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Synthesis of Oligonucleotides Carrying Inter-nucleotide *N*-(Benzoazole)-phosphoramide Moieties

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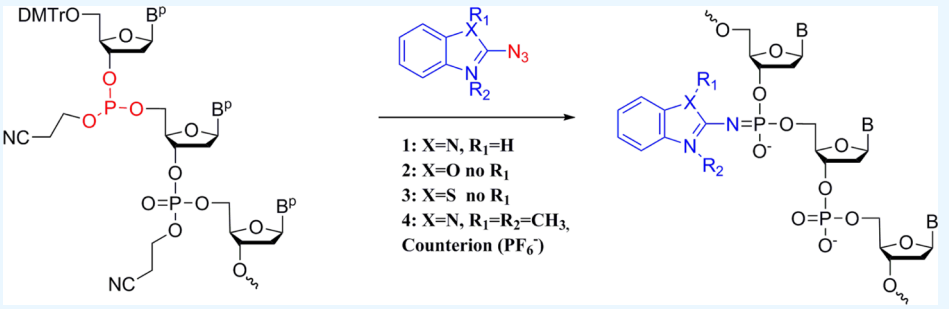

ABSTRACT: In this work, we present new oligonucleotide derivatives containing inter-nucleotide *N*-benzimidazole, *N*-benzoxazole, *N*-benzothiazole, and 1,3-dimethyl-*N*-benzimidazole (benzoazoles) phosphoramide groups. These modifications were introduced via the Staudinger reaction with appropriate azides during standard automated solid-phase oligonucleotide synthesis. The principal structural difference between the new azido modifiers and those already known is that they are bulk heterocyclic structures, similar to purine nucleoside bases. Modified oligonucleotides with one and two modifications at different positions and multiple modified heteronucleotide sequences were obtained with high yields. The possibility of multiple modifications in the process of automatic DNA synthesis is fundamental and critical for further application of our oligonucleotide derivatives. Initial studies on the properties of new oligonucleotides were carried out. The stability of the oligodeoxyribonucleotide duplex containing phosphoramide groups of *N*-benzoazoles with complementary DNA or RNA is slightly lower than that of native complexes.

1. INTRODUCTION

Currently, the synthesis of oligonucleotides is of great importance in the field of life sciences. Synthetic oligonucleotides are used as polymerase chain reaction (PCR) primers in diagnostics, sequencing, and gene synthesis for synthetic biology. They are promising tools for the development of drugs to treat cancer, various genetic diseases, and viral and bacterial infections.[1,2] Oligonucleotide therapies such as antisense oligonucleotides, siRNAs, and CRISPR-Cas9 are the booming market.[3−5] Solid-phase chemical synthesis of oligonucleotides with reagent substitution in any of the cycles allows the introduction of a huge number of functional groups and structural changes, for example, changing the base and sugar and introducing noncanonical nucleobases. An inter-nucleotide phosphate is an attractive site for chemical functionalization of nucleic acids due to the phosphite intermediate formed in the synthesis cycle. The classical modification is the production of phosphorothioates.[6] Phosphate-modified oligonucleotides meet the key requirements for therapeutic oligonucleotides. They can form a stable complex with the complementary RNA chain and are resistant to cleavage by nucleases.

An interesting and rapidly developing method for obtaining new modifications of the inter-nucleotide bond is the one with the use of the Staudinger reaction.[7] This reaction between the esters of 3′,5′-dinucleoside phosphites and organic azides has been used previously to introduce *N*-sulfonylphosphoramide groups[8−11] and *N*-alkylphosphoramide groups[12,13] into the oligonucleotide. The phosphoryl guanidine and mesyl phosphoramidate oligonucleotides are particularly important as potential splice-switching agents.[14]

More recently, chimeric stereopure oligonucleotides with phosphorothioate (PS) and phosphorylguanidine (PN) backbone modifications were obtained. These oligonucleotides have been shown to have markedly improved pharmacology and efficacy compared to PS-derived oligonucleotides.[15] In

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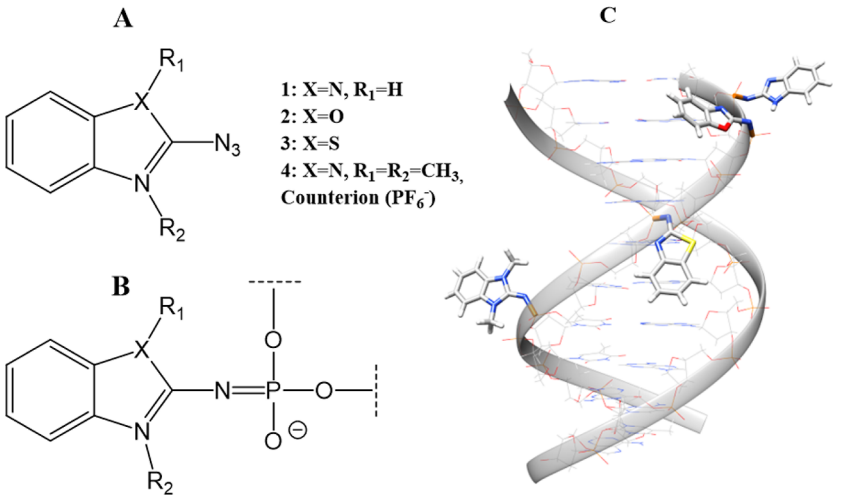
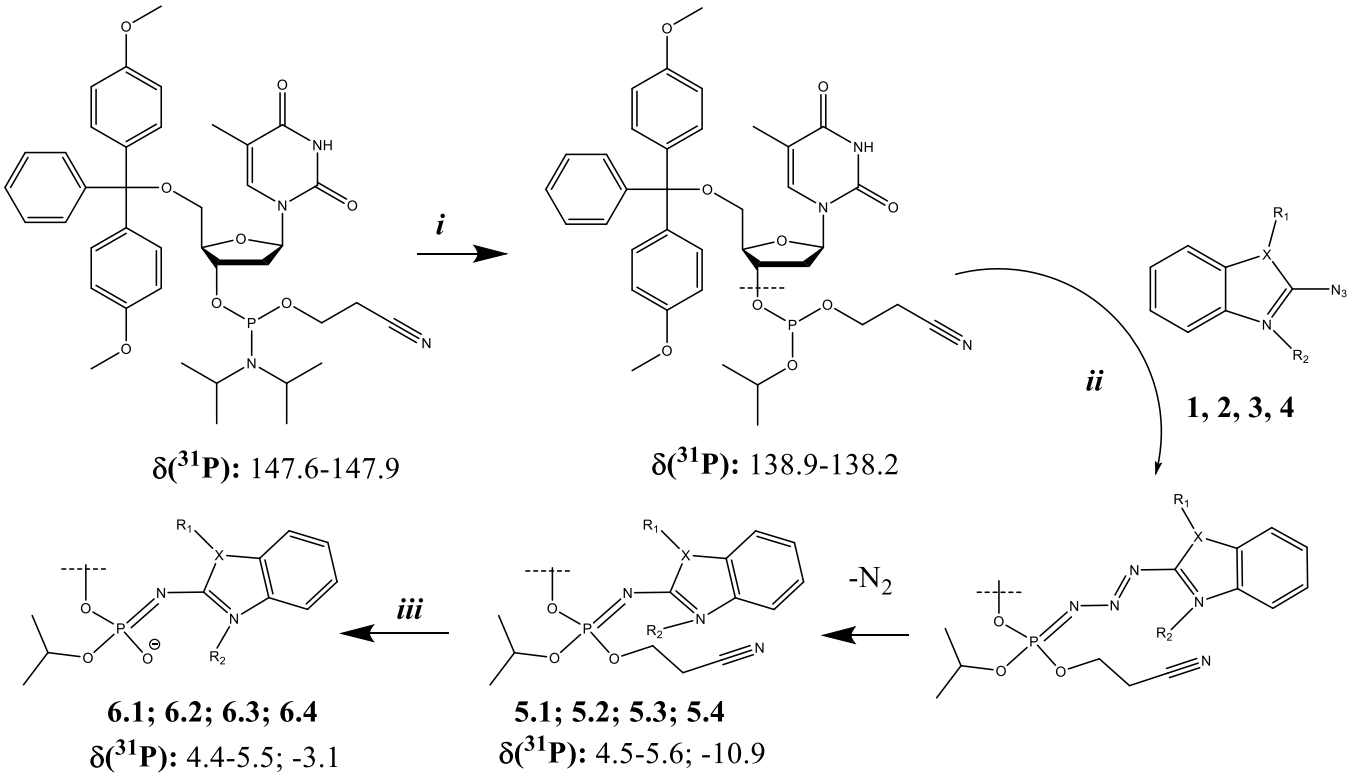
Scheme 1. (A) General Structure of Benzoazole Azides; (B) Modified Inter-nucleotide Phosphate Group; (C) Schematic Representation of the DNA Duplex with *N*-Benzimidazole, *N*-Benzoxazole, *N*-Benzothiazole, and 1,3-Dimethyl-*N*-benzimidazole Phosphoramide Groups



Scheme 2. Schematic Representation of the Studied Transformations Using Azides 1−4[a]



[a](i) CH_3CN solvent, isopropyl alcohol, ethylthiotetrazole; (ii) CH_3CN solvent, azides **1−4** ambient temperature, 5 min; and (iii) triethylamine, 10 min. For compounds characterized by NMR, the characteristic chemical shifts or their ranges for ^{31}P nuclei are noted.

addition, it has been shown that the incorporation of PN linkages into stereopure oligonucleotides with chimeric backbone modifications has the potential to render regions of the brain beyond the spinal cord more accessible to oligonucleotides and, consequently, may also expand the scope of neurological indications amenable to oligonucleotide therapeutics. Incorporation of various types of PN linkages to a stereopure oligonucleotide backbone can increase the potency of silencing in cultured neurons under free-uptake conditions 10-fold compared with similarly modified stereopure phosphorothioate (PS)- and phosphodiester (PO)-based molecules.[16]

Recently, the advantages of PN applications in an allele-specific real-time PCR analysis have been shown. The introduction of phosphorylguanidine modification(s) leads to a significant increase in the selectivity and a decrease in the concentration detection limit of the mutant DNA.[17,18]

Currently, the choice of modifiers to investigate the impact of phosphoramidate (PN) containing backbone linkages such as those generated with phosphoryl guanidine is limited. In this

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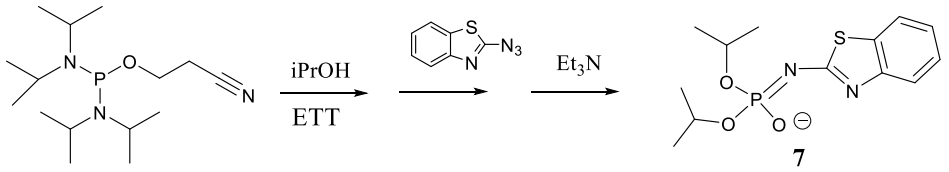
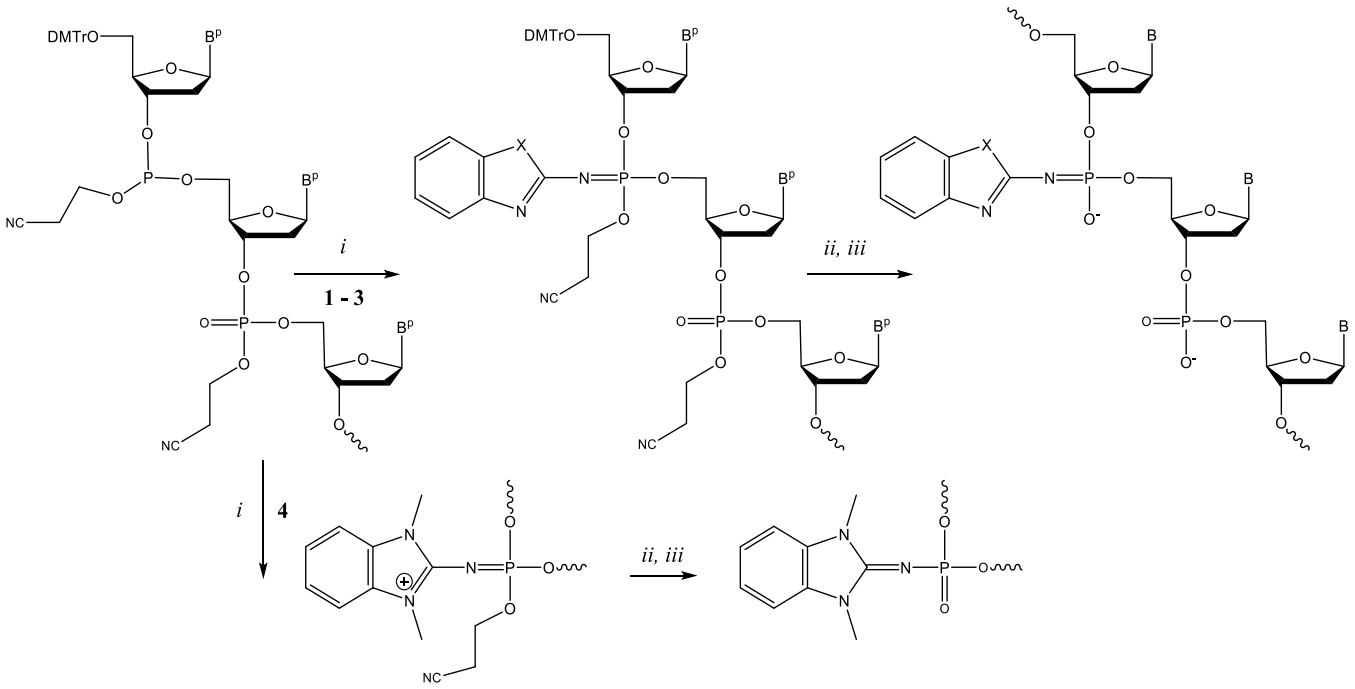
Scheme 3. Synthesis of Bisisopropyl-benzothiazol-2-yl-phosphoramidate 7



Scheme 4. Scheme for the Preparation of Oligodeoxyribonucleotides with *N*-(Benzoazole)-phosphoramide Groups According to the Staudinger Reaction with the Corresponding Azides[a]



[a](i) 0.25 M **1**−**4**, MeCN, 25 °C, and 15 ÷ 45 min; (ii) DNA synthesis; and (iii) concentrated aqueous NH_3, 55 °C, and 1−12 h. B is a nitrogenous base and ^{P} is an N-protecting group.

connection, the inter-nucleotide benzoazole (benzo-imidazoles, -oxazoles, and -thiazoles) phosphoramide (PN) substituents look interesting and promising (Scheme 1). In addition, the benzo-imidazoles, -oxazoles, and -thiazoles are of particular interest among the most highly recognized pharmacophores.[19] They are well known to exhibit a broad range of biological activities; these include anti-HIV, antibacterial, antitumor, and neuroprotective activities.[20,21] They also show interesting advantages in advanced materials science such as nonlinear optics[22] and organic light-emitting diodes.[23] The main idea of this work is to expand the variety of modifications with PN linkages in an oligonucleotide backbone using new azide modifiers.

2. RESULTS

In this work, we present benzo-imidazole, -oxazole, and -thiazole azides for the synthesis of new oligonucleotide derivatives using the Staudinger reaction (Scheme 1A,B). The molecular structure of a DNA duplex with *N*-benzoazole phosphoramide groups is schematically shown in Scheme 1C (the molecular structure is prepared with the use of UCSF Chimera software[24]).

2.1. Synthesis of Benzoazole Azides. 2-Azidobenzimidazole **1**, 2-azidobenzoxazole **2**, and 2-azidobenzothiazole **3** (Scheme 1) were obtained from the corresponding chlorides according[25] to minor changes: the reactions were carried out in dimethylformamide (DMFA) under heating at 60 °C overnight (see in the Experimental Section). The 2-azido-1,3-dimethylbenzimidazole hexafluorophosphate (ADMBI) **4** was obtained in three steps from 2-hydroxybenzimidazole according to the described methods.[26,27] Formation of azides was confirmed by IR, UV, and NMR spectroscopies, which were in accordance with the previously published data[25−32] (Figures S1−S9).

2.2. Study of Azides' Reactivity with Trivalent Phosphorus (Staudinger Reaction) on the Example 3′-Phosphitetriester Thymidine. Reactions of azides **1**−**4** with a triester were carried out under the control of ^{31}P NMR as described earlier[33] (Scheme 2). Initially, 5′-*O*-DMT-thymidine-3′-CE phosphoramidite was converted to a phosphite triester. The chemical shift of the phosphorous atom in the phosphoramidite is close to 148 ppm, and for the phosphite triester, this value was close to 139 ppm (Figure S10). The reaction of the phosphite intermediate with azides **1**−**4** proceeded very quickly; in 5 min, signals from phosphorus(III) disappear in all cases. Visually, this transformation is characterized by the appearance of a yellowish color of the solution, which disappears after a few minutes. NMR analysis showed complete conversion of P(III) to P(V). For iminophosphorane **5.1**−**5.3**, the value of $\delta(^{31}P)$ reaches 4.5−

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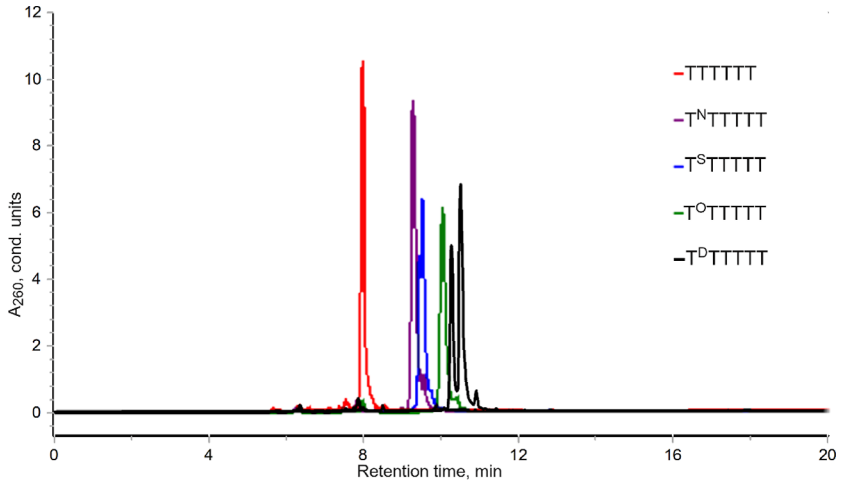

Figure 1. HPLC elution profiles of reaction mixtures for TTTTTT oligonucleotide (red) without modification, (purple) T^{N}TTTTT, with *N*-benzimidazole, (blue) T^{S}TTTTT with *N*-benzothiazole, (green) T^{O}TTTTT—*N*-benzoxazole, and (black) T^{D}TTTTT 1,3-dimethyl-*N*-benzimidazole phosphoramide groups. Conditions: see in the Experimental Section.

5.6 ppm and for **5.4** −10.9 ppm. The addition of a base (NEt_3) to the reaction mixture containing iminophosphorane **5** naturally turns it into the corresponding phosphoryl derivative **6** by the *β*-elimination reaction. The value of $\delta(^{31}P)$ for **6.1**−**6.3** is close to the value of 4.4 to 5.5 ppm; for **6.4**, the value of $\delta(^{31}P)$ is −3.1 (Figures S11−S18). The successful results of the experiment allowed us to proceed to the study of oligonucleotide modification by the azides.

2.3. Determination of the Modifying Group Contribution to the Extinction Coefficient of the Oligonucleotide. To determine a contribution of the modifying group to the extinction coefficient of some modified oligonucleotides, bisisopropyl-benzothiazol-2-yl-phosphoramidate **7** (as the closest in structure to that introduced into the oligonucleotide modification) was obtained (Scheme 3 and Figures S19 and S20). The extinction coefficient of the *N*-benzothiazole modifying group was defined according to the UV spectrum of **7** ($C = 8 \times 10^{-5}$ M, 50% EtOH−H_2O): ε = 7125 (260 nm) (Figures S21 and S22). Subsequently, the assumption was made that the contribution to the extinction coefficient made by the *N*-benzoxazole, *N*-benzimidazole, and 1,3-dimethylbenzimidazole groups was approximately the same.

2.4. Modification of the Model T6-Oligonucleotide by the Staudinger Reaction Using Benzoazole Azido Reagents. Initially, the modification was carried out at the 5′-end of the T6 oligonucleotide (Scheme 4). At the last stage, instead of the oxidation reaction (manually in a test tube), the Staudinger reaction was carried out. For modification, 0.25 M solutions of azides **1**−**4** were used, what are approximately 10-fold excess. The reaction was carried out under stirring at 25 °C for 30 min; then, the solid-phase polymer was treated with anhydrous TEA in AN for 10 min, and the carrier was placed back into the reactor for the removal of dimethoxytrityl protection. Cleavage from a solid-phase support was carried out with concentrated ammonia at 55 °C for 40 min.

It should be noted that the modification with azide **4** after deprotection results in an uncharged phosphate group (Scheme 4) similar to the dimethylimidazole one.[13]

The Staudinger reaction yield was >90% for all azides according to analytical high-pressure liquid chromatography (HPLC) (Figure 1). As can be seen, the modifications are hydrophobic in nature. Due to a chiral center at the phosphorus atom of the *N*-benz-imida(oxa/thia)zole phosphoramide group, HPLC did result in the separation of diastereomers for *N*-benzothiazole and 1,3-dimethyl-*N*-benzimidazole modifications and did not result in the separation of diastereomers for *N*-benz-imida(oxa)zole, which supported the insignificant difference in the hydrophobicity of the diastereomeric oligonucleotides. Mass spectra of the reaction mixtures confirm the structure and good purity of the obtained oligonucleotides (Table 1, nos. 1−4, Figures S23−S26).

Table 1. Sequences and Molecular Weights of Modified Oligonucleotides Obtained Using Azides 1−4

		molecular weight[a]	
no.	5′-3′ oligonucleotide sequence	calc. *m*/*z*	experimental, *m*/*z*
T6	TTTTTT	1762.3	1763.2
1	T^{N}TTTTT	1876.4	1878.4
2	T^{O}TTTTT	1877.8	1879.2
3	T^{S}TTTTT	1893.3	1895.4
4	T^{D}TTTTT	1905.4	1906.4
5	$T^{S}T^{S}$TTTT	2026.4	2027.4
6	TTTTT^{S}T	1893.4	1895.4
7	TTT^{S}T^{S}T	2026.4	2027.4
8	T^{S}TTT^{S}T	2026.4	2027.4
9	TTTTT^{N}T	1877.4	1877.4
10	TTTTT^{O}T	1878.4	1878.2
11	G^{S}CGCCAAACA	3137.6	3136.8
12	GCGCC^{S}AAACA	3137.6	3136.4
13	GCGCCAAAC^{S}A	3137.6	3136.4
14	G^{S}CG^{S}CC^{S}AA^{S}AC^{S}A	3665.6	3665.4

[a]Based on ESI LC−MS/MS in the negative-ion mode; ^{N}—position of *N*-benzimidazole, ^{O}—*N*-benzoxazole, ^{S}—*N*-benzothiazole, and ^{D}—1,3-dimethyl-*N*-benzimidazole phosphoramide groups.

2.5. Automatic Synthesis of Modified Oligothymidylates Using Azides 1−3. The capability of single and double modifications of oligomers to be synthesized in the automatic mode on an ASM-800 DNA/RNA synthesizer (Biosset, Russia) was investigated. The synthesis of oligonucleotides was performed according to the standard phosphoramidite chemistry protocol and the inter-nucleotide phosphate modification; the oxidation step was replaced by the

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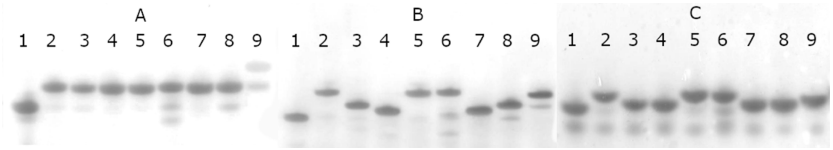

Figure 2. Electrophoretic analysis of the mobility of modified oligonucleotide reaction mixture in a 15% denaturing gel: (A) pH = 4.5, (B) pH = 8.3, (C) pH = 10. (Line 1) 5′-TTTTTT-3′, (line 2) 5′-T^{N}TTTTT-3′, (line 3) 5′-T^{O}TTTTT-3′, (line 4) 5′-T^{S}TTTTT-3′, (line 5) 5′-T^{D}TTTTT-3′, (line 6) 5′-TTTTT^{N}T-3′, (line 7) 5′-TTTTT^{O}T-3′, (line 8) 5′-TTTTT^{S}T-3′, and (line 9) 5′-T^{S}TTTT^{S}T-3′.

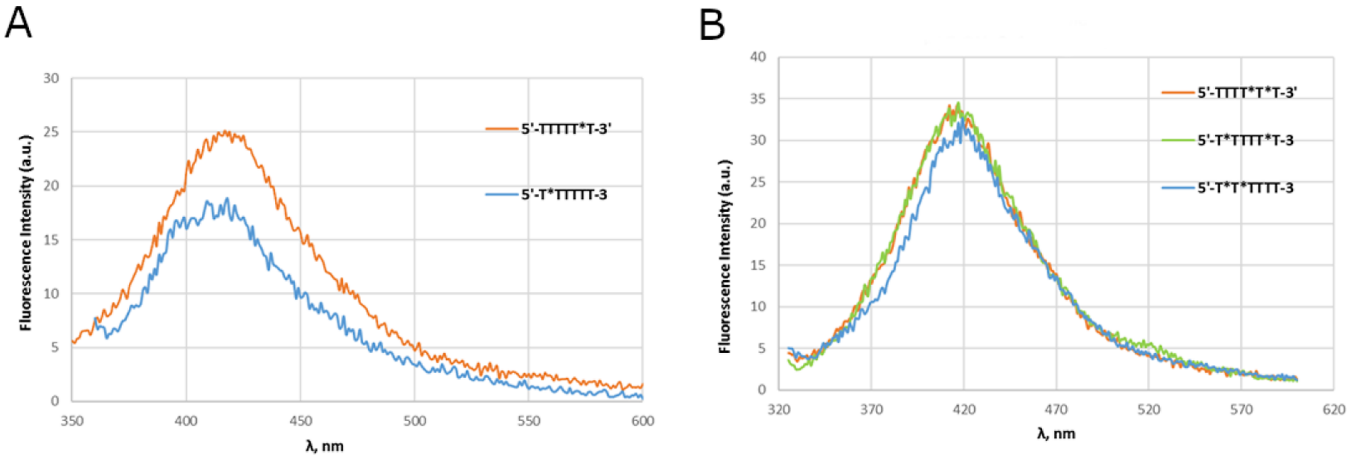

Figure 3. Fluorescence emission spectra of oligonucleotides: (A) with one benzothiazole modification at 3′- and 5′-phosphate (nos. 3 and 6, Table 1), λ_{Ex} = 350 nm; (B) with two benzothiazole modifications at 3′-, 5′-, and 3′,5′-phosphates (nos. 5, 7, and 8, Table 1), λ_{Ex} = 315 nm. Spectra are registered in 50% EtOH (10^{-4} M concentration).

Staudinger reaction with 2-azidobenzimidazole **1** or 2-azidobenzoxazole **2** or 2-azidobenzothiazole **3** (0.25 M solution in acetonitrile). After the completion of the synthesis, the solid-phase polymer was transferred to a tube and treated with anhydrous triethylamine. Oligonucleotides were removed from the solid-phase carrier with concentrated ammonia at 55 °C for 45 min. The benzimidazole or benz(oxa/thia)zole phosphoramidate group was stable under the conditions of DNA synthesis including acidic detritylation and concentrated aqueous ammonia treatment to cleavage from the solid support. T6-oligonucleotide sequences with one and two modifications at different positions were synthesized (Table 1, nos. 5−10). The mass spectra of the reaction mixtures confirm the structure and good purity of the obtained oligonucleotides (Table 1, nos. 5−10, and Figures S27−S32).

In addition, the possibility of modification of the heteronucleotide sequence was shown. One benzothiazole phosphoramidate group each at the 5′ and 3′ ends and in the middle and five groups were introduced into the 10-linked sequence containing G, C, and A nucleotides in the process of automatic DNA synthesis. The experiment had two goals: to investigate whether automatic synthesis of modified hetero-sequences is possible and whether it is possible to introduce a large number of modifications with a frequency of modified phosphates, for example, through one. The benzothiazole phosphoramidate groups were stable under the conditions of DNA synthesis including acidic detritylation and concentrated aqueous ammonia treatment at 55 °C for 12 h to remove protecting groups and cleavage from the solid support. Oligonucleotides 11−14 (Table 1) were synthesized with an adequate yield of ≈72% according the HPLC elution profile of oligonucleotide 14 reaction mixture (Figure S33). The mass spectra confirm the structure of the desired products (Figures S34−S37).

2.6. Study of the Modified Oligonucleotide Properties. *2.6.1. Electrophoretic Analysis of Reaction Mixtures.* Electrophoretic analysis of reaction mixtures was carried out in denaturing 15% polyacrylamide gel under different pH conditions: at pH 4.5, 8.3, and 10 (Figure 2A−C). Visualization was performed by staining gel via Stains-All dye. It can be seen that there are practically no side products in the reaction mixture, and the introduction of modifications took place in all cases.

Modified oligonucleotides have reduced charge than unmodified T6. Dimethylbenzimidazole (DMBI) modifying group results in uncharged nucleotide similar to the dimethylimidazole one[13] while being the closest in the mass and structure to *N*-benz-imida(oxa/thia)zole groups. The *N*-benzimidazole group (line 2) appears to be protonated under all studied pH (Figure 2A−C). It actually gives the uncharged nucleotide like DMBI.

N-Benz-imida(oxa/thia)zole groups are protonated under pH 4.5. This fact is according to changes in the adsorption spectra at acidic pH for the *N*-benzothiazole group (Figure S21). In pH 8.3, *N*-benzoxa/thiazole groups (lines 3, 4, 7, 8, and 9, B) are protonated partially. Moreover, for a single modification, its localization near 3′ or 5′ end is important in some cases (line 4 and 8, B). In pH 10, *N*-benzoxa/thiazole groups are deprotonated.

2.6.2. Stability under Acidic Hydrolytic Conditions. The modified oligonucleotides 1−3 (Table1) have been treated at pH 1 (1 N HCl) at 37 °C for 1 h. The HPLC elution profiles of treated oligonucleotides showed that they are practically not subject to acidic hydrolysis (Figure S38).

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Table 2. Thermodynamic Stability of the Complex of Modified and Corresponding Native Oligonucleotides with DNA

		[a]T_m (°C)	
no.	5′-3′ oligonucleotide sequence	dA_{12}	Poly(dA)
T6	TTTTTT	14.0 ± 1.0	21.9 ± 0.2
1	T^NTTTTT	9.0 ± 1.0	13.7 ± 1.0
2	T^OTTTTT	10.0 ± 1.0	17.9 ± 0.1
3	T^STTTTT	11.0 ± 1.0	17.3 ± 0.4
4	TTTTT^{S}T		17.8 ± 0.1
5	T^{S}T^{S}TTTT		13.4 ± 0.9
6	TTTT^{S}T^{S}T		13.0 ± 0.8
7	T^{S}TTTT^{S}T		13.1 ± 0.8
8	TTTTT^{N}T		13.3 ± 0.7
9	TTTTT^{O}T		13.0 ± 1.5
	DNA/DNA		
no.	5′-3′ oligonucleotide sequence	$\Delta H°$ (kcal/mol)[b]	$\Delta G°_{37}$ (kcal/mol)[b]	T_m (°C)[b]
10	GCGCCAAACA	−77.7 ± 0.9	−12.8 ± 0.1	61.5 ± 0.1
11	G^{S}CGCCAAACA	−74.5 ± 0.8	−11.7 ± 0.1	57.5 ± 0.1
12	GCGCC^{S}AAACA	−74.8 ± 1.9	−11.4 ± 0.1	56.1 ± 0.1
13	GCGCCAAAC^{S}A	−75.8 ± 0.6	−12.0 ± 0.0	58.7 ± 0.1
14	G^{S}CG^{S}CC^{S}AA^{S}AC^{S}A	−35.9 ± 9.0	−6.3 ± 0.1	30.5 ± 0.6
	DNA/RNA			
no.	5′-3′ oligonucleotide sequence	$\Delta H°$ (kcal/mol)[c]	$\Delta G°_{37}$ (kcal/mol)[c]	T_m (°C)[c]
10	GCGCCAAACA	−80.4 ± 0.7	−11.5 ± 0.1	55.1 ± 0.2
11	G^{S}CGCCAAACA	−74.1 ± 4.8	−10.4 ± 0.2	51.7 ± 0.1
12	GCGCC^{S}AAACA	−65.7 ± 0.4	−9.6 ± 0.1	49.2 ± 0.1
13	GCGCCAAAC^{S}A	−76.0 ± 3.1	−10.8 ± 0.1	52.9 ± 0.1
14	G^{S}CG^{S}CC^{S}AA^{S}AC^{S}A	−40.7 ± 0.3	−5.4 ± 0.1	24.2 ± 0.2

[a]The melting temperatures of the complexes are measured in 1 M NaCl, 10 mM sodium cacodylate, pH 7.2, at 0.1 mM per nucleotide concentration as in the case of analysis with dA_{12} and in the case analysis with poly(dA). [b]Thermal stability for the complexes with a complementary oligonucleotide 5′-TGTTTGGCGC-3′ at 20 μM (0.2 mM/per nucleotide) concentration. [c]Thermal stability for the complexes with a complementary oligoribonucleotide 5′-UGUUUGGCGC-3′ at 5 μM (0.05 mM/per nucleotide) concentration and at 10 μM (0.1 mM/per nucleotide) concentration for oligonucleotide 14 with the multiple modification.

2.6.3. Absorption and Fluorescence Properties. The UV−visible absorption spectra of modified oligonucleotides 1−3, 5−8 ($C = 10^{-5}$ M), and 14 ($C = 4.7 \times 10^{-6}$ M) were registered in H_2O (Figures S39−S41). As expected, the spectra of modified oligonucleotides, compared with those of original T6, contain the absorbance bands of the corresponding azide heterocyclic system (Figure S8) in the form of a weakly or well-defined shoulder (λ_{max} 283, 294 nm) to the maximum (λ_{max} 267−270 nm) of the oligonucleotide itself. An increase in the number of modifications in the oligonucleotides (nos. 5, 7, 8, and 14, Table 1) leads to a more pronounced shoulder in the spectrum.

In addition, we found that oligothymidylates modified with benzothiazole exhibit a low fluorescence efficiency. Emission spectra were taken for oligonucleotides 3 and 6 (Table 1) with one modification at 3′- and 5′-phosphate (Figure 3A) and for oligothymidylates 5, 7, and 8 (Table 1) with two modifications at 3′-, 5′-, and 3′,5′-phosphates (Figure 3B).

The following regularities were revealed. The fluorescence intensity is higher for single modification at the 3′-terminal inter-nucleotide phosphate (λ_{Ex} = 350 nm, λ_{Em} = 418 nm, and I = 25 a.u.) than at the 5′-end. The introduction of two modifications leads to an increase in intensity, leveling the influence of their location (λ_{Ex} = 315 nm, λ_{Em} = 420 nm, and I = 34 a.u.).

The results show promise for new fluorescent markers based on *N*-(benzoazole)-phosphoramide moieties. For this purpose, the corresponding aryl or methine groups can be inserted as substituents in the benzene ring (Scheme 1A).

2.6.4. Thermal Denaturation Studies. The possibility and efficiency of formation complexes of modified oligonucleotides with DNA was investigated. Hexathymidylates do not form stable complexes with the complementary DNA chain, so we decided to consider tandem oligomer complexes with dA_{12} and poly(dA). The presence of a cooperative contact between the duplex structures in the nick stabilizes tandem complexes.[34] In the case of formation of complexes with a polymer chain, a more thermostable complex should be formed due to the greater contribution of cooperative interactions.

The melting experiments were performed at equal concentrations of dA and dT nucleotides that should lead the formation of double-stranded DNA. The melting curves in all cases have a single transition and the same at different wavelengths. All the above facts indicate the formation of tandem complexes.[34] The melting temperatures (T_m) of the complexes of hexamers modified at the 5′-end with a dodecameric chain are in the range of 9−11 °C. This is slightly lower than the thermostability of the native chain, which is 14 °C (Figure S42), that is in agreement with previous results.[34] In this case, the accuracy of the determined values of melting temperatures is not high due to the lack of a low-temperature baseline. For a more reliable analysis, the thermostability of tandem complexes of hexamers with a poly(dA) polymer chain was investigated (Figure S43). The trends in the thermal stability of the native and modified

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complexes with dA_{12} and poly(dA) are the same (Table 2). The introduction of one *N*-benzothiazole modification in the hexamer near the 5′- or 3′-end reduces its thermostability in such a complex from 22 °C (for the native chain) to ∼17.5°. The two modifications have an almost additive effect and reduce the T_m to 13 °C. A single *N*-benzimidazole modification near the 5′- or 3′-end significantly reduces the stability to ∼13.5°. *N*-benzoxazole modifications near the 5′- or 3′-end affect the thermal stability in a different manner: in the first case, the decrease is minimal—by 4°, and in the second case—by 9°. The obtained data indicate that the introduction of these modifications reduces the thermal stability of complementary complexes under standard conditions, and the magnitude of the reduction depends on both the type of modification and its position.

Duplexes of dodecamers were studied to determine the thermodynamic effects associated with the modifications. Duplexes with one modification of *N*-benzothiazole in the region of 5′- or 3′-end and in the middle (Table 2, nos. 11−13) and the same native duplex (Figure S44 and Table 2) were studied. The decreases in the thermal stability of the duplex by 5° in the case of modification near the 5′-end, by 5.4° in the case of the middle modification, and by 2.8° in the case of the 3′-end modification indicate a sequence-dependent effect. Small changes in thermodynamic parameters was observed. The Gibbs free energy change differs from the native duplex by 1.1, 1.4, and 0.8 kcal/mol in the case of 5′-end, middle, and 3′-end modifications, respectively. It has both enthalpy and entropy contributions (Table 2).

To determine the thermodynamic effects associated with the multiple modifications of the duplex, the one chain containing five modifications of *N*-benzothiazole (no. 14, Table 2) and the same native duplex were studied (Figure S44 and Table 2). The effect of multiple modifications (through a single phosphate) of a 10-linked sequence on the stability of the complex was significant. Indeed, there is a decrease in the thermal stability of such a complex by almost 30°. The thermodynamic effect has both enthalpic and entropic contributions. The increase in enthalpy from −77.7 to −35.9 kcal/mol and in Gibbs free energy by 6.8 kcal/mol for DNA with multiple modifications is very significant and indicates a probable change in the structure.

We observed the same trends for complexes of modified heteronucleotide oligomers with RNA. The decrease in the T_m of complexes with a single modification at the 5′ end, in the middle, and at the 3′ end was 3.5, 6.0, and 2.3 °C, respectively. Five modifications reduce the thermal stability by 30.9°. The increase in enthalpy from −80.7 to −40.7 kcal/mol and the change in Gibbs free energy by 6.1 kcal/mol are almost the same as for duplexes with DNA for an oligomer with five modifications.

In the case of phosphorylguanidine modifications, we have not previously observed such significant changes even for fully modified complexes. There was practically no change in enthalpy, and the value of ΔG°_{37} increased by only 1.2 kcal/mol.[35] A more detailed study of the effect of the modified oligonucleotide sequence on the thermodynamic stability of the complexes and the determination of the cause of the observed thermodynamic effects will be presented in our next papers. The obtained data indicate the possibility of using the modifications for a targeted oligonucleotide design in various applications (e.g., PCR, hybridization analysis, etc.).

3. CONCLUSIONS

In summary, new derivatives of oligodeoxyribonucleotides containing inter-nucleotide *N*-benzimidazole, *N*-benzoxazole, *N*-benzothiazole, and 1,3-dimethyl-*N*-benzimidazole (benzoazoles) phosphoramide moieties are first obtained. Hexathymidilates with one and two modifications at different positions and multiple modified heteronucleotide sequences have been synthesized with high yields in the process of automatic solid-phase oligonucleotide synthesis. The benzimidazole and benz(oxa/thia)zole phosporamide groups are stable under the conditions of DNA synthesis and purification. It was shown that hexathymidylates modified with benzothiazole exhibit low-efficiency fluorescence. Perhaps, the introduction of appropriate substituents in the benzene ring will make benzoazoles as fluorescent markers for oligonucleotides. Also, the obtained oligonucleotides are promising in the study of their membrane permeation properties since *N*-benzoazole phosphoramide groups have a hydrophobic nature and are partially or completely protonated at physiological pH. The duplex stability of oligodeoxyribonucleotides containing *N*-benzoazole phosphoramide groups with complementary DNA or RNA is somewhat lower than that of native complexes.

4. EXPERIMENTAL SECTION

4.1. Materials and Methods. Acetonitrile for HPLC (Panreac, US), 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl diamidophosphite, 2-chlorobenzimidazole, 2-chlorobenzoxazole, 2-chlorobenzothiazole, *N*,*N*-diisopropylethylamine, DMFA, pyridine, dichloromethane, chloroform, isopropyl alcohol, hexane, tetramethylethylenediamine, sodium azide, iodine, 0.25 M 5-ethylthio-1*H*-tetrazole in anhydrous acetonitrile, and the Stains-All dye were purchased at Sigma-Aldrich (USA); methylamine 40% aqueous solution (Reakhim, Russia), ammonia concentrated solution (Reakhim), and 2 M TEAA, pH 7.0, were prepared from triethylamine (ACS grade, Panreac, Spain) and glacial acetic acid (extra-pure grade, Reakhim, Russia); and high-purity argon (according to TS 2114-002-49632579-2006) and 5′-*O*-dimethoxytrityl-3′-[(*β*-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite were purchased from Glen Research (United States). Organic solvents were dried by standard methods, followed by exposure over molecular sieves or calcium hydride. Gel electrophoresis was performed using a Bio-Rad (United States) apparatus. Small volumes of oligonucleotide solutions (up to 1.5 mL) were concentrated on a Savant SpeedVac DNA120OP (Thermo Fisher Scientific, United States) vacuum concentrator. Oligonucleotides were obtained on an automatic ASM-800 DNA/RNA synthesizer (Biosset, Russia) on the basis of the protocols of phosphoramidite synthesis in containers that are 25 mL in volume on a 0.2 *μ*mol scale from the corresponding 2′-deoxyribonucleoside *β*-cyanoethyl *N*,*N*-diisopropyl phosphoramidates and polymeric supports with immobilized 2′-deoxyribonucleosides (Sigma-Aldrich, United States).

Analytical HPLC was performed on a reversed-phase HPLC Milichrom A-02 (Econova, Russia) using a ProntoSil 120-5-C18 column (Econova, Russia) with gradient elution of acetonitrile (0−50%) in 0.02 M TEA-Ac (pH 7.0) over 30 min at an elution rate 0.15 mL per min and UV detection at a wavelength of 260, 280, 330 nm. Water was purified by a Simplicity 185 water system (Millipore, United States) and had a resistivity of 18.2 MΩ·cm at 25 °C. The deionized water was used to prepare buffers and oligonucleotide solutions. For thin-

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layer chromatography (TLC), Alufolien Kieselgel 60 F254 TLC plates (Merck, Germany) were used.

NMR spectra were recorded on spectrometer Bruker AV-400 or AV-300 (Bruker Daltonics, Germany) at the Center for Analysis of Organic Compounds and Materials of the Institute of Organic Chemistry SB RAS. The chemical shifts (δ) are given in ppm. For the ^{1}H NMR spectra, tetramethylsilane was used as an internal standard; for the ^{31}P NMR spectra, 85% H_3PO_4 in D_2O was used as an external standard. For NMR control of reactions directly in ampoules, the Spinsolve 80 MHz spectrometer (Magritek, New Zealand) was used.

Mass spectra analysis (ESI) of the compounds was carried out at the Center for Mass Spectrometric Analysis of the Institute of Chemical Biology and Fundamental Medicine SB RAS using ESI LC/MS/MSD XCT equipment (Agilent Technologies, United States). UV absorption spectra were recorded on a UV-1800 spectrophotometer (Shimadzu, Japan). The presence of the azide functional group was registered on a Vector 22 spectrometer (Bruker, Germany). The concentration of oligonucleotide solutions was determined by the optical density of the solution using a NanoDrop 2000c UV spectrophotometer (Thermo Fisher Scientific, United States). Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, United States) at 10^{-4} M concentrations in 50% EtOH. The molecular structure was prepared using UCSF Chimera software.[24]

4.2. Syntheses. *4.2.1. General Procedure for Obtaining Benzimidazole and Benzoxa/(thia)zole Azides (**1**−**3**, Scheme 1).* The synthesis was performed similarly to the previously proposed methods[20,25,28,31] with modifications. Sodium azide (1.43 equiv, 10 mmol) was added to the solution of the corresponding chloride: 2-chlorobenzimidazole or 2-chlorobenzoxa/(thia)zole (1 equiv, 7 mmol) in DMFA (6 mL), and the reaction mixture was placed in a thermostat under stirring and an inert atmosphere (60 °C, 24 h). Then, it was diluted with chloroform to 60 mL, and the organic phase was washed with saturated NaCl solution (60 mL) and then with $NaHSO_4$ (60 mL) and water (60 mL). The organic phase was dried over anhydrous sodium sulfate, and then the solvent was evaporated at a rotary evaporator. If necessary, the product was further purified by column flash chromatography (silica gel 60, 0.04−0.063 mm; 10% ethyl acetate in hexane).

*4.2.1.1. 2-Azidobenzimidazole **1**.* TLC (hexane/ethyl acetate = 7.5:2.5): R_f Cl = 0.2, R_f N_3 = 0.31. The yield 386 mg, 2.42 mmol (37%). NMR and IR spectroscopy data were in accordance with the literature.[21,24] IR (KBr) N_3 υ [cm^{-1}] 2140 (Figure S1). UV (50% EtOH) λ_{max}: 240, 275, 281, 290 nm (Figure S8). ^{1}H NMR ($(CD_3)_2SO$, 600 MHz, δ, m.d.): 8 (s, 1H, NH), 7.45−7.43 (m, 2H, CH-Ar), 7.16−7.15 (m, 2H, CH-Ar). ^{13}C NMR ($(CD_3)_2SO$, 150 MHz, δ, m.d.): 147.7, 122, 114.2.

*4.2.1.2. 2-Azidobenzoxazole **2**.* TLC (hexane/ethyl acetate = 9.5:0.5): R_f Cl = 0.61, R_f N_3 = 0.45. The yield 631 mg, 3.94 mmol (61%). NMR and IR spectroscopy data were in accordance with the literature.[30] IR (KBr): N_3 υ [cm^{-1}] 2150 (Figure S2). UV (50% EtOH) λ_{max}: 255, 282 nm (Figure S8). ^{1}H NMR ($CDCl_3$, 600 MHz, δ, m.d.): 7.56 (d, 1H-Ar), 7.40 (d, 1H-Ar), 7.29 (t, 2H-Ar). ^{13}C NMR ($CDCl_3$, 150 MHz, δ, m.d.): 156.9, 149.7, 141.1, 124.9, 124.1, 118.5, 110.2.

*4.2.1.3. 2-Azidobenzothiazole **3**.* TLC (hexane/ethyl acetate = 8.2:1.8): R_f Cl = 0.71, R_f N_3 = 0.16 and 0.74. The yield 788 mg, 4.48 mmol (76%). NMR and IR spectroscopy data were in accordance with the literature.[30] UV (50% EtOH) λ_{max}: 238, 284, 290 nm (Figure S8). ^{1}H NMR ($CDCl_3$, 80 MHz, δ, m.d.): 8.20−7.49 (m, 4H). IR (KBr): N_3 no band 2130−2150 (Figure S3). A mixture of benzo[4,5]thiazolo[3,2-*d*]tetrazole and 2-azidobenzo [*d*]thiazole is obtained in accordance with refs 32 and 36, herein recovered mostly as benzo[4,5]thiazolo[3,2-*d*]tetrazole.

*4.2.2. Synthesis of 2-Azidobenzodimethylimidazole (**4**, Scheme 1). 4.2.2.1. 1,3-Dimethylbenzimidazole (DMBI).* To a solution of 2-hydroxybenzimidazole (1 equiv, 2.535 g, 18 mmol) in 13 mL of toluene, tetrabutylammonium bromide (0.05 equiv, 303.8 mg) and an aqueous solution (40% w/w) of potassium hydroxide (4 equiv, 4.211 g) were added. The reaction mixture was heated in an oil bath to 60 °C, and iodomethane (2.3 equiv, 6.08 g, 2.67 mL) was added in portions over an hour. The reaction mixture was left for 24 h at 58 °C under stirring. The completion of the reaction was controlled by TLC (ethyl acetate/hexane = 1:1). Then, 75 mL of 1 N HCl was added to the reaction mixture. The organic phase was washed with 75 mL of $NaHCO_3$ and water and dried over sodium sulfate, and then the solvent was evaporated at a rotary evaporator. The product was recrystallized from acetone/hexane = 3:2. The yield was 1.4438 g, 8.9 mmol (50%).

4.2.2.2. 2-Chloro-1,3-dimethylbenzimidazole Chloride. To a solution of DMBI (1 equiv, 1.44 g, 8.9 mmol) in 11 mL of toluene, 2.26 mL of oxalyl chloride was added at 40 °C. The reaction mixture was heated to 70 °C and left for 3 days under stirring; then 1.13 mL of oxalyl chloride was added and kept for another 2 days at 70 °C. The heating was turned off, and the reaction mixture was cooled to room temperature and then on ice. The precipitate was filtered on a glass filter, washed with cold toluene, and dried in the desiccator in vacuo. The yield was 0.47 g, 2.2 mmol (25%).

*4.2.2.3. ADMBI **4**.* The compound was obtained according to the procedure described in ref 27. At first, the chlorine anion was replaced with a hexafluorophosphate anion because this procedure improves the solubility of the product in acetonitrile and promotes greater stability during storage. Chloride (1 equiv, 0.4694 g, 2.2 mmol) and potassium hexafluorophosphate (1 equiv, 2.2 mmol, 409.4 mg) were placed in a round-bottom flask with a stir bar and blown with argon. The flask was closed with a septa. Using a syringe, 10 mL of dry acetonitrile was added and stirred at room temperature overnight. The reaction mixture was filtered on a glass filter with a 0.5 cm silica gel layer. The substrate was evaporated on a rotary evaporator (heating below 50 °C) and dissolved in a small amount of acetonitrile (2 mL), and the product was precipitated with diethyl ether (15 mL × 2). The resulting 584.7 mg of the product was dissolved in 5 mL of dry acetonitrile, and sodium azide (1.5 equiv, 2.6 mmol, 171 mg) was added. The reaction was left to stir overnight at room temperature under argon. The reaction mixture was filtered on a glass filter with a 0.5 cm silica gel layer. The substrate was evaporated at a rotary evaporator (heating below 50 °C) and dissolved in a small amount of acetonitrile (2 mL), and the product was precipitated with diethyl ether (15 mL × 2). The yield was 442.8 mg, 1.3 mmol (60%). IR (KBr): N_3 υ [cm^{-1}] 2140 (Figure S4), UV (water/acetonitrile) λ_{max}: 200 ÷ 300, 370 ÷ 443 nm (Figure S9). ^{1}H NMR ($CDCl_3$, 80 MHz, δ, m.d.): 7.63−7.49 (m, 4H, 4, 5, 6, 7), 2.64 (s, 6H, $2CH_3$). ^{31}P NMR ($CDCl_3$, 32 MHz, δ, m.d.): −101.13, −122.916,

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−144.694, −166.479, −188.263. ^{19}F NMR ($CDCl_3$, 75 MHz, δ, m.d.): −66.49, −75.86 (Figures S5−S7).

*4.2.3. Study of the Reactivity of Azides **1**−**3** with Trivalent Phosphorus (Scheme 2).* The reactivity was tested similarly to the method described in ref 33. In screw-tight plastic tubes, ETT (0.08 mmol) and isopropanol (abs.) (4 mmol) were added to a solution of 5′-*O*-dimethoxytrityl-3′-[(β-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (0.02 mmol) in deuterated acetonitrile. The reaction mixture was placed in a thermostat (30 °C, 10 min). Most of the solvent was evaporated in a vacuum concentrator; the residual volume was 90 μL. Further reactions were carried out in NMR ampoules. The 5′-*O*-dimethoxytrityl-3′-[(βcyanoethyl)-alkyl]-phosphite triether was used in further synthesis without purification (Figure S10).

In each ampoule, the corresponding ∼0.1 M solution of azide in deuterated acetonitrile (0.031 mmol, 4.9 mg of **1** in 220 μL; 0.033 mmol, 5.4 mg of **2** in 220 μL; 0.02 mmol, 5.3 mg of **3** in 200 μL; and 0.015 mmol of **4** in 153 μL) was added to the phosphite triether. After azide addition, all the reaction mixtures were turned yellow (the strongest color was in the case of azide **3**), and the gas emission was observed in the process. ^{31}P NMR spectra (Figures S11−S14) demonstrated that the reaction occurred in 5 min for all azides. Triethylamine (10 μL) was then added, and the reaction mixtures were left overnight on a thermostat at 30 °C. ^{31}P NMR (R. mix. of **6.1**−**6.4**) showed complete conversion of trivalent phosphorus to a pentavalent one (Figures S15−S18).

*4.2.4. Synthesis of Diisopropyl Benzo[d]thiazol-2-ylphosphorimidate **7** (Scheme 3); Definition of the Modifying Group Contribution to the Extinction Coefficient of a Modified Oligonucleotide.* Reaction of 2-azidobenzothiazole **3** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl diamidophosphite was carried out in an ampoule under NMR control similar to the experiment described in ref 33. To a solution of 0.5 M ETT (4 equiv, 0.112 mmol, 240 μL) in dry acetonitrile was added isopropanol (abs.) (100 equiv, 2.8 mmol, 595 μL) and phosphitylation reagent (1 equiv, 0.028 mmol, 9 μL). The triester formation occurs instantaneously, which was confirmed by ^{31}P NMR. After addition of 2-azidobenzothiazole (1 equiv, 0.028 mmol, 280 μL of 0.1 M solution), the reaction was carried out within an hour. Then, 250 μL of TEA was added to remove the cyanethyl protection. The reaction mixture was diluted with water (∼10-fold). White precipitate was centrifuged and dried by co-evaporation with acetonitrile (after decanting the super). The structure of compound **7** was confirmed by ^{31}P and ^{1}H NMR spectra (Figures S19 and S20). The UV absorption spectra of **7** were registered at the concentration $C = 1.57 \times 10^{-4}$ M under different pH conditions: in 30% EtOH−0.1 M HCl, pH = 2; 30% EtOH in acetate buffer, pH = 3.8; 30% EtOH−H_2O, pH = 6−7; 30% EtOH−0.1 M NaOH, pH = 10 (Figure S21). The extinction coefficient of the *N*-benzothiazole group was defined according to the UV spectrum ($C = 8 \times 10^{-5}$ M, 50% EtOH−H_2O): ε = 7125 (260 nm) (Figure S22). The concentration of **7** was calculated from the weight of the sample. In approximation of additive impact of modification, the extinction of the oligonucleotide derivate should be calculated as a sum of extinction of a native oligomer and a number of modification multiplied on its extinction.

4.2.5. General Procedure for the Preparation of Oligodeoxyribonucleotides with Inter-nucleotide N-Benzimidazole, N-Benzoxa/(Thia)zole, and 1,3-Dimethyl-N-benzimidazole Bonds (Scheme 4). Oligodeoxyribonucleotides were synthesized by the phosphoramidite approach using an automatic DNA synthesizer. *N*-(Benzoazole) phosphoramide groups were introduced as in a manual mode and in the automatic synthesizer mode. In the case of manual mode, the automatic synthesis was terminated, and the 25 μL container for the solid-phase synthesis with a polymer support was pulled out of the synthesizer. The support (∼5 mg) was transferred to a 1.5 mL plastic tube, ∼0.25 M azide (**1**−**4**) in anhydrous acetonitrile (80 μL) was added, and the air was replaced by argon. The tube was thoroughly shaken for 1 min, shaken on a shaker at 800 rpm for 30 min, and then centrifuged at 14 500 rpm for 30 s. The supernatant was taken out, the precipitate was washed with anhydrous acetonitrile (3 × 200 μL) and treated with a mixture of triethylamine and acetonitrile (1:1) under stirring for 10 min, then centrifuged, and washed with anhydrous acetonitrile (3 × 100 μL); the supernatant was removed each time. The polymer was placed into a 25 μL container to complete the synthesis. The oligonucleotide deblocked with concentrated ammonia (∼28%) (200 μL per ∼5 mg of the polymer) at 55 °C for 45 min. The solution was decanted, the glass was washed once with 50% acetonitrile (200 μL), and the solvent was evaporated on a rotary concentrator. Oligonucleotides were dissolved in 150 μL of water.

Automated oligonucleotide synthesis was performed according to the standard protocol. When introducing the inter-nucleotide phosphate modification, a protocol was used in which the oxidation step was replaced by the Staudinger reaction with azides **1**−**3** (0.25 M solution in acetonitrile). After completion of the synthesis, the solid-phase polymer was transferred into a screw-cap Eppendorf and treated with a mixture of triethylamine and acetonitrile (1:1) under stirring for 10 min. The solution was decanted, and the polymer was washed with acetonitrile and treated with conc. ammonia (500 μL) under stirring on a thermostatically controlled shaker at 55 °C for 45 min for homothymidylates or at 55 °C for 12 h for the heterooligonucleotide. The solution was decanted, the glass was washed once with 50% acetonitrile (200 μL), and the solvent was evaporated on a rotary concentrator. Oligonucleotides were dissolved in 200 μL of water.

4.2.6. Thermal Denaturation of Oligonucleotide Duplexes. Thermal denaturation studies of oligonucleotide complexes were carried out in 0.2 cm path length quartz cells using a Cary 300-Bio Melt spectrophotometer (Varian, Australia) equipped with a Peltier thermostabilized multicell holder (6 × 6). Each melting curve was recorded at a heating rate of 0.5 °C/min in the range of 5−95 °C. The components of the DNA/DNA duplexes in equimolar per nucleotide amounts were placed in a buffer solution at 0.1 mM per nucleotide concentration as in the case analysis with dA_{12} and with poly(dA) and at 20 μM (0.2 mM/per nucleotide) concentration in the case analysis of heteronucleotide oligomers. The thermal stability for the DNA/RNA duplexes with complementary oligoribonucleotide 5′-UGUUUGGCGC-3′ was studied at 5 μM (0.05 mM/per nucleotide) concentration and at 10 μM (0.1 mM/per nucleotide), the concentration for oligonucleotide 14 with the multiple modification. Denaturation melting curves were recorded in a multi-wavelength mode by automatic switching of the monochromator between three wavelengths (260, 270, and 330 nm) with 1 nm bandwidth. The profile of optical density at 330 nm was used for the baseline correction of

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thermal denaturation curves. The values of thermodynamic parameters obtained by fitting denaturation curves at 260 and 270 nm using two-state model were averaged.[37] Thermodynamic parameters were calculated in MS Excel by fitting procedure.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07083.

NMR, IR, UV, and mass spectra of the obtained compounds (reaction mixtures) and differential curves of thermal denaturation of complementary DNA/DNA and DNA/RNA duplexes (PDF)

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Notes

The authors declare no competing financial interest.

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