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# The Synthesis and Mass Spectrometry of Oligonucleotides Bearing Thiophosphoryl Modifications of the Predetermined Localization

**T. A. Luk'yanova, M. A. Zaitseva, V. A. Karpov, and G. E. Pozmogova[1]**

*State Research Institute of Physicochemical Medicine, Agency of Health and Social Development, ul. Malaya Pirogovskaya 1a, Moscow, 119435 Russia*

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**Abstract**—The technique of parallel automated synthesis of oligodeoxynucleotides bearing various local thiophosphoryl internucleotide bonds was optimized using assembling in the standby mode and creation of special program blocks. The selected conditions of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS) provided an increase in the method sensitivity (up to 1–10 fmol of oligonucleotide in sample) and registration of reliable spectra of oligodeoxynucleotide thiophosphoryl analogues. This enables to reliably prove the presence of the specified number of thiophosphoryl bonds within synthetic sequences. A series of oligodeoxynucleotides, thioanalogues of d(GGTTGGTGTGGTTGG), a known G-quadruplex antithrombin aptamer, were obtained.

*Key words: automated synthesis, mass spectrometry, thiophosphoryl oligodeoxynucleotides*

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## INTRODUCTION

Despite a great number of studies supporting a high therapeutic potential of RNA and DNA fragments, the use of oligonucleotides in medical practice is still limited. One of the most essential obstacles is their rapid biodegradation. Therefore, the development of methods of synthesis and evaluation of properties of structural oligonucleotide analogues bearing internucleotide bonds differing from natural ones [1–3] or containing modified sugar backbones [4–8] provoke interest. In addition, modified oligonucleotides are more and more widely used for diagnostics of diseases and in molecular biological research.

Specific aspects of use and metabolism of thiophosphoryl oligonucleotides was studied more completely. It was shown that hybridization properties of these analogues are close to those of natural oligonucleotides. At the same time, they are rather stable in biological liquids, are membrane-acting agents and, therefore, are effective antisense oligonucleotides [6, 8, 9]. On the other hand, thiophosphoryl oligonucleotides and their metabolites display high systemic toxicity [7–9]. Partially modified effective and less toxic sequences are only searched for. For example, it was shown that the substitution of two 3'-terminal internucleotide bonds by thiophosphoryl bonds increased in vivo half-life of oligonucleotides [9]. Thiophosphoryl oligonucleotides offer some advantages when used in gene diagnostics and rapid amplification of plasmid DNA [10, 11]. The thiophosphoryl modification of sugar backbone may provide functionally significant variations in the oligomer tertiary structure [12–14]. For example, a thio analogue of G-quadruplex oligonucleotide composed of telomeric repeats $d(TTAGGG)_4$ has a configuration of a melted chain [13, 14]. The study of the effect of thiophosphoryl replacements on the conformation of oligonucleotide aptamers is especially important as their spatial organization often determines their biological role [15, 16]. Therefore, with the goal to study the influence of thiophosphoryl replacements in oligonucleotides on their conformations, stability toward nucleases, and biological activity, we used in this work the sequence of the known G-quadruplex antithrombin aptamer TBA15 (thrombin binding aptamer) d(GGTTGGTGTGGTTGG) [16–18].

Comparative studies require synthesis of a wide set of partially modified oligonucleotides. Currently, for introduction of thiophosphoryl bonds within a traditional reaction cycle of oligonucleotide phosphoramidite method, oxidation of trivalent phosphorus in the presence of 3*H*-1,2-benzodithiol-3-one-1,1-dioxide is conducted. Preparation of highly purified partially thiomodified oligonucleotides is hampered because the

[1] Corresponding author; phone: +7 (495) 246-4310; e-mail: pozmge@gmail.com.

control of conversion of the reaction is unavailable and purification of oligomer sequences from potential phosphodiester admixtures is not always effective. In general, the data confirming the composition of synthetic oligomer thioanalogues are not available from publications. Only changes in properties of oligomers imply that some modifications took place [7, 8].

For screening and structure–function studies, a large series of variably modified oligonucleotides is required. The most economical approach is an automated parallel synthesis using multichannel synthesizers, both optimization of the synthetic strategy for locally modified oligonucleotides and the development of reliable methods of corroboration of their composition are most important.

## RESULTS AND DISCUSSION

### *Oligonucleotide Synthesis*

In this work, we carried out an automated synthesis of oligonucleotides using a traditional reaction cycle of oligonucleotide phosphoramidite synthesis. In the case of introduction of thiophosphoryl units, the oxidation step was carried out before capping to decrease the probability of formation of phosphodiester admixtures. For the formation of thiophosphoryl *O*,*O*,*O*-triesters, a standard freshly prepared solution of 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in acetonitrile was used. Technical characteristics of many modern synthesizers provide a possibility of a parallel synthesis of several oligomeric sequences as well as independent use of two different methods of organization of the reaction cycle in the process of elongation of each chain. Simultaneous assembling of exhaustively modified oligonucleotides or sequences bearing only terminal thiophosphates in the same positions does not cause any technical or program difficulties. The difference in the alternation of phosphotriester and thiophosphotriester internucleotide bonds in the sequence for the oligomers synthesized in parallel requires other organization of reaction cycles.

The synthetic program of automated multichannel synthesizers that describes the oligomer assembling procedure is composed of several subprograms, blocks that preset parameters of operation of the instrument for performing certain stages of the reaction cycle (for each monomer, deblocking, oxidation, capping, and coupling). For each channel, the same operations (washing, detritylation, capping, etc.) and parallel addition of identical nucleotide units in the sequences of the synthesized oligonucleotides are simultaneously carried out. Reduction of the time for oligomer assembling depends on the quantity of simultaneous and parallel stages of synthesis. A single method cannot contain different subprograms for carrying out identical reactions, e.g., oxidation reaction. The use of combination of other subprograms implies the employment of another method. The number of different methods and the number of their reversals is determined by the potential of the specific synthesizer model, but it does not change the principles of organization of the synthesis suggested in this work. For example, for ASM-800, two methods alternated seven times can be used for a parallel synthesis of eight sequences.

Flexible software of some synthesizers, including DNA ASM-800, supports realization of another approach based on the reprogramming or integration of blocks. Two following approaches proved to be most convenient and universal in this work:

1. “Standby mode”. In the process of simultaneous synthesis of all (eight for ASM-800) oligonucleotides bearing less than 4–5 sulfuration sites (without regard to their length), the introduction of the sign “0” (zero) into the specified nucleotide sequences provides operation of two programs within one parallel synthesis which differ by the order of capping and oxidation and, hence, by the composition and the input line for the oxidizing agent. In this case, the consumption of reagents does not increase, whereas the reaction time is only insignificantly grown. Noteworthy that, due to apparatus–software specific features, the sequences synthesized in parallel must bear identical 5'-terminal internucleotide bonds. Therefore, for example, oligonucleotides shown in Table 1 were synthesized by two individual parallel processes (series 1 and series 2).

2. For introduction of more than 4–5 thiophosphoryl bonds into oligomers, a deeper modification of the procedure is rational. For each nucleotide unit, two protocols are created, individual capping and oxidation blocks being normally included in the coupling block. Thus, the sequence is set with eight symbols rather than with four, where “G”, for example, corresponds to the phosphotriester coupling of a guanine unit and symbol “[g]”, to *O*,*O*,*O*-thiophosphotriester one. In this case, the oligomer P4 sequence (Table 1) containing four 3'-G-thiophosphoryl modifications should be specified as G[g]GTT[g]GTGT[g]GTT[g]GG.

This mode provides an unlimited number of internucleotide modifications, and it compensates a 2–3-fold increase in the time and reagent consumption.

The yields of routinely deblocked and purified oligomers were not affected by the assembling regime, other conditions being equal. The choice of the synthetic strategy of partially thiomodified oligonucleotides in favor of one of the described above approaches or their combination is determined by a specific task and composition of the target oligonucleotides.

### *Mass Spectrometry*

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is one of the most widely used and informative methods of analysis of oligonu-

**Table 1.** Sequences and assembling scheme in a standby mode of thioanalogues of oligodeoxyribonucleotide GGTTGGTGTGGTTGG (TBA15)

| Name* | Sequence | Series 1** |
|---|---|---|
| P111 | gGTTGGTGTgGTTGG | G 000000 000 0000 0GTT G 0 GTG T G G TTG 0 G |
| P4 | gGTT gGTGTgGTTgG | G 000000 000 0000 00GT T G GTG T G 0 GTT G G |
| TBAs | g g t t g g t g t g g g t t g g | GGTTGGTGTGG G 000000 000 0000 0000 0 T 000 0 T 0 000 G G |
| | | Series 2 |
| L1 | GGTTGGTGTGGtTGG | GGT 000 0TGG 0000 T G TGG 0 0 T 00T G G |
| L11 | GG tTGGTGTGGtTGG | GG 00T 0TGG 0000 T G TGG 0 0 T 00T G G |
| L121 | GG tTGG t gTGGtTGG | GG 00T 0TGG 00TG 0 0 TGG 0 0 T 00T G G |
| L343 | GG t t gG t g t g Gt t g G | GG TTG 000G TGTG 0 0 00G T T G 000 0 G |
| L3 | GG t t gGTGTGGTTGG | GG 0TTG G T GTG 0 0 0 GTT G G |
| P1415 | GGTTGGTGTGGTT gG | GGTT 000 GGTG 0000 T G GTT 0 0 G 000 0 G |
| P1011 | GGTTGGTGT gGTTGG | GGTT 0000 G G TGT 0 0 G GTT G G |
| P511 | GGTT gGTGTgGTTGG | GGTT 000G 0 G TGT 0 0 G GTT G G |

Notes: * Oligomers L contain thiomodifications in loops and oligomers P, in the planes of G-quadruplex TBA.

** Oligonucleotides of the series were synthesized under conditions of one parallel synthesis. Oligomers of series 1 contain thiophosphoryl 5'-terminal internucleotide bonds; series 2, phosphodiester bonds. 3'-Thiomodified units are designated with lower case characters. "0" means omission of the cycle; blocks for software for introduction of thiphosphoryl internucleotide bonds are in grey.

cleotide structures including those with various modifications and terminal functions (e.g., [19, 20]). There are some publications on MALDI MS of oligonucleotides bearing only 1 or 2 terminal thiophosphoryl modifications [7, 8, 21]. Our efforts to obtain mass spectra of the synthesized oligonucleotide thio analogues at good resolution using the standard protocol failed. Therefore, we analyzed the influence of preparation conditions of the matrix working solution, sampling, and some other parameters. Optimization experiments were carried out using phosphodiester oligomer TBA15 at the concentrations varied from 5 to 1000 fmol/μl on MALDI TOF spectrometers at a matrix/sample ratio of 2 : 1 (0.4 : 0.2 μl). The following parameters were compared: uniformity of crystallization of 3-hydroxypicolinic acid, distribution of the sample on the matrix, signal/noise ratio, peak intensity, the presence of peaks of adducts of alkaline metal ions, and reproducibility.

The quality of solvents (mixtures of water and acetonitrile) of various grades and manufacturers substantially affected the quality of mass spectra. It turned out that high quality mass spectra of oligonucleotide diluted solutions (50 fmol/μl, 100 : 1 signal/noise) allowed five of twelve combinations (see the Experimental section) and only three of them (water, Sigma LC-MS–acetonitrile, Fluka for mass spectrometry; water, Sigma LC-MS–acetonitrile, Merck for spectroscopy; and water, Panreac Hiperpur-plus–acetonitrile, Panreac Hiperpur-plus) gave suitable results for 20 fmol/μl solution (10 : 1 signal/noise). Note that a variation of exposure time of matrix crystallization (10–45 min) only negligibly affected the result. An addition of 20–60% acetonitrile into a sample water solution proved to be a good approach, because it provided a reproducible crystallization picture with the characteristic type of crystals (see Fig. 1), improvement in the uniformity of sample distribution, and increase in peak height in the spectra as well as their better resolution. For example, under the optimized conditions, the spectra with a 5 : 1 signal/noise ratio were obtained even for 5 nM TBA15 (1 fmol in the aliquot).

The developed procedures of matrix preparation and sample loading resulted in reliable mass spectra of thiophosphoryloligonucleotides, particularly, of analogues of antithrombin aptamer, whose structures are given in Table 1, with a 50 : 1 signal/noise ratio and dominating peak of molecular ion. Typical spectral patterns

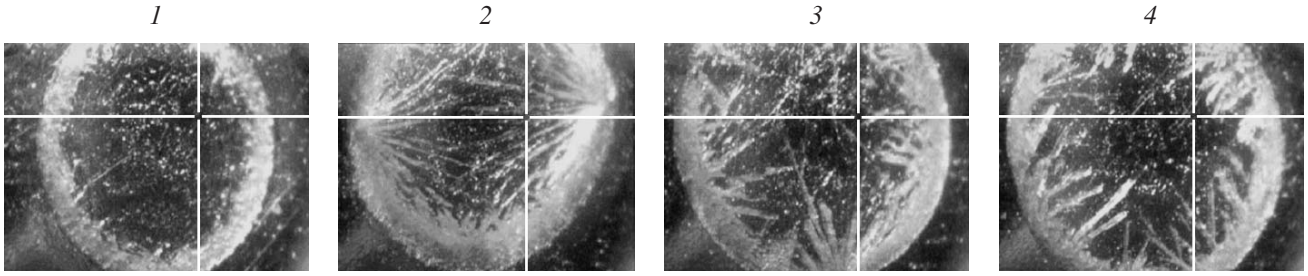


**Fig. 1.** A typical appearance of MSP target polished steel with the sample when oligonucleotide is loaded on 3-hydroxypicoline matrix as a solution (*1*) in water and in water in the presence of (*2*) 20, (*3*) 40, and (*4*) 60% acetonitrile on a MicroFlex mass spectrometer.

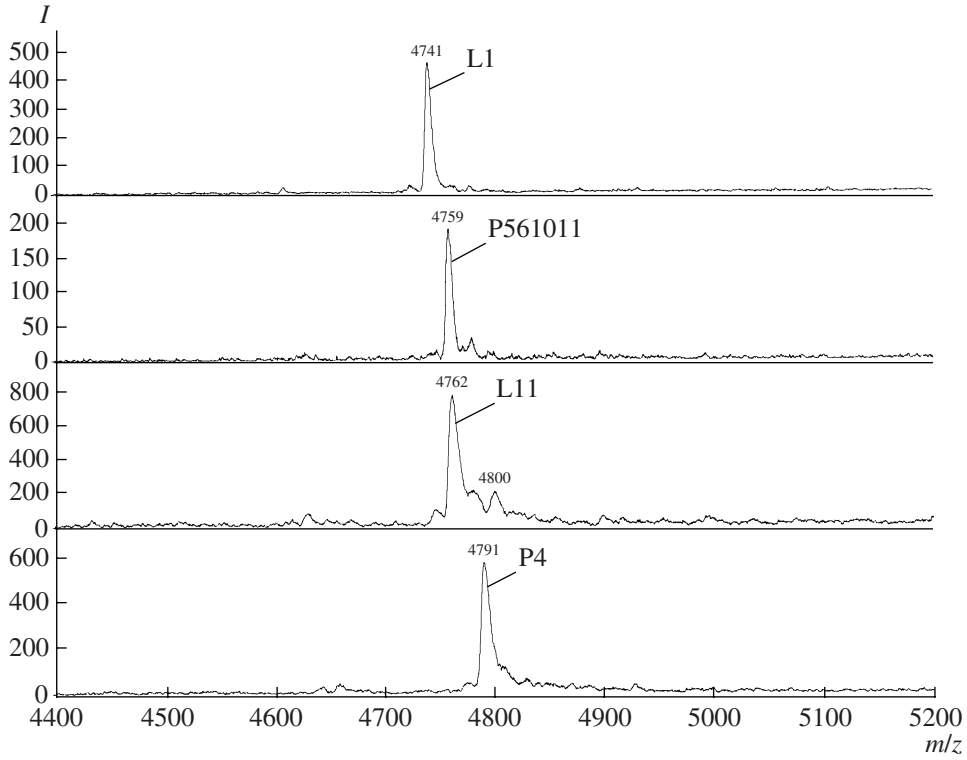


**Fig. 2.** Mass spectra of oligonucleotides L1, P561011, L11, and P4.

obtained on a MicroFlex spectrometer are shown in Fig. 2.

Based on the results, the number of sulfur atoms (*n*) in the oligonucleotide molecule is easily calculated by the difference in the found and calculated molecular masses for the corresponding phosphodiester oligomer. The calculations confirmed the composition of the synthesized sequences at a high precision (see Table 2).

To summarize, we managed to develop conditions for MALDI TOF mass spectrometry ensuring an increase in the method sensitivity and registration of positive characteristics of oligonucleotide thiophosphoryl analogues reliably confirming the presence of a preset number of thiophosphoryl bonds within the target sequences.

We proposed some approaches to the parallel automatic synthesis of oligonucleotides bearing various local thiophosphoryl internucleotide bonds based on the design of special software blocks or oligonucleotide assembling in a standby mode. The developed schemes of organization of reaction cycles can be successfully used on modern synthesizers as well as for the development of new models.

Parallel synthesis yielding highly purified thioanalogues of antithrombin aptamer TBA15 bearing a preset number of modifications demonstrated efficiency of the developed methods of oligonucleotide assembling.

**Table 2.** The number of sulfur atoms in oligonucleotide molecules based on the analysis of mass spectra

| Oligonucleotide | Molecular mass, Da | | *n** |
|---|---|---|---|
| | calc. | found | |
| TBA15 | 4726 | 4727 | 0 |
| TBAs | 4950 | 4949 | 14 |
| L1 | 4742 | 4741 | 1 |
| L11 | 4758 | 4762 | 2 |
| L121 | 4790 | 4789 | 4 |
| L343 | 4886 | 4885 | 10 |
| L3 | 4774 | 4774 | 3 |
| P1415 | 4742 | 4742 | 1 |
| P1011 | 4742 | 4740 | 1 |
| P121011 | 4758 | 4758 | 2 |
| P561011 | 4758 | 4759 | 2 |
| P4 | 4790 | 4791 | 4 |

Note: * The number of sulfur atoms ($n$) in oligonucleotide molecules is accurate within integers using the following formula: $n = [M_F - M_C]/16$, where $M_F$ is the oligonucleotide molecular mass found by mass spectrometry; $M_C$, calculated molecular mass of the corresponding phosphodiester, in this case, the mass of TBA15 is 4726 Da. 16 is the difference between molecular masses of sulfur and oxygen.

The developed methods make partially modified oligonucleotides available, which supports their wide use as research tools for molecular biology and components of diagnosticums as well as instruments for search for new oligonucleotide-based drugs.

## EXPERIMENTAL

**Oligonucleotides were synthesized** by the solid phase phosphoramidite method on an automated DNA ASM-800 synthesizer (Biosset, Russia) with the use of standard commercial reagents: nucleoside–phosphoramidite derivatives, polymers–nucleosides, 3*H*-1,2-benzodithiol-3-on-1,1-dioxide (Glen Research, United States), and solvents (Lekbiopharm, Russia, and Panreac, Spain) under the conditions of modified software for reaction cycles (not listed because of a large volume).

For reaction cycles with introduction of 3'-thiophosphoryl units, a freshly prepared 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile was used instead of a standard oxidizing agent containing iodine and water. Deblocking and degradation of oligonucleotide–support bonds were carried out by ammonolysis (28% ammonia, 5 h, 50°C). 5'-*O*-Dimethoxytrityl oligonucleotide derivatives were purified by reversed-phase HPLC in a linear gradient of acetonitrile in 0.1 M ammonium acetate (pH 7.0, 50°C). The dimethoxytrityl group was removed with 80% acetic acid (20 min). Deblocked oligonucleotides were rechromatographed to give 15-mers in yields of 10–12 $OU_{260}$ (62-75 nmol) on a scale of 0.1 μmol (Table 1). The resulting oligomer preparations were analyzed by HPLC, MALDI TOF mass spectrometry, UV spectrophotometry, and PAG electrophoresis. According to the HPLC data, the purity of the resulting oligomers was at least 97%.

**Mass spectra** were obtained on time-of-flight UltraFlex and MicroFlex mass spectrometers (Bruker, Germany) supplied with UV lasers (Nd, 354 nm, and $N_2$, 337 nm) using a standard MSP target polished steel (Bruker, Germany). Mass spectra were obtained in a linear regime with detection of positive ions; the accuracy of measured masses was 0.1%.

For sampling, a solution of 3-hydroxypicolinic acid (Fluka, United States) (0.4 μl) was loaded on a target, dried on air, and the formed crystals were covered with a sample oligonucleotide solution (0.2 μl) in water or acetonitrile at varied concentrations, and dried again. Saturated solutions of 3-hydroxypicolinic acid in 50% acetonitrile were obtained using 12 variants of solvent combinations of various grades: water, Sigma LC-MS (United States), Sigma BPC Grade, Panreac Hiperpur-Plus (Spain), and Merck Ultrapur (Germany); acetonitrile, Panreac Hiperpur-Plus, Fluka (for mass spectroscopy), and Merck (for spectroscopy, Uvasol). After 10% ammonium citrate (10 vol %) was added, the resulting matrix solution was stored at –20°C no longer than for 2 months.

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