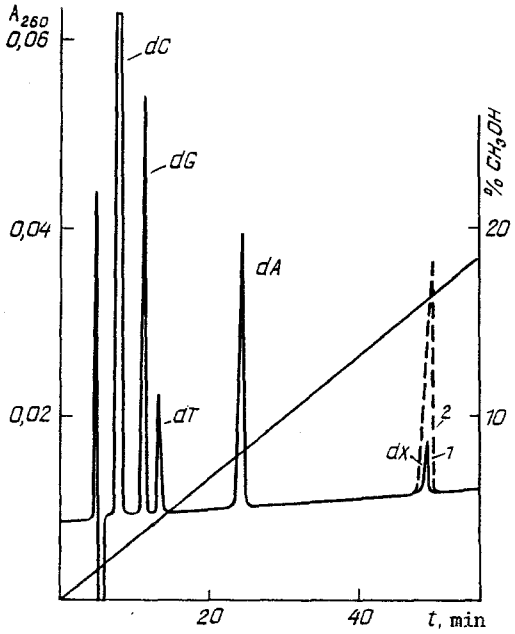


Fig. 2

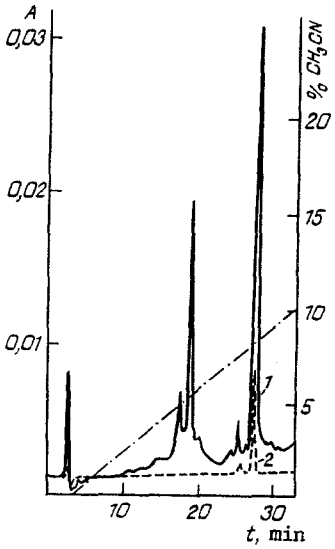


Fig. 3

Fig. 2. Analysis of the nucleotide composition of oligodeoxyribonucleotide (II). Chromatograms: 1) products of the exhaustive hydrolysis of compound (II) by a mixture of PME and PDE from snake venom; 2) hydrolysate of the oligonucleotide (II) with the addition of 4-N-(ω-aminohexyl)-5-methyldeoxycytidine (dX).

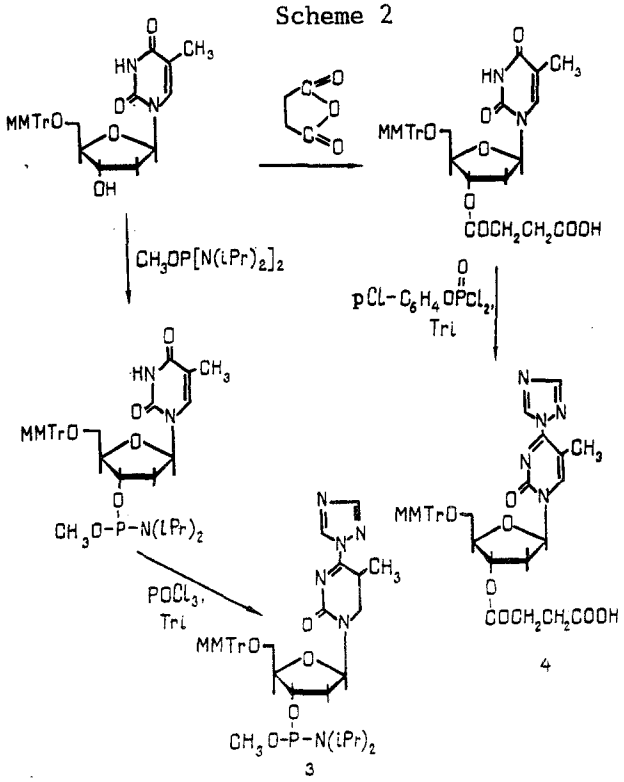
Fig. 3. Chromatogram of the products of the reaction of the oligodeoxyribonucleotide (II) with FITC. Detection: 1) at 260 nm; 2) at 493 nm.

It followed from the ratio of the areas of the peaks that the nucleotide compositions in all cases corresponded to the expected ones. Each of the hydrolysates of the oligodeoxyribonucleotides (I-IV) gave a peak identical in its retention time with that of the 4-N-(ω-aminohexyl)-5-methyldeoxycytidine that we had synthesized. The comparison was made with controlled mixtures of nucleosides made up in proportions corresponding to their amounts in the oligonucleotides (I-IV). The presence of a free aliphatic amino group in the modified oligodeoxyribonucleotides was also confirmed by the reaction with fluorescein isothiocyanate (FITC). Fluoresceinated oligodeoxyribonucleotides (I-IV) were obtained which had characteristic absorption spectra with maxima at 260 and 493 nm corresponding to the absorption of the oligonucleotide and fluorescein fragments (Fig. 3), and also fluorescence spectra with an excitation maximum at 492 nm and an emission maximum at 516 nm, which agrees with literature information [7].

In the synthesis of the oligodeoxyribonucleotide (II) we also used another method of introducing an aliphatic amino group attached to a heterocyclic base. Initially, the 3'-position of monomethoxytritylthymidine was protected by a methyl N,N-diisopropylphosphoramidite group (which gave the finished monomeric component for oligonucleotide synthesis) or by a succinate group, which was subsequently used for the immobilization of the modified nucleoside on a polymeric support (synthesis of oligodeoxyribonucleotides with a 3'-terminal modified unit). Then, by the procedure described in [8, 9], a triazolyl grouping, which is a "good" leaving group, was introduced into the fourth position of the thymine (Scheme 2, compounds 3 and 4) (see top of following page).

After the synthesis of the oligodeoxyribonucleotides by the phosphoramidite method the reaction mixture was treated with hexamethylenediamine, and then the protective groups were eliminated by a standard procedure [4]. The replacement of the triazole group in the oligodeoxyribonucleotide by a hexamethylenediamine residue did not apparently take place. Two compounds were isolated from the reaction mixture after the elimination of the protective groups, and these had to be identified for further use. Analysis of the nucleotide compo-

Scheme 2



sitions and primary structures of these oligodeoxyribonucleotides (after the elimination of the dimethoxytrityl groups) by the method of chemical modification and degradation [5, 6] and the exhaustive hydrolysis by a mixture of PME and PDE from snake venom showed that one compound was the unmodified ribonucleotide d(AGAGCCGCCACCCTCT) while the other apparently contained 5-methyldeoxycytidine. It did not interact with FITC and, consequently, did not contain a free aliphatic amino group. Furthermore, this compound and the oligodeoxyribonucleotide (II) synthesized by the first method differed in their retention times in the ion-pair variant of HPLC.

Thus, the use of the prepared monomeric component of type of (2) (Scheme 1) is a simpler and more reliable method of synthesizing oligodeoxyribonucleotides containing an aliphatic amino group linked to a heterocyclic base.

EXPERIMENTAL

We used methyl N,N-diisopropylphosphoramidite derivatives of 5'-O,N-protected nucleosides from Biosearch (USA), deoxythymidine derivatives from the Omutninsk chemical factory (USSR), N-methylimidazole, trimethylchlorosilane, and N,N-diisopropylethylamine from Fluka (Switzerland), 1,2,4-triazole and hexamethylendiamine from the Biokhimreaktiv Scientific Production Combine (USSR), snake venom phosphodiesterase (EC 3.1.4.1) from Worthington Biochemical Corp (USA), and phosphomonesterase (EC 3.1.3.1) from Sigma (USA).

Absorption spectra were recorded on a Hitachi 150-20 spectrophotometer (Japan), fluorescence spectra on a Hitachi MPF-4 spectrofluorimeter, and ^{1}H NMR spectra on a Varian VXP-400 instrument (USA) in deuterated chloroform, using chloroform as internal standard.

Thin-layer chromatography (TLC) was conducted on Kieselgel 60 F_{254} plates (Merck, FRG) in the chloroform–ethanol (95:5) or (9:1) system; and column chromatography on silica gel L 40/100 (Chemapol, Czechoslovakia). The course of the process was followed with the aid of an ISCO UF-5 flow-through microspectrophotometer (USA) and by TLC.

The oligodeoxyribonucleotide were synthesized by the procedure of [4]. The amount of the first nucleoside on the polymers and the yields per stage in the growth of the oligonucleotide chain in the 3' → 5' direction were determined from the amounts of mono- or dimethoxytrityl cation [10].

Synthesis of 5'-O-Monomethoxytrityl-4-N-(ω-trifluoroacetylaminohexyl)-5-methyldeoxycytidine. MMTrT (1.03 g, 2 mmole) was dissolved in 5 ml of absolute Py (5 × 5 ml) [sic], and N,N-diisopropylethylamine (1.7 ml, 10 mmole) and trimethylchlorosilane (0.63 ml, 5 mmole)

were added. The reaction mixture was kept at 20°C for 30 min and was then concentrated in vacuum. A mixture of 2.6 g (37 mmole) of Tri, 6 ml (43.4 mmole) of triethylamine, and 0.8 ml (8.7 mmole) of phosphorus oxotrichloride was prepared in 60 ml of acetonitrile, and after 30 min this was added to the nucleoside. After another 30 min, the resulting mixture was poured into 200 ml of a 5% solution of sodium bicarbonate and was extracted with chloroform (2 × 100 ml). The chloroform was evaporated off, and the residue was treated with 20 ml of 0.2 M hexamethylenediamine in dioxane–water (9:1). After 12 h, the reaction mixture was diluted with 100 ml of chloroform and was washed with water (3 × 100 ml). The organic layer was dried over calcined sodium sulfate, and the solvent was evaporated off until a foam had been formed. The course of the reaction was monitored at each stage by TLC in the chloroform–ethanol (95:5) system.

The compound (1) obtained as a result (Scheme 1) was silylated with trimethylchlorosilane by the procedure described above. Then 0.42 ml (3 mmole) of trifluoroacetic anhydride and, after 30 min, 1 ml of absolute methanol were added to the reaction mixture. After another 30 min, the mixture was poured into 200 ml of a 5% solution of sodium bicarbonate and was extracted with chloroform (2 × 50 ml). The organic layer was dried over sodium sulfate and evaporated. To eliminate the residual silyl protective groups, the modified nucleoside was treated with 6 ml of a 1 M solution of tetrabutylammonium fluoride in absolute Py at room temperature for 20 min. After evaporation, the substance was dissolved in 200 ml of chloroform and the organic layer was washed repeatedly with a 5% solution of sodium bicarbonate (200 ml) and with water (200 ml). The organic layer was dried over sodium sulfate and evaporated.

The desired substance (2) (Scheme 1) was isolated by column chromatography on silica gel in a concentration gradient of ethanol (0-10%) in chloroform. The yield of compound (2) after column chromatography was 420 mg (30%, calculated on the initial MMTrT). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 6.44 (t, 1, H-1', $J_{1',2'}$ = 6.6 Hz); 5.0 (t, 1, NH); 4.52 (m, 1, H-3'); 4.08 (m, 1, H-4'); 3.8 (s, 3, OCH_3); 3.45; 3.3 (two m, 6, CH_2NH, CH_2NH, H-5'); 2.55 (m, 1, H-2'e); 2.2 (m, 1, H-2'a); 1.48 (s, 3, CH_3–C=); 1.58; 1.35 (two m, 8, $(CH_2)_4$).

Addition of the Modified Nucleoside (2) to the Polymeric Support. The polymeric support $Si-(CH_2)_3-NHCOCH_2CH_2COOH$ obtained according to [4] (0.5 g; 0.1 mole) and 70 mg (0.1 mmole) of the modified nucleoside (2) were evaporated repeatedly with absolute Py (5 × 5 ml) and were suspended in 4 ml of absolute Py. To the mixture so obtained were added 70 μl (1 mmole) of N-methylimidazole and 100 mg (0.3 mmole) of triisopropylbenzenesulfonyl chloride and the mixture was left at room temperature for 2 h and was then treated with 1 ml of absolute methanol and allowed to stand for another 30 min. The polymer was washed with absolute Py (3 × 20 ml), with ethanol (3 × 20 ml), and with diethyl ether (3 × 20 ml), and was dried in vacuum. The loading of the polymer with the modified nucleoside (2) amounted to 45 μmole/g. The synthesis of all the oligodeoxyribonucleotides (I)-(IV) (Table 1) was carried out on this support.

5'-O-Monomethoxytrityl-4-triazolyl-5-methyl-4-deoxythymidine (methyl N,N-diisopropylphosphoramidite) (compound 3, scheme 2) was obtained by the procedure of [8].

The synthesis of 5'-O-monomethoxytrityl-4-triazolyl-5-methyl-4-deoxythymidine 2'-O-succinate (compound 4, scheme 2) and its addition to the polymeric support $Si-(CH_2)_3NHCO-(CH_2)_2COO(CH_2)_3OH$ was performed by the procedures of [11]. The loading of the polymer calculated to the first nucleotide was 32 μmole/g. The synthesis of the oligodeoxyribonucleotide (II) was performed on this polymer. After the end of the synthesis the oligodeoxyribonucleotide attached to the polymeric support was treated with 200 ml of a 0.2 M solution of hexamethylenediamine in dioxane–water (9:1, by volume), at 20°C for 2 h. The elimination of the protective groups was carried out as described in [4].

The analysis and isolation of the desired oligodeoxyribonucleotide were performed as described in [4].

The hydrolysis of the modified oligodeoxyribonucleotides with a mixture of phosphodiesterase and phosphomonoesterase from snake venom was carried out in 0.2 M Tris-HCl buffer (pH 8.5) containing 0.04 M $MgCl_2$ at 50°C for 16-18 h. The hydrolysate was analyzed by reversed-phase HPLC in a Tracor chromatograph (Holland). Column 4.6 × 250 mm containing Ultrasphere TM-octyl (5 μm); eluent – 0.1 M ammonium acetate with a linear concentration gradient of methanol of from 0 to 20% in 60 min; rate of flow of eluent 1 ml/min. Column temperature 45°C.

Preparation of Fluoresceinated Oligodeoxyribonucleotides. A solution of 1 mg of FITC in 150 µl of 1 M sodium carbonate–water–dimethylformamide (5:8:2) was added to 150 µl of an aqueous solution of the modified oligodeoxyribonucleotide (2 OE_{260}). The reaction was conducted at 20°C in the dark for 24 h. After its completion, the excess of FITC and salts were eliminated by gel filtration on a column of Bio-Gel P-2, particle size 200-400 mesh (Bio-Rad, USA). The reaction mixture was analyzed by reversed-phase HPLC on an Altex chromatograph (USA) using a 4.6 × 250 mm column containing Zorbax ODS (5 µm); detection was carried out at wavelengths of 260 and 493 mm (Fig. 3). The eluent was 0.1 M ammonium acetate for 5 min, and then a linear concentration gradient (0-20%) of acetonitrile in 30 min at a rate of flow of eluent of 1 ml/min. The column temperature was 45°C. The reaction product was isolated by preparative HPLC under similar conditions with detection at 493 nm. The yield of fluoresceinated oligodeoxyribonucleotides (I-IV) was approximately 60%.

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LITERATURE CITED

1. J. M. Coull, H. L. Weith, and R. Bischoff, Tetrahedron Lett., 27, 3991 (1986).
2. P. Li, P. P. Medon, D. C. Skingle, J. A. Lanser, and R. H. Symons, Nucleic Acids Res., 15, 5275 (1987).
3. B. Arcangioli, S. Pochet, R. Sousa, and T. Haynh-Dinh, Eur. J. Biochem., 179, 359 (1989).
4. S. M. Gryaznov, I. P. Chernov, V. K. Potapov, A. A. Purmal', V. G. Metelev, A. A. Elov, and Z. A. Shabarova, Bioorg. Khim., 12, 1604 (1986).
5. A. M. Maxam and W. Gilbert, Methods Enzymol., 65, 499 (1980).
6. A. Rosenthal, F. Schubert, D. Cech, T. S. Orezkaja [Oretskaya], S. A. Kuznezova, and Z. A. Shabarova, Biomed. Biochim., Acta, 44, 75 (1985).
7. S. Agrawal, C. Christodoulou, and M. J. Gait, Nucleic Acids Res., 14, 6227 (1986).
8. T. R. Webb and M. D. Matteucci, Nucl. Acids Res., 14, 7661 (1986).
9. W. L. Sung, J. Org. Chem., 47, 3623 (1982).
10. T. Atkinson and M. Smith, in: Oligonucleotide Synthesis: A Practical Approach, J. J. Gait (ed)., IRL Press, Oxford (1984), pp. 35-81.
11. E. M. Volkov, E. A. Romanova, A. Krug, T. S. Oretskaya, V. K. Potapov, and Z. A. Shabarova, Bioorg. Khim., 14, 1034 (1988).