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Optimization of Automatic Synthesis of Benzoxazole Phosphoramidate Oligonucleotides

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Abstract. Phosphoramidate benzoazole oligonucleotides (PABAO) are a newly developed oligonucleotides derivatives that carrying internucleotide *N*-(benzoazole)-phosphoramidate moieties. They can be obtained by the classical method of automated solid-phase phosphoramidate synthesis, in which the oxidation step is replaced by a Staudinger reaction with an azide modifier. In this work, optimization of the synthetic protocol of *N*-benzoxazole oligonucleotides was carried out. The optimization consisted of reducing the concentration of the azido reagent and shortening the synthesis time. With the adjustment, optimized synthesis conditions allow for a 2.5-fold decrease in benzoxazole azide consumption as well as a 20-min reduction in the time required for single modification introduction in oligonucleotide synthesis. The obtained advantages will be used for the scaled-up production of PABAOs in order to study them in biomedical applications.

Keywords: phosphoramidate benzoazole oligonucleotide, PABAO, automatic synthesis, Staudinger reaction.

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Introduction

Phosphoramidate benzoazole oligonucleotides (PABAO) are a newly developed derivative of nucleic acids that can be synthesized using the classical automatic solid-phase phosphoramidate method with some modification [1, 2]. The synthesis of four homological types of PABAO containing *N*-benzimidazole, *N*-benzoxazole, *N*-benzothiazole, or 1,3-dimethyl-*N*-benzimidazole phosphoramide groups has been demonstrated [1]. Studies of their physico-chemical properties have shown their ability to form stable complexes with complementary DNA and RNA chains [1, 3]. It was found that when PABAO duplexes are formed with DNA, a B-form of double-stranded nucleic acid is preserved [3]. Previously proposed phosphoryl guanidine oligonucleotides (PGOs) [4–6] have shown promise in various biomedical applications [7, 8]. Later developed PABAOs

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have a number of advantages with respect to PGOs. They are more hydrophobic and, unlike PGOs, are able to change charge depending on the pH of the medium [1, 3]. Studies of the usage of PABAO as primers in PCR diagnostic systems have shown the more prospects of their application in allele-specific PCR (AS-PCR) when detection of a low concentration of mutant DNA against a large number of wild-type DNA [9]. The developed PABAOs allow for the construction of oligonucleotides with specified physicochemical and biological properties. This can be achieved by simply changing the benzoazole azides used in the synthesis. As a result, PABAO can be used to address a wide range of tasks in fundamental research and applications.

The aim of the research is to optimize the synthetic protocol of the PABAO. The Staudinger reaction of benzoazole azides is used at the oxidation stage to introduce modifications into the oligonucleotide structure [1]. In previous studies, we used concentrations of 0.25 M solutions of benzoazole azides and an oxidation time of 40 min [1, 9]. The latter is 60 times greater than the phosphite oxidation stage by iodine in the optimized protocol of native oligodeoxyribonucleotides. Moreover, the long time required to obtain modified oligonucleotides is associated with the long duration of the Staudinger reaction. Also, the high cost of benzoazole azides makes it necessary to reduce the consumption of these compounds. Here, we searched for optimal conditions for the PABAO synthesis by varying the concentration of azide and the time of the Staudinger oxidation stage. A *N*-benzoxazole azide was used as an example of an azido reagent for the introduction of the *N*-benzoxazole modification at the 5′-end phosphate residue of oligonucleotide. The fraction of the modified oligonucleotide in the reaction mixture was determined by the methods of reverse-phase high-efficiency liquid chromatography (RP-HPLC). Based on the obtained data, optimal conditions for PABAO synthesis were determined in terms of the duration of the oxidation stage and the concentration of azide.

1. Materials and methods

1.1. Materials

Acetonitrile (CH_3CN, RCI Labscan Limited, Thailand), tetrahydrofurane (THF, RCI Labscan Limited, Thailand), propionic anhydride (Chemical line, Russia), *N*-methylimidazole (Sigma Aldrich, USA), pyridine (Sigma Aldrich, USA), dichloromethane (CH_2Cl_2, RCI Labscan Limited, Thailand), trichloroacetic acid (Sigma Aldrich, USA), iodine (RIAKHIM, Russia), 5-ethylthio-1*H*-thetrazole (Sigma Aldrich, USA), concentrated ammonia solution (RIAKHIM, Russia), DMTr-deoxyhymidine-CE-phosphoramidite (Biolabmix, Russia), triethylamonium acetate (Sigma Aldrich, USA) were used in the study. *N*-Benzoxazole azide has been synthesized from *N*-bensoxazole chloride (Sigma Aldrich, USA), as described in ref. [1].

1.2. Synthesis of modified oligonucleotide

Oligodeoxyribonucleotide synthesis was carried out using the automatic DNA synthesizer ASM-800E (Biosset, Russia) according to the standard phosphoramidate synthetic protocol. In the case of introducing a phosphate modification, the oxidation stage in the synthetic protocol was replaced by the Staudinger reaction of the last internucleosidic phosphate residue at the 5′-end of hexatymidylate with *N*-benzoxazole azide.

Oligonucleotide synthesis was performed at a scale of 0.16 μM with a 3-min coupling step for DMTr-dT-CE-phosphoramidite (0.06 M in CH_3CN) and 5-ethylthio-1*H*-thetrazole (0.25 M in CH_3CN) as activating agent. A mixture of propionic anhydride in THF and *N*-methylimidazole

in THF were utilized as capping reagents. The oxidizing agents were iodine 0.02 M in pyridine/water/THF (1/9/90) or *N*-benzoxazole azide (see concentration in the Results section) in CH_3CN. Trichloroacetic acid (3%) in CH_2Cl_2 was used as a detritylating reagent. A universal polymer support (controlled pore glass, CPG) with pores of 500 $\AA$ was used in the oligonucleotide synthesis (Lumiprobe, Russia).

After synthesis, the polymer was treated with concentrated ammonia at 55°C for 18 h to separate the oligonucleotide from the polymer carrier and remove the phosphate protecting groups. The reaction mixtures were then evaporated on a rotavapor concentrator, and the oligonucleotide was dissolved in 50 μl of milliQ water and analyzed.

1.3. Reverse-phase high-pressure liquid chromatography (RP-HPLC)

Synthesized oligonucleotides were analyzed on HPLC chromatograph Shimadzu (Shimadzu, Japan) using a Zorbax SB-C18 (5 μm, 4.6×150 mm) column (Agilent, United States) with a 0 to 30% gradient of acetonitrile in 20 mM triethylammonium acetate (pH 7.0) for 30 min; flow rate 1.5 mL/min.

1.4. Mass spectra analysis

Mass spectra analysis of the compounds was carried out at the Center for Mass Spectrometric Analysis of the Institute of Chemical Biology and Fundamental Medicine SB RAS. A liquid chromatography with tandem mass spectrometry with electrospray ionization (ESI LC-MS/MS) was performed in the negative ion mode using ESI LC/MS/MSD XCT equipment (Agilent Technologies, United States).

2. Results

To investigate the effectiveness of the inclusion of *N*-benzoxazole modification into oligodeoxyribonucleotide in the process of solid-phase amidophosphite synthesis, we used the model of hexathymidylate. The using of oligothymidylate avoids the need to take into account the efficiency of removal of heterocyclic bases protective groups when assessing the overall efficiency of synthesis. Also, such relatively short native oligomers are synthesized with a yield close to 100%, which in turn makes the quantitative estimation of the output of PABAO synthesis easy and reliable. The introduction of the modification in the last internucleoside phosphate residue avoids any effect of modification on the subsequent synthesis.

Our previous studies have shown that concentration of 0.25 M of benzoazole azides and reaction time of 40 min is sufficient for high yields (>95%) of multiply modified PABAOs [1]. These durations and concentrations are excessive to obtain modified oligonucleotides without an unmodified one in the reaction mixture. For the standard oxidation of phosphates, a 0.02 M iodine solution is used for 40 sec, while the synthesis of PG-modified oligonucleotides involves oxidation in a 0.1 M azide solution [10]. In the case of phosphorothioate oligonucleotides, oxidation using sulfurizing reagent II is carried out for 1 min at a concentration of 0.05 M [11]. In order to test the possibility of reduction of azide consumption, we chose concentrations of *N*-benzoxazole azide: 0.15, 0.1, 0.05, and 0.01 M. We tested the oxidation time of the Staudinger reaction 40, 30, 20, or 10 min to increase the productivity of PABAO synthesis. This is especially important in the synthesis of long oligonucleotides containing a large number of modified phosphate residues.

Synthesis was carried out automatically using standard reagents and synthetic protocols, with the only difference being that the last stage of oxidation by iodide was replaced by the Staudinger reaction with *N*-benzoxazole azide. After synthesis, the oligonucleotide was separated from the polymer carrier, as described in the materials and methods section. The analysis of the synthesis efficiency was conducted using the RP-HPLC method in a gradient of acetonitrile. The introduction of *N*-benzoxazole group leads to an in higher the hydrophobicity of the oligonucleotide resulted in increase the retention time of the PABAO in comparison with native hexathymidylate [1]. We varied the concentrations of azide from higher to lower and change the oxidation stage time. Initially, the concentration, reduced relative to the previously used (0.25 M), was taken as 0.15 M, and the presence of the incomplete oxidation product was assessed. Such a by-product in small quantities appears only at 10 min of the duration of the Staudinger reaction. In the chromatographic profiles of the reaction mixtures, the presence of additional peaks with retention times (τ_R) of 11.7 and 12.1 min in addition to the main peaks with τ_R 14.5 min, indicates an incomplete phosphite oxidation in the Staudinger reaction (Fig. 1b). The notable fractions of the by-products were found only at a reaction time of 10 min when the concentration was reduced to 0.1 M (Fig. 1c, peaks at τ_R of 11.7 and 12.1 min). Therefore, conversion completeness analysis at 0.05 M azide concentration was performed at oxidation phase times of 20 min or more. We detected the formation the products of partial oxidation at 0.05 M of azide concentration and reaction time of 20 min (Fig. 1d). Similarly, oxidation analysis at 0.01 M concentration was performed at 30 and 40 min. In this case, for both reaction times considered, we predominantly observed by-products (Fig. 1e).

It can be seen that the product with a τ_R of 15 min is imaged by two peaks indicating its two diastereomers. The proof that there are indeed diastereomers was conducted using ESI LC-MS/MS analysis of the mixture of both peaks in negative ion mode, which showed the presence of only peaks (ions of different charges) corresponding to the mass of only one modified oligonucleotide. The calculated mass is 1877.8, while the experimentally determined by ESI MS analysis value was 1878.0. This analysis was performed for the reaction mixture for 0.15 M concentration of azide and 40 min of oxidation time. It should be noted, the quantity of Rp and Sp diastereomer is different. The estimated of the fraction of oligonucleotide by the area of the peaks gives the following values. The fraction of a faster product with a retention time of 14.38 min on the column is 40%, and a slower one is 14.42 and 60%, respectively. In contrast, the synthesis of phosphate-modified oligonucleotides such as PGO [12] or methylphosphonate [13] usually results in the formation of a racemic mixture.

A quantitative analysis of the chromatograms presented in the Fig. 1 was carried out. Product yield was calculated as the ratio of areas under peak with retention times 14.1 – 15.6 min to the total area of peaks from 11.4 to 15.6 min. The results of the analysis are presented in Tab. 1.

Table 1. Yields of the *N*-benzoxazole containing hexathymidylate d(T*TTTTT) depending on the azide concentration and the duration of the oxidation phase using the Staudinger reaction

	0.15 M	0.1 M	0.05 M	0.01 M
10 min	94.1%	86.6%	n/d[#]	n/d
20 min	97.9%	97.2%	85.9%	n/d
30 min	97.2%	98.8%	95.9%	36.0%
40 min	98.1%	98.7%	97.6%	45.1%

[#] — n/d — not determined

Since the method error is 2%, the obtained data indicates that the optimal azide concentration

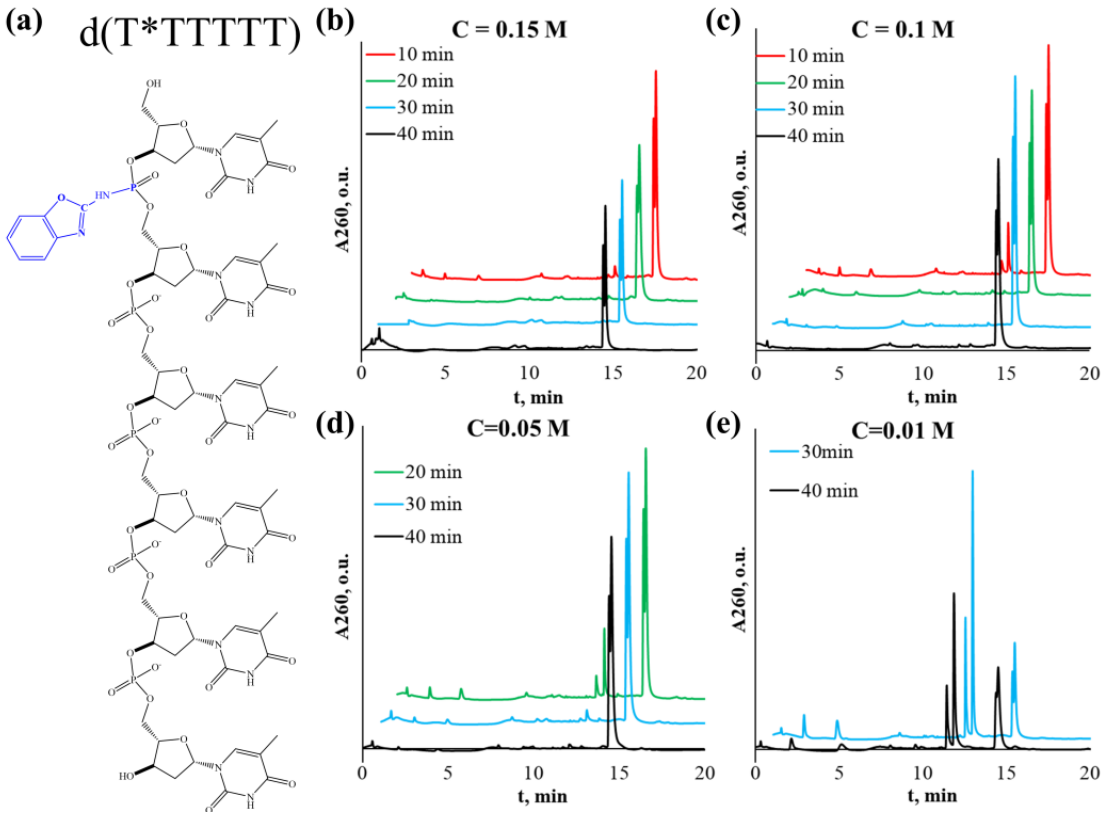

Fig. 1. (a) Structure of the *N*-benzoxazole phoshoramide hexathymidylate d(T*TTTTT). Benzoxazole phoshoramide group shown in blue. RP-HPLC profiles of the reaction mixture of d(T*TTTTT) obtained at different concentration of *N*-benzoxazole azide in the synthesis: (b) 0.15 M; (c) 0.1 M; (d) 0.05 M; and (e) 0.01 M. The oxidation stage time correspond to the color of the lines: 40 min — black, 30 min — blue, 20 min — green, 10 min — red. Chromatograms corresponding to 30, 20 and 10 min of the oxidation time are shifted on x-axis on 1, 2, and 3 min, respectively, and on y axis for convenient visualization

for routine synthesis is 0.1 M solution and the oxidation time at the Staudinger reaction is 20 min. This reduces reagent consumption by two and a half times and reduces the synthesis time of oligonucleotides, especially those containing multiple modifications. If it is necessary to accelerate the synthesis, for example, when synthesizing long oligonucleotides with multiple modifications, the concentration of azide can be increased up to 0.15 M with reducing the oxidation time. In the case where researchers have a small amount of *N*-benzoxazole azide at introducing a single modification into the oligonucleotide, a solution concentration of 0.05 M can be used, while it is necessary to extend the oxidation time to 40 min.

In this work, the optimization of the phosphoramidate synthetic protocol of *N*-benzoxazole oligonucleotides was carried out. The possibility of reducing in the concentration of *N*-benzoxazole azide used in the oxidation stage and the shortening of the phosphate oxidation time in the Staudinger reaction is justified. Using *N*-benzoxazole azide as an example, we demonstrated that optimizing the PABAO synthetic protocol not only reduces reagent consumption by 2.5 times but also decreases the time for each modification by 20 min.

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Оптимизация условий автоматического синтеза бензоксазольных фосфорамидных олигонуклеотидов

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Аннотация. Фосфорамидные бензоазольные олигонуклеотиды (ФАО) — недавно разработанные производные нуклеиновых кислот, несущие межнуклеотидные *N*-(бензоазол)-фосфорамидные остатки. Их можно получить классическим методом автоматического твердофазного фосфитамидного синтеза, в котором стадия окисления заменена реакцией Штаудингера с азидным модификатором. В данной работе проведена оптимизация протокола синтеза *N*-бензоксазольных олигонуклеотидов. Она заключалась в снижении концентрации азидо-реагента и сокращении времени синтеза. Оптимизированные условия синтеза позволяют снизить расход бензоксазолазида в 2.5 раза, а также сократить на 20 мин время, необходимое для введения единичной модификации при синтезе олигонуклеотида. Полученные преимущества будут использованы для масштабированного синтеза ФАО с целью изучения их в биомедицинских приложениях.

Ключевые слова: фосфорамидные бензоазольные олигонуклеотиды, ФАО, автоматический синтез, реакция Штаудингера.