Russian Journal of Bioorganic Chemistry, Vol. 31, No. 2, 2005, pp. 139–145. Translated from Bioorganicheskaya Khimiya, Vol. 31, No. 2, 2005, pp. 151–158.

Determination of Oligonucleotide Molecular Masses by MALDI Mass Spectrometry

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Received October 5, 2004; in final form, November 1, 2004

Abstract—MALDI mass spectrometry (MS) of 14- to 42-mer homogeneous oligonucleotides and their mixtures was carried out using a Vision 2000 instrument (Thermo BioAnalysis, Finnigan, United States). Conditions for the determination of oligonucleotide molecular masses were optimized by applying various matrices and operation modes. The most reproducible results with minimal uncontrolled decomposition of the oligonucleotides including their apurinization during the MALDI MS registration were obtained using 2,4,6-trihydroxyacetophenone as a matrix instead of 3-hydroxypicolinic acid usually employed in the mass spectrometry of oligonucleotides. Our approach allows the determination of molecular masses of oligonucleotides obtained by chemical synthesis and the evaluation of their component composition and purity. It was applied to the mass spectrometric analysis of oligonucleotides containing a 3'-(methyl-*C*-phosphonate) group or a modified 1,N^6-ethenodeoxyadenosine unit.

Key words: MALDI mass spectrometry, modified oligonucleotides, oligonucleotides

INTRODUCTION

Mass spectrometry is one of physicochemical methods used for the elucidation of structures of organic compounds.[2] This method is based on the generation in gas phase of positively or negatively charged ions from electrically neutral organic molecules, the determination of their masses (mass to charge ratio, *m*/*z*, mass number) and abundance (by ionic current), and the study of their fragmentation. Currently, various MS methods are successfully applied to the analysis of natural and synthetic oligonucleotides, in particular, the method of MALDI MS [1–4].

A correct choice of matrix and the registration mode of mass spectrum, which provide a minimal fragmentation of molecular ions, are crucial for the successful analysis of organic compounds by MALDI MS. The main objectives of MS analysis of oligonucleotides are the following: the reliable determination of oligonucleotide molecular masses, the evaluation of quality of synthetic oligonucleotide products, the step-by-step monitoring of the oligonucleotide synthesis, and structural study of the oligonucleotides using their fragmentation patterns.

HPA in combination with such additives as PA and ammonium citrate is believed to be an optimal matrix, which ensures good results upon the registration of mass spectra of both positively and negatively charged oligonucleotide ions [5–7]. In some works, THA, ANP, and DHB had been successfully used as matrices [8, 9].

In this work, 14–42-mer homogeneous oligonucleotides and mixtures of oligonucleotides were analyzed by MS in order to choose an optimal matrix and an optimal registration mode that allow one to completely exclude an uncontrolled decomposition of oligonucleotides and to obtain the MALDI mass spectra containing only the peaks corresponding to ions with the molecular masses of compounds present in the preparations under study.

RESULTS AND DISCUSSION

Oligonucleotide Synthesis

Synthetic modified oligonucleotides now find a wide application not only as tools in scientific research, but also as diagnostic and therapeutic agents in medicine. MALDI MS is one of the efficient methods (and

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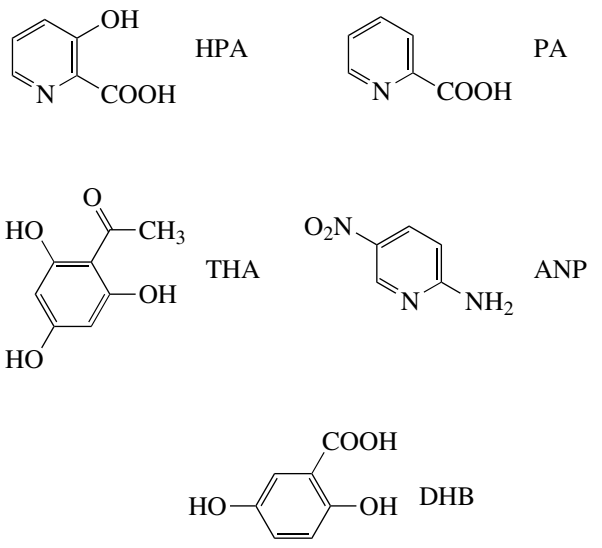
[2] Abbreviations: εA, 1,N^6-ethenodeoxyadenosine; εAde, 1,N^6-ethenoadenine; ANP, 2-amino-5-nitropyridine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DHB, 2,5-dihydroxybenzoic acid; HPA, 3-hydroxypicolinic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PA, picolinic acid; TEA, triethylamine; and THA, 2,4,6-trihydroxyacetophenone. Prefix *d* in oligodeoxyribonucleotide abbreviations is omitted.)



sometimes the only possible method) that can be used to confirm the structure and to evaluate the purity of oligonucleotide preparations. In this work, we used a number of model oligonucleotides (**1**)–(**3**), a modified oligonucleotide (**4**) containing 3'-(methyl-*C*-phosphonate) group –P(O)(CH_3)(OH), an oligonucleotide (**5**) containing the modified εA base, and common oligonucleotides (**6**)–(**21**) (the table). All the oligonucleotides were synthesized on an automatic ASM102U synthesizer (Novosibirsk, Russia). The reaction products were isolated by reverse phase HPLC, and their homogeneity was checked by ion exchange HPLC and gel electrophoresis.

3'-Phosphorylated oligonucleotide (**3**) was obtained by the procedure proposed by Krynetskaya *et al.* [10] with some modifications; oligonucleotide (**4**), by a solid phase phosphoramidite method on a LCAA-CPG support[3] using dinucleoside phosphonate 5'-*O*-(4,4'-dimethoxytrityl)thymidilyl-(3'-methyl-*C*-phosphonate)-5'-uridine (**III**) as the first unit; it was synthesized according the procedure [11]. Cleavage of the *cis*-glycol grouping of uridine at the last stage with subsequent β-elimination of the nucleoside fragment resulted in the formation of target oligonucleotide 3'-methyl-*C*-phosphonate (**4**) (Scheme 1).

The interaction of deoxyadenosine with chloroacetaldehyde leads to εA, which has fluorescent properties [12–14]. It was protected at 5'-hydroxyl by 4,4'-dimethoxytrityl group and then converted into 2-cyanoethyl-*N*,*N*-diisopropylamidophosphite according to standard procedure. This synthon was used for the incorporation of εA unit into the growing chain of oligonucleotide (**5**). A methanol solution of DBU was used for the removal of protecting groups and cleavage from the carrier [15].

[3] Long chain alkylamino controlled-pore glass.

Optimization of Conditions of the Registration of Oligonucleotide Mass Spectra

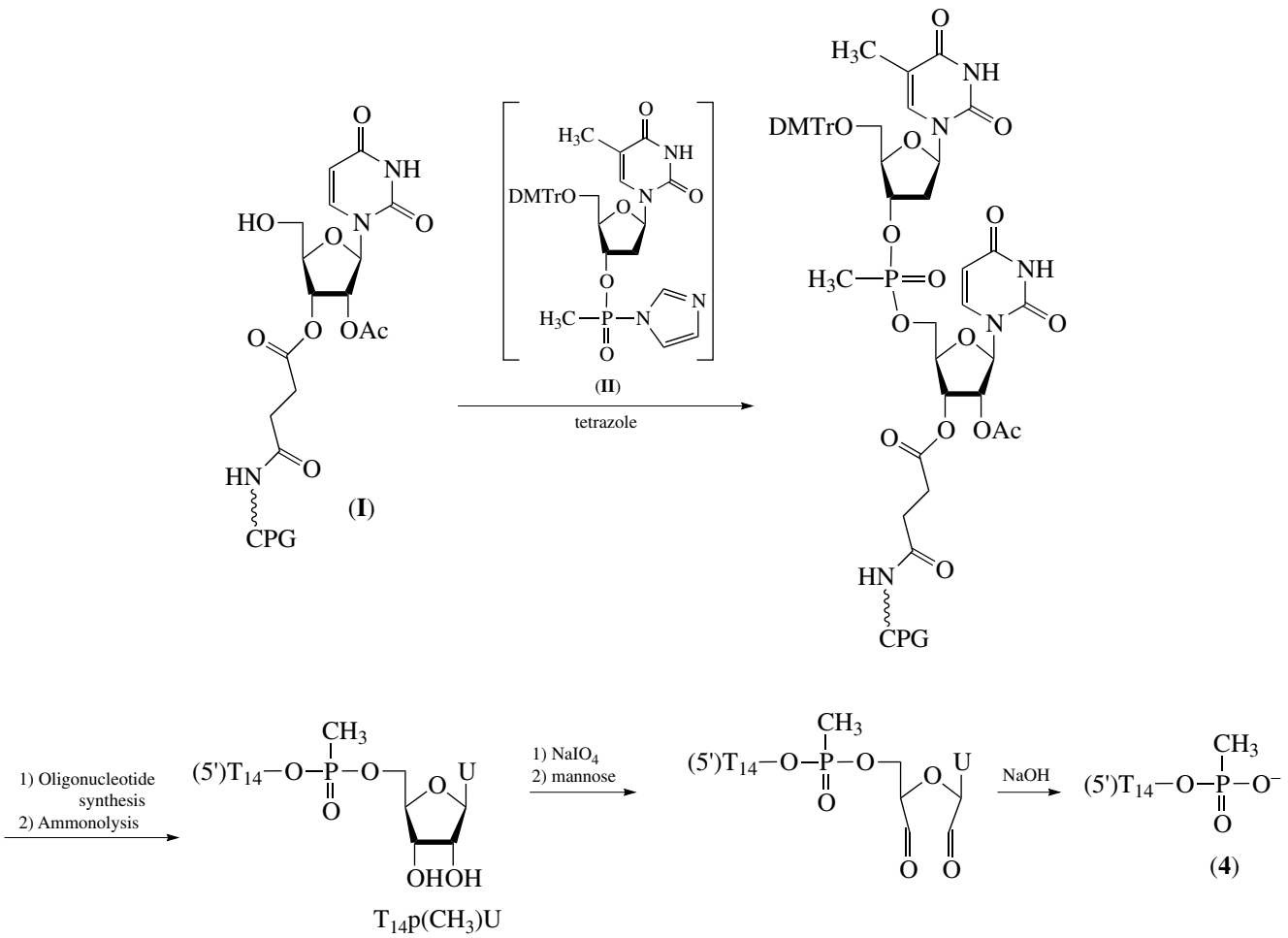
Oligonucleotide samples were prepared using the HPA, PA, THA, ANP, and DHB matrices proposed previously. An analysis of the oligonucleotides mass spectra obtained with such matrices as HPA/PA and ANP showed that these matrices provide a significantly lower accuracy of molecular mass determination than THA. The signal resolutions (widths at half height) of positively charged ions were five times lower than those of negatively charged ions. Therefore, the use of THA as a matrix for the determination of oligonucleotide molecular masses was preferable when negatively charged ions were registered.

We determined molecular masses of a number of oligonucleotides (**7**)–(**21**) (table). For example, Fig. 1 shows the mass spectra of negatively charged ions of oligonucleotide (**21**) obtained with THA and DHB matrices. We did not observe significant apurinization of oligonucleotides with THA (Fig. 1a), whereas apurinization occurred when DHB was used (Fig. 1b).

We developed a technique of MALDI MS that was based on the technical potentialities of a Vision 2000 instrument and the specially optimized conditions of sample preparations. It allowed us to obtain the mass spectra that mainly exhibit peaks corresponding to the molecular masses of oligonucleotides present in the analyzed sample. This is important for the quality monitoring of oligonucleotide preparations. For example, the mass spectrum given in Fig. 2a shows that, in addition to the target product (molecular peak with *m*/*z* 4197), the preparation of oligonucleotide T_{14} (**1**) contains impurities of T_{13}- and T_{12}-mer oligonucleotides whose molecular ions exhibit peaks at *m*/*z* 3893 and 3586, respectively. The peaks of double-charged ions of these oligonucleotides are seen in the area of *m*/*z* 2000. The mass-spectrometric data are in a good agreement with the results of analysis of this oligonucleotide by ion-exchange chromatography (data not shown).

The same was observed in the mass spectrum of oligonucleotide A_{14}(6) (peak of molecular ion with *m*/*z* 4321) (data not shown). The preparation of this oligonucleotide contained impurities of A_{13}- and A_{12}-mer oligonucleotides (peaks at *m*/*z* 4011 and 3695, respectively), which was also confirmed by chromatography.

The mass spectrum of oligonucleotide $T_{14}U$ (**2**) (Fig. 2b) exhibits the peak of molecular ion shifted by one uridine fragment (ions with *m*/*z* 4502 $[T_{14}U]^-$ and 2251 $[T_{14}U]^{2-}$) in comparison with the peak of molecular ion of oligonucleotide T_{14} (**1**) (Fig. 2a). The peak of molecular ion with *m*/*z* 4276 in the mass spectrum of oligonucleotide $T_{14}p$ (**3**) (Fig. 2c) is shifted by a value corresponding to a residue of phosphoric acid. The $T_{14}p$ preparation contains the impurities of oligonucleotides T_{14}, $T_{13}p$, and T_{13} with *m*/*z* 4197, 3968, and 3896, respectively.



Scheme 1. Scheme of synthesis of modified oligonucleotide $T_{14}pCH_3$ (**4**)

The intensities of peaks of both mono- and multicharged ions decrease upon passing from short to longer oligomers. However, the accuracy of mass spectrometric determination of oligonucleotide molecular masses remains sufficiently high within the range of 4000–13 000 amu, and the error does not exceed 0.1%, which agrees with literature [16].

A high informativity and unambiguity of the information obtained from MALDI MS are demonstrated by the given below examples of molecular mass determination of the modified oligonucleotides containing a 3'-terminal (methyl-*C*-phosphonate) group (**4**) [17, 18] or a modified, εAde, unit (**5**).

The mass spectrum of oligonucleotide $T_{14}pCH_3$ (**4**) (Fig. 2d) exhibits the base peak corresponding to the molecular ion with *m/z* 4277 and minor peaks of impurities. Figure 3 shows the mass spectrum of products obtained in the synthesis of oligonucleotide T_{12}εAT (**5**). In addition to the peak of molecular ion of the target product (*m/z* 4229), the spectrum contains the peak of molecular ion of oligomer T_{11}εAT (*m/z* 3923) truncated by one thymidine unit, the peaks of molecular ions of T_{13} and T_{12} (*m/z* 3894 and 3585), and the peaks of molecular ions $[T_{12}εAT - εAde]^-$ and $[T_{11}εAT - εAde]^-$ (*m/z* 4086 and 3776) of the oligomers in which the modified εAde base is absent. These oligomers are the side products of the synthesis, and peaks of the corresponding molecular masses observed in the mass spectrum unambiguously confirm their presence in the preparation.

EXPERIMENTAL

The following chemicals were used: 2,5-dihydroxybenzoic acid, 2,4,6-trihydroxyacetophenone, picolinic acid, and 3-hydroxypicolinic acid (Fluka Chemicals); 2-amino-5-nitropyridine and methyldichloro-*C*-phosphonate (Aldrich). 1,N^6-Ethenodeoxyadenosine was donated by Yu.V. Khropov (Problem Research Laboratory of Enzyme Chemistry, Moscow State University).

TLC was carried out on Kieselgel 60 F 254 DC-Alufolien (Merck); the developing systems were (A) 8 : 2 : 0.1 chloroform–methanol–TEA and (B) 45 : 45 : 10

Oligonucleotides studied by MALDI MS

Code	Oligodeoxyribonucleotide (5' ⟶ 3')*	$[M–H]^-$ value	
		calculated	experimental
(**1**)	TTTTTTTTTTTTTT	4195.8	4197
(**2**)	TTTTTTTTTTTTTTU	4501.9	4502
(**3**)	TTTTTTTTTTTTTTp	4275.8	4276
(**4**)	TTTTTTTTTTTTTTpCH_3	4273.8	4277
(**5**)	TTTTTTTTTTTTεAT	4222.0	4229
(**6**)	AAAAAAAAAAAAAA	4322.0	4321
(**7**)	TCTTATCTCCACCCACCAGA	5956.0	5962
(**8**)	CCTGGATGTTGAGCTTCCTA	6098.0	6102
(**9**)	GCAGTCAGACGTTGCCTATT	6107.0	6116
(**10**)	GTGGTCTTCTACTTGTGTCAATAC	7323.8	7325
(**11**)	CAGAACCAGCAGAATCTTTGC	6398.2	6401
(**12**)	CTGGAGACCACTCCCATCCTTTCT	7198.7	7204
(**13**)	CAAGTCTTTCACTGATCTTC	6017.0	6021
(**14**)	GCCTGGCGCCATTAAAGAA	5820.9	5824
(**15**)	GTCGACCTGCAAGTTGATTCTGTATG	7991.3	8000
(**16**)	TAAAGAAAGACCCCACCAGTC	6376.2	6377
(**17**)	ATGAATCGCATGACGTTCGG	6156.1	6163
(**18**)	CCGCGTCGACTTATTTGTCGCATTTCTTC	8775.7	8791
(**19**)	CCGGAATTCGACCCGTGGTCTACTATCGTTAAGTTGAC	11643.6	11659
(**20**)	CGCCCAAGCTTTCGACCCGTGGTCTACTATCGTTAAGTTGAC	12815.4	12827
(**21**)	CGCTTGACATGGCCCTC	5121.4	5117

* Prefix d in oligonucleotide abbreviations is omitted.

chloroform–ethyl acetate–TEA. The spots were visualized under UV light or by charring.

Column absorption chromatography was performed on Kieselgel 60 0.063-0.2 mm in system (B).

Oligonucleotide synthesis was achieved on an automated ASM102U synthesizer (Novosibirsk, Russia). LCAA-CPG (Cruachem, UK) with an immobilized first units was used as a support. The concentration of oligonucleotides was spectrophotometrically determined on a Unicam spectrophotometer (UK). Reverse phase HPLC was carried out on a Beckman chromatograph (United States) using a Silosorb C18 column (4.6 × 250 mm) in the following systems: (A) linear acetonitrile gradient in 0.1 M ammonium acetate buffer (pH 7.5) from 25 to 40% for 20 min; and (B) that from 5 to 25% for 20 min.

Measurement of ^{31}P NMR spectra was achieved on a Bruker MSL-200 pulse Fourier transform spectrometer in DMSO-d_6 at a working frequency of 81 MHz using 85% H_3PO_4 as an external standard (δ 0.000 ppm) with or without decoupling from protons. A Vision 2000 time-of-flight mass spectrometer (Thermo Bio-Analysis, Finnigan, United States) was used for mass spectrometric analyses.

5'-*O*-(4,4'-Dimethoxytrityl)thymidine 3'-*O*-methylphosphonoimidazolide (II) [10]. Methyl dichloro-*C*-phosphonate (33 μl, 0.36 mmol) was added to a solution of imidazole (125.3 mg, 1.84 mmol) in anhydrous acetonitrile (400 μl). The mixture was stirred for 1 h to yield a white precipitate of methylphosphonodi(imidazolide) hydrochloride. A solution of 5'-dimethoxytritylthymidine (216 mg, 0.396 mmol) in dry THF (400 μl) was then added to a stirred solution of methylphosphonodi(imidazolide). The completeness of reaction was checked by TLC in system A according to disappearance of starting nucleoside (**I**) (R_f 0.74); (**II**): R_f 0.81; ^{31}P NMR (δ, ppm): 33.520, 33.730. The untreated reaction mixture was directly used in the synthesis of modified support without the isolation of (**II**).

5'-*O*-[5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-yl methylphosphono]-2'-*O*-acetyluridin-3-ylsuccinyl-LCAA-CPG (III). 5'-Dimethoxytrityluridine-3'-acetyl-2'-succinylyl-LCCA-CPG (DMTrU-LCAA-CPG) (**I**) (100 mg, support with a load of 31 μmol/g) was treated with 2.5% solution of dichloroacetic acid in dichloromethane, successively washed with methanol and diethyl ether, and dried in an oil pump vacuum for 5 h. Tetrazole (20 mg, 0.3 mmol) was dissolved in the

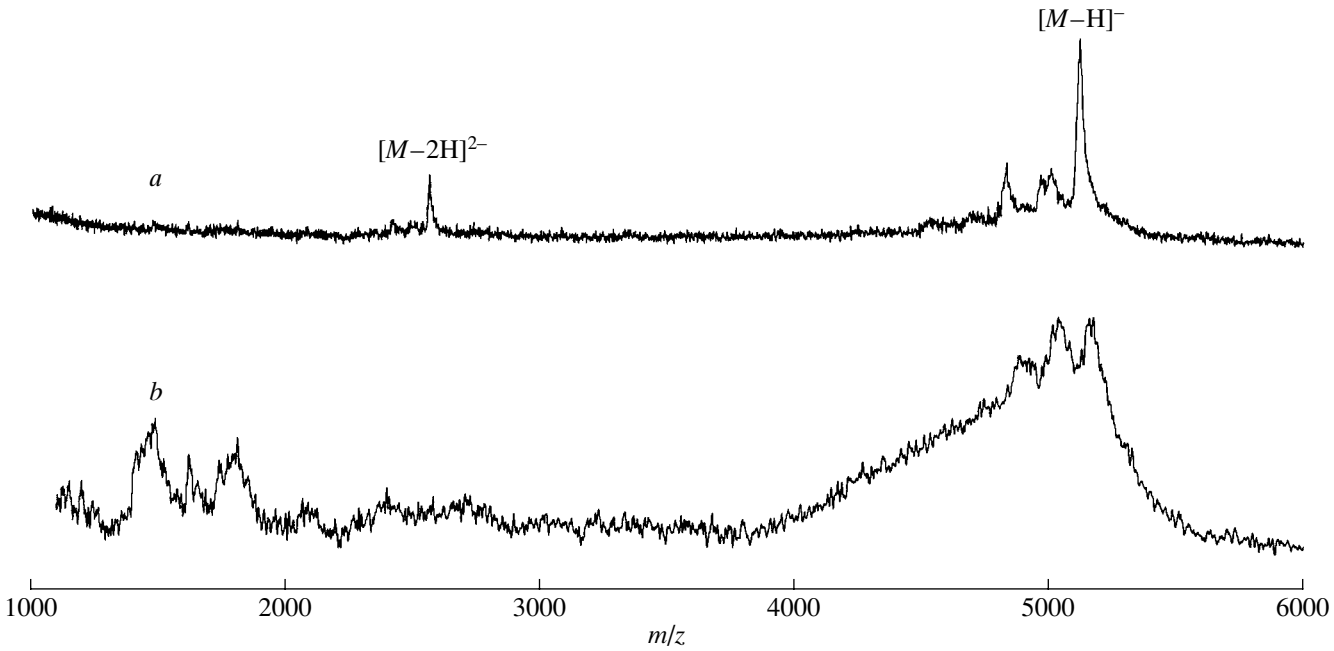


Fig. 1. MALDI MS of oligonucleotide CGCTTGACATGGCCCTC (**21**) obtained with (*a*) THA and (*b*) DHB matrices.

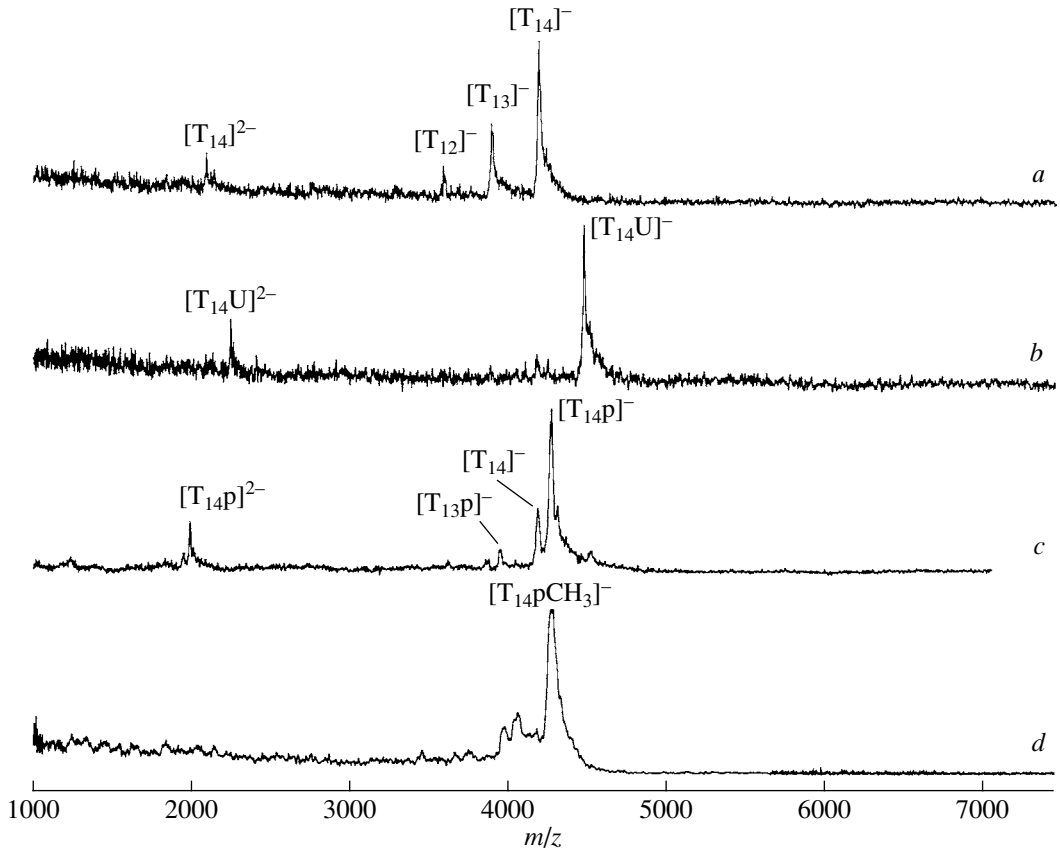


Fig. 2. MALDI MS of oligonucleotides (*a*) T_{14} (**1**), (*b*) $T_{14}U$ (**2**), (*c*) $T_{14}p$ (**3**), and (*d*) $T_{14}pCH_3$ (**4**) obtained using THA matrix.

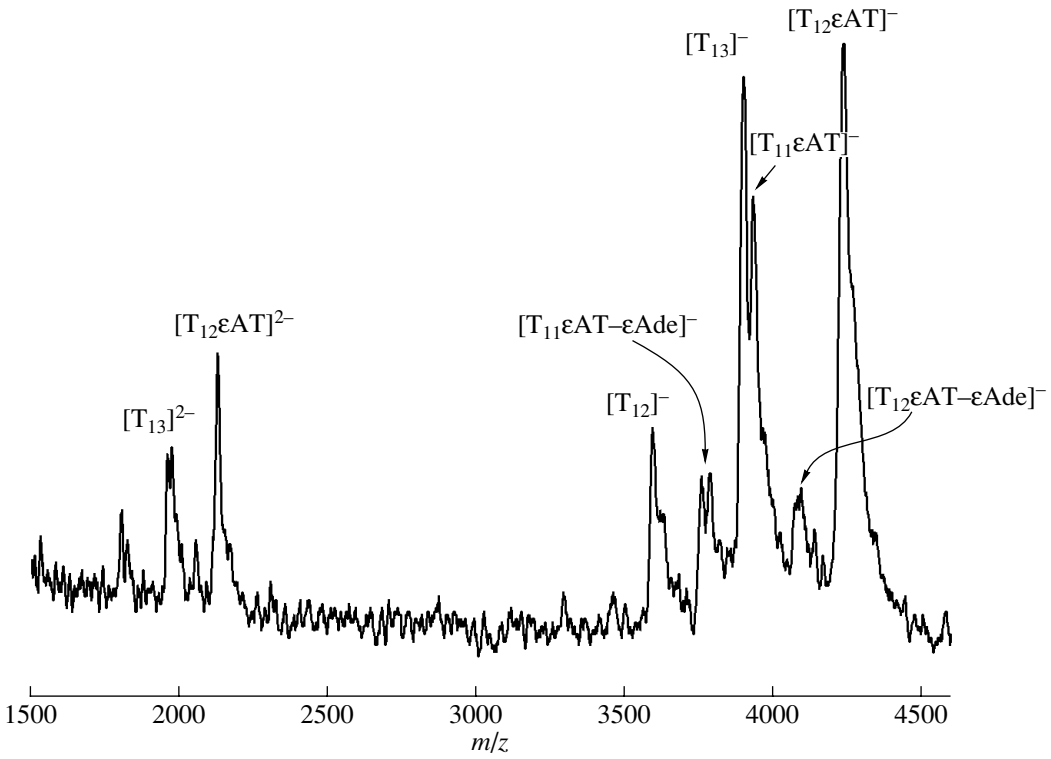


Fig. 3. MALDI MS of products obtained in the synthesis of oligonucleotide T_{12}εAT (**5**) (THA matrix).

reaction mixture (400 μl) of 5'-*O*-(4,4'-dimethoxytrityl)thymidine-3'-*O*-methylphosphonoimidazolide obtained as described above, added to the support, and stirred for 4 h. The support was then washed with methanol and diethyl ether and dried in an oil pump vacuum for 4 h. The load of dinucleotide in DMTrTp(CH_3)U-LCAA-CPG (**III**) was 8.8 μmol/g.

5'-*O*-(4,4'-Dimethoxytrityl)-1,N^6-etheno-2'-deoxydenosine was obtained according to a standard procedure as described in [19].

5'-*O*-(4,4'-Dimethoxytrityl)-1,N^6-etheno-2'-deoxydenosine-3'-*O*-(*N*,*N*-diisopropyl) cyanoethylphosphoramidite. A solution of diisopropylammonium tetrazolide (0.14 g, 0.78 mmol) and bis(diisopropylamido) cyanoethylphosphoramidite (0.59 g, 1.95 mmol) was added to a solution of 5'-*O*-(4,4'-dimethoxytrityl)-1,N^6-etheno-2'-deoxydenosine (0.45 g, 0.78 mmol) in anhydrous acetonitrile (10 ml). The progress of reaction was monitored by TLC in system B. After disappearance of the starting compound, the reaction mixture was treated with methanol (1 ml), kept for 10 min, and evaporated to yield oil, which was dissolved in ethyl acetate (20 ml) containing 10% TEA. The solution was washed with 10% sodium bicarbonate. The aqueous layer was extracted with ethyl acetate–TEA 9 : 1, and the combined organic layer was dried with anhydrous Na_2SO_4, evaporated to dryness, and coevaporated with acetonitrile. The residue formed a foam in an oil pump vacuum. The product was purified by column chromatography. The yield was 0.50 g (82%).

Synthesis of oligonucleotides. Oligonucleotides were synthesized using the standard solid-phase phosphoramidite method. A modified support was prepared for the synthesis of oligonucleotide T_{14}p(CH_3)U. A support DMTrU-LCAA-CPG (Cruachem) was used for the synthesis of oligonucleotide T_{14}U.

Oligonucleotides T_{14}p (3) and T_{14}pCH_3 (4). Oligonucleotide with 3'-terminal uridine (T_{14}U or T_{14}p(CH_3)U, respectively) was dissolved in distilled water (100 μl) and mixed with 0.1 M $NaIO_4$ (100 μl). The reaction mixture was incubated in dark at 60°C for 0.5 h, mixed with 1 M mannose (20 μl) and again incubated in dark at 60°C for 0.5 h. It was then mixed with 1 M NaOH (22 μl), again incubated at 60°C for 0.5 h, diluted with distilled water to the volume of 10 ml, and passed through a DEAE-Sephadex layer (~100 μl). The oligonucleotide was eluted with 3 M $LiClO_4$ (200 μl) and reprecipitated with cooled acetone (1.5 ml). The products of reaction were isolated by reverse phase HPLC.

Oligonucleotide T_{12}εAT (5) was synthesized using the standard solid-phase phosphoramidite method. Deprotection and cleavage from the support were carried out by incubation of the support with synthesized oligonucleotide in 10% DBU in absolute methanol for 5 days. Methanol was then evaporated in a vacuum, the

reaction mixture was diluted with distilled water to a volume of 500 μl. The target product was isolated using reverse phase HPLC.

Mass spectrometric analysis of oligonucleotides. 2,5-Dihydroxybenzoic acid or a 10 : 1 3-hydroxypicolinic–picolinic acids mixture was dissolved in 7 : 3 acetonitrile–water (25 mg per ml of solvent). 2,4,6-Trihydroxyacetophenone and 2-amino-5-nitropyridine were dissolved in a 1 : 1 0.1 M ammonium citrate–acetonitrile mixture (15 mg per ml). Oligonucleotides to be analyzed were dissolved in 1 : 1 acetonitrile–water at concentrations varying from 10^{-5} to10^{-6} M.

A solution of the analyzed sample was mixed with a matrix solution at a molar ratio of 1 : 1000 to 1 : 10000. No more than 1 μl of the mixture was applied on a target and dried in air. The mass spectra of nucleotides were registered in a reflection mode. The sample was irradiated on the target with N_2 laser (λ 337 nm) at a pulse length of approximately 9 ns. In order to decrease the destructive effect of laser irradiation, its intensity was chosen according to a minimum at which the ions of analyzed compounds were formed. The mass spectra of analyzed samples were registered for both positively and negatively charged ions. Each mass spectrum was obtained through averaging the ion signal obtained at more than 50 laser pulses. The oligonucleotides of known composition, T_{14} (*m/z* 4195.8), T_{15} (*m/z* 4500.0), and T_{23} (*m/z* 6933.6), were used for the mass calibration.

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