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LETTER
TO THE EDITOR

A Convenient Method for the Synthesis of Phosphoramidite Non-Nucleotide Inserts for Preparation of Functionalized Oligonucleotides

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Abstract—A simple approach to the synthesis of amidophosphite synthons of achiral non-nucleotide inserts using 4-(2-(4,4'-dimethoxytrityloxy)ethyl)morpholine-2,3-dione as a backbone of key precursor was suggested. Non-nucleotide synthons were synthesized using this approach that were suitable for synthesis of acridine-containing oligonucleotide derivatives, as well as oligonucleotides with branched carbohydrate-phosphate backbone.

Keywords: branched DNA, non-nucleotide insert, modified oligonucleotides, fluorescent probes, thermal stability

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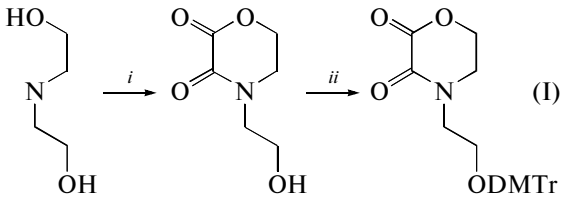
Amidophosphite monomers, including those of a non-nucleotide nature, are widely used for the preparation of a variety of oligonucleotide derivatives in the context of automatic DNA synthesis. However, the synthesis of required synthons, such as non-nucleotide inserts for the introduction of functional groups into various positions of the oligonucleotide chain, is a separate synthetic problem for each case. Hence, the development of a convenient method for the synthesis of non-nucleotide inserts carrying different functional residues is an actual problem.

Based on a series of certain synthetic approaches suggested by various authors [1, 2], a simple method of synthesis of achiral non-nucleotide inserts for development of oligonucleotide derivatives using automatic amidophosphite protocol was proposed. This methods includes a parallel synthesis of two functionally different building blocks: an universal building block, 4-(2-(4,4'-dimethoxytrityloxy)ethyl)morpholine-2,3-dione (**I**) (Scheme 1), and a functionalized building block, substituted aliphatic amine (for example compounds (**II**) and (**III**)). Combining the two building blocks into a unified structure is conducted in the stage prior to the final phosphitylation step providing the final product (Scheme 2). The universal building block (**I**) is a common reagent for the synthesis of inserts with this method, which allows the production of a series of various non-nucleotide inserts with structures defined by the chosen functionalized building block carrying the required group (groups).



Reagents and conditions:
(*i*) diethyl oxalate, isopropanol, 12 h, 70%. ^{1}H NMR: δ 3.43 (t, 2 H, >N–CH_2–C**H_2**–OH, *J* 5.5 Hz); 3.53 (t, 2 H >N–C**H_2**–CH_2–OH, *J* 5.5 Hz); 3.71 (t, 2 H, –O–CH_2–C**H_2**–N<, *J* 5.1 Hz); 4.49 (t, 2 H, –O–C**H_2**–CH_2–N<, *J* 5.1 Hz). ^{13}C NMR δ: 45.99, 49.45, 58.25, 65.84, 154.01, 157.77; (*ii*) DMTr-Cl, pyridine, 2 h, (**I**): above 90%. ^{1}H NMR: δ 3.16 (t, 2 H, >N–CH_2–C**H_2**–ODMTr, *J* 5.3 Hz); 3.59 (t, 2 H, >N–C**H_2**–CH_2–ODMTr, *J* 5.3 Hz); 3.68 (t, 2 H, –O–CH_2–C**H_2**–N<, *J* 4.9 Hz); 3.73 (s, 3 H, OMe); 4.49 (t, 2 H, –O–C**H_2**–CH_2–N<, *J* 4.9 Hz); 6.85–8.66 (m, 13 H, aromatic).

Scheme 1. Synthesis of a universal building block (**I**) on the basis of substituted morpholine-2,3-dione.

Abbreviations: TBDMS, *tret*-butyldimethylsilyl; DMTr, dimethoxytrityl; CEP, 2-cyanoethoxy diisopropylamino phosphinyl; Lev, levulinyl; DIPEA, diisopropylethylamine; TEA, triethylamine; DMAP, 4-*N,N*-dimethylaminopyridine.

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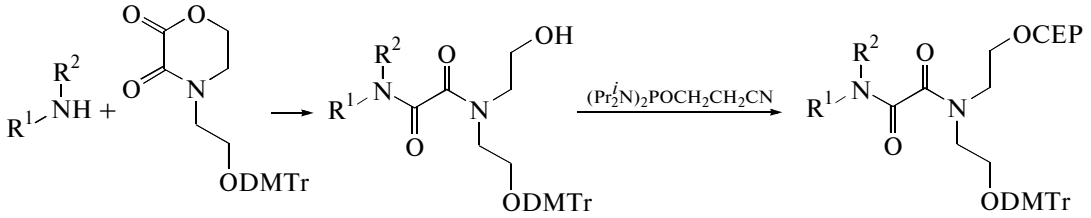
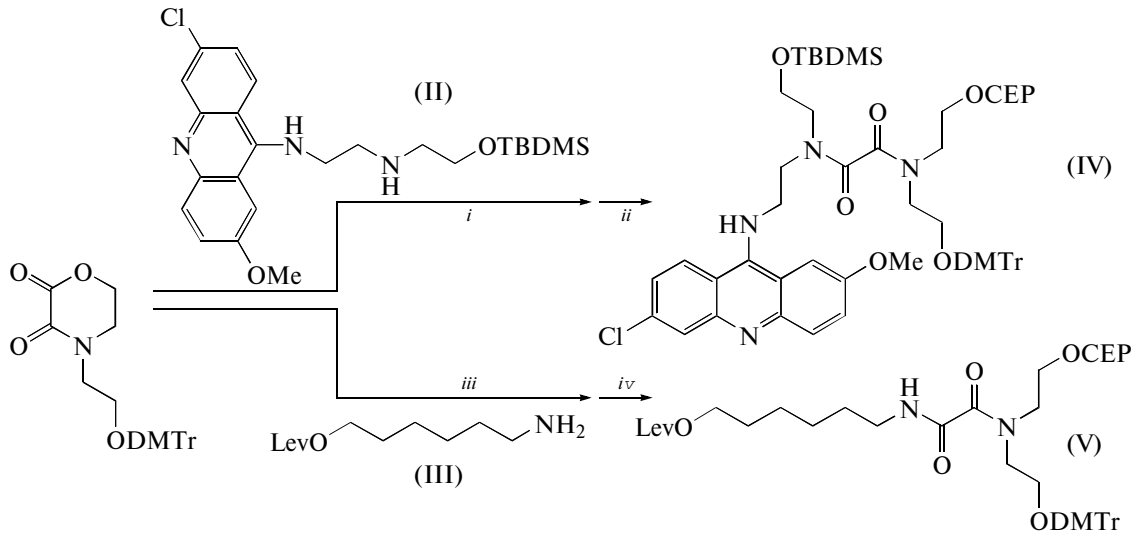


Scheme 2. General scheme for preparation of non-nucleotide synthon insert from 'functionalized' and 'universal' building blocks.

A series of non-nucleotide inserts was synthesized using derivatives of the primary amine (**III**) (one functional group) and secondary amine (**II**) (two functional groups) as functionalized building blocks to demonstrate the possibilities of the method (Scheme 3).



Reagents and conditions:
(*i*) , DMAP, pyridine, 45°C, 12 h, 93%; (*ii*) $(Pr^i_2N)_2POCH_2CH_2CN$, DIPEA, tetrazole, acetonitrile, 1 h, (**IV**): 88%. ^{1}H NMR: δ –0.25–0.00 (m, 6 H, $(CH_3)_2Si$); 0.63–0.68 (m, 9 H, $(CH_3)_3CSi$); 0.97–1.29 (m, 12 H, $(CH_3)_2CH$–); 2.65–2.81 (m, 2 H, –CH_2–CN); 3.05–3.25 (m, 2 H, –CH_2–O–DMTr); 3.45–4.00 (m, 24 H, –OCH_3, –CH_2–CH_2–, –CH_2–CH_2–CN); 6.75–8.39 (m, 19 H, aromatic). ^{31}P NMR: δ 147.74; 147.84; (*iii*), DMAP, pyridine, 60°C, 8 h, 40%; (*iv*) $(Pr^i_2N)_2POCH_2CH_2CN$, DIPEA, tetrazole, acetonitrile, 1 h, (**V**): 77%. ^{1}H NMR: δ 1.1–1.6 (m, 20 H, (CH_3)CH–, –CH_2–CH_2–CH_2–); 2.1 (s, 3 H, CH_3–C(O)–); 2.45 (m, 2H, –CH_2–CH_2–C(O)–O–); 2.68–2.81 (m, 4 H, –CH_2–CN, CH_3–C(O)–CH_2–CH_2–); 3.16 –3.05 (m, 4 H, –CH_2–NH–C(O)–, –CH_2–ODMTr); 3.5–3.8 (m, 16 H, >N–CH_2–CH_2–O–, $(CH_3)CH$–, –CH_2–CH_2–CN, –OCH_3); 3.93–4.00 (m, 2 H, –C(O)–O–CH_2–CH_2–); 6.85–8.66 (m, 13 H, aromatic). ^{31}P NMR: δ 147.96, 148.06.

Scheme 3. Synthesis of bi- and monofunctionalized non-nucleotide synthon inserts (**IV**) and (**V**), respectively.

A structure of amidophosphite synthon of achiral acridine-containing non-nucleotide insert that was suggested previously was synthesized in seven stages with the total yield of only ~10% [3]. A compound (**IV**) similar to that described in [3] was synthesized in four stages with a total yield of approximately 60% with the method of synthesis suggested earlier for N^1-(2-(*tert*-butyldimethylsiloxy)ethyl)-N^2-(6-chloro-2-methoxyacridine-9-yl)ethylene diamine (**II**) as a functionalized amine. The compound (**IV**) differs from the analogue synthesized earlier in the shorter linker between diethanolamine fragments of the insert backbone.

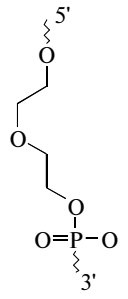
It was established that inserts with a linker fragment on the basis of an oxalate residue are significantly more effective in the production of oligonucleotide derivatives when compared with analogues based on a glutarate linker [3]. Thus, a monomer (**IV**) is inserted

Properties of oligo-thymidylate derivatives and their DNA complexes

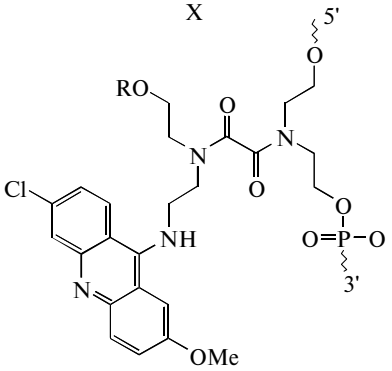
Compound	$\Delta S°$, cal/(mol K)	$\Delta H°$, kcal/mol	$\Delta G°_{37}$, kcal/mol	T_m[a], °C	ΔF, %[b]	m/z ([M − H]$^-$)[c] calc/exp
T_6	−117.6	−38.8	−2.4	6.8		
T_3DÒ$_3$	−114.0	−35.4	0.0	−9.5 [d]		
XT_6	−171.2	−58.0	−4.9	30.8	−12.9	2443.4/2443.5
X$^1{}_2T_6$	−135.4	−47.9	−5.9	35.8	−13.5	2896.1/2896.9
T_5XT	−89.9	−32.3	−4.4	21.6	25.4	2443.4/2443.5
T_3XT_3	−63.6	−23.6	−3.8	9.7	24.0	2443.4/2443.8
$(T_3)_2$X$^2T_3$	–	–	–	–	–	3242.8/3246.5
T_2XT_4	−93.3	−33.3	−4.4	21.6	18.6	2443.4/2443.5
T_3X*T_3	−113.8	−39.6	−4.3	20.5	57.5	2485.5/2484.5
X*T_6	−144.5	−50.1	−5.2	29.1	18.6	2485.5/2483.7

D, X, X* are non-nucleotide inserts of the following structure:

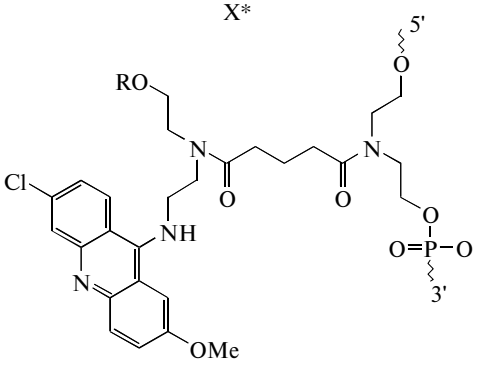
D



X



X*



where in inserts X: R = −TBDMS; X^1: R = −H; X^2: R = oligonucleotide fragment (here T_3);
X*: R = −TBDMS.

[a] Total concentrations of oligonucleotides taken at a stoichiometric ratio is 2×10^{-4} M.

[b] ΔF is a relative change in fluorescence at λ_{em} = 500 nm (λ_{ex} = 420 nm) as a result of hybridization with DNA matrix CA_6C at 6°C, $\Delta F(\%) = 100\%(F_{ss} - F_{ds})/F_{ss}$. Concentration of acridine-containing nucleotides was 10^{-6} M. A 20-fold excess of DNA matrix, CA_6C, was added. All measurements conducted in a buffer containing 1 M NaCl, 10 mM sodium phosphate (pH 7.3), 0.1 M Na_2EDTA.

[c] Obtained by V. V. Koval with Autoflex-III MALDI TOF (Bruker Daltonics, Germany) using linear regime of negative ions registration, N_2 laser (337 nm), matrix, 3-hydroxypicolinic acid.

[d] Thermodynamic parameters of formation of the complex containing insert based on diethylene glycol ether **D** were calculated according to the data in [4].

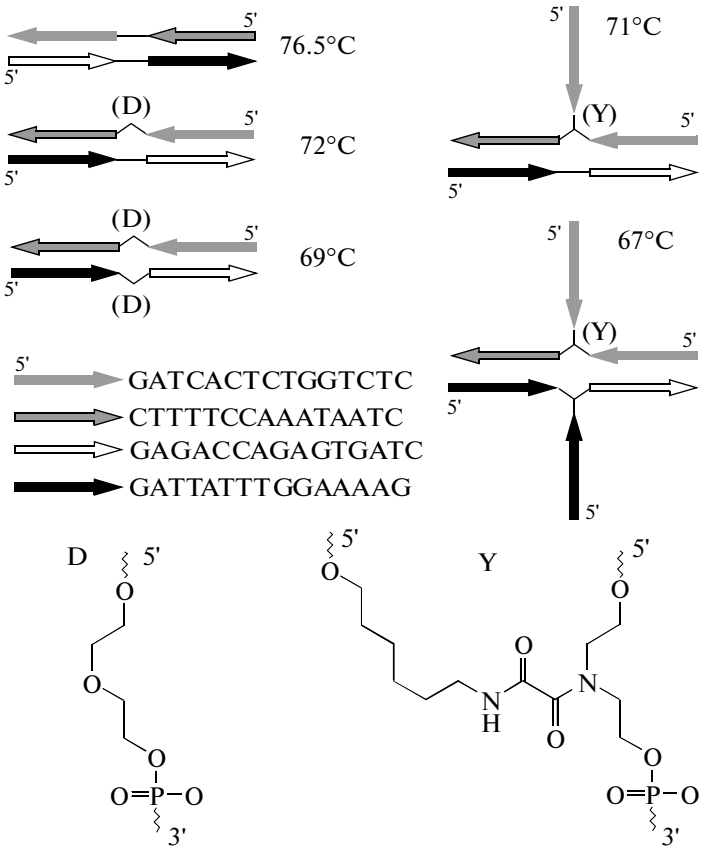
in a growing chain with an efficiency close to the efficiency of insertion of a standard amidophosphite monomer, and a non-nucleotide unit obtained with its help is stable in conditions of post-synthetic deprotection (*tert*-butylamine in an aqueous methanol solution).

In addition, the simplified non-nucleotide insert (**V**), lacking the acridine residue derivative obtained with the help of compound (**III**), was found to be sufficiently stable in standard conditions of oligonucleotide deprotection (concentrated aqueous ammonia).

Compounds (**I**), (**IV**), and (**V**) are characterized with ^{1}H NMR (300 MHz, DMSO-d_6) and ^{32}P NMR (DMSO-d_6) spectra.

A series of linear and branched oligonucleotides containing the developed non-nucleotide inserts (**X** and **Y**, respectively, Table) in various positions of carbohydrate-phosphate backbone was synthesized with an automatic DNA synthesizer ASM-800 (Biosset, Novosibirsk) using the amidophosphites (**IV**) and (**V**). Furthermore, mass spectra and data on the thermodynamic stability of duplexes formed with complementary DNA probes were obtained (table, figure).

It can be seen from the Table that the acridine derivative residue in an insert composition with oxalate linker **X** containing intercalator compensates its destabilizing effect caused by the distortion of the regular structure of the carbohydrate-phosphate backbone in the duplex composition. The observed melting



Structures of DNA duplexes and their melting points. Buffer: 1M NaCl, 0.01 M sodium phosphate (pH 7.3), 0.1 mM Na_2EDTA, total concentration of oligonucleotide components taken in stoichiometric ratio is 10^{-5} M.

point of DNA duplex ($\mathbf{T_3XT_3}/CA_6C$) with insert **X** is, however, lower than the melting point of $\mathbf{T_3X^*T_3}/CA_6C$ complex carrying the similar insert with the glutarate linker (**X***). It is likely that the short, rigid linker does not provide an optimal interaction with the intercalating residue with internal base pairs in the modified duplex composition. The inserts at the end of duplexes stabilize the double helix by a practically equal extent independent of the length of the linker fragment (see table, $\mathbf{XT_6}/CA_6C$ and $\mathbf{X^*T_6}/CA_6C$).

A significant effect of the nature of combining the linker in the insert can be seen with an analysis of the fluorescence change of oligonucleotide conjugates on transfer from the single-stranded to the double-stranded state (see ΔF in table). As would be expected, the formation of a complex of acridine-containing oligonucleotides with a complementary DNA probe results in the change of fluorescence of the intercalating residue. Fluorescence quenching occurs on the formation of the double-stranded complex in the case of internal location of fluorophore residue, independent of the combining linker length. Formation of the duplex with terminal location of acridine derivative is characterized by different changes of fluorescence efficiency of the intercalating residue depending on the length of combining linker. Acridine residue demonstrates a fluorescence decrease in the binding of modified oligonucleotide with DNA (for example $\mathbf{X^*T_6}$) when in the insert with the glutarate linker, and an increase when in the insert with the oxalate linker ($\mathbf{XT_6}$). Hence, even so, insignificant changes of insert structure, such as the structure of the linker fragment combining different branches of the non-nucleotide unit, could significantly affect the properties of the respective oligonucleotide derivatives.

Two extended branched oligonucleotides (45 nt) carry three pentadecanucleotide fragments: two identical 5'-end fragments and one 3'-end were obtained using synthon (**V**). It was shown that the oligomers are capable of duplex formation with a melting point (T_m) significantly lower (T_m = 71 and 67°C) than for an unmodified duplex of 30 bp length (T_m =76.5°C), but comparable with the melting point of the duplexes of the same length carrying insert(s) based on the phosphodiester of diethylene glycol (**D**) in the central part of one or both chains. Hence, the availability of the second extended single-stranded fragment in the branching point does not produce a significant destabilizing effect on the DNA-duplex.

The present work demonstrated that a convenient approach to the preparation of amidophosphite synthons, allowing the creation of non-nucleotide inserts of different functionalities with minimal synthetic steps, was developed.

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REFERENCES

1. Dioubankova, N.N., Malakhov, A.D., Stetsenko, D.A., Korshun, V.A., and Gait, M.J., *Org. Lett.,* 2002, vol. 4, pp. 4607–4610.
2. Morocho, A.M., Karamyshev, V.N., and Polushin, N.N., *Bioconjugate Chem.,* 2004, vol. 15, pp. 569–575.
3. Kupryushkin, M.S. and Pyshnyi, D.V., *Russ. J. Bioorg. Chem.,* 2012, vol. 38, pp. 625–638.
4. Lomzov, A.A., Pyshnaya, I.A., Ivanova, E.M., and Pyshnyi, D.V., *Dokl. Biochem. Biophys.,* 2006, vol. 409, pp. 211–215.