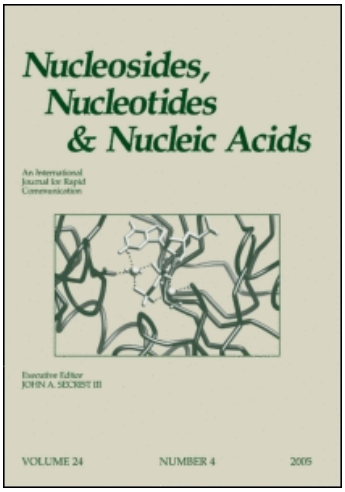

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Synthesis and Properties of Modified Oligodeoxyribonucleotides Containing 9-(2-Amino-2-deoxy-β-D-arabinofuranosyl)adenine

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SYNTHESIS AND PROPERTIES OF MODIFIED OLIGODEOXYRIBONUCLEOTIDES CONTAINING 9-(2-AMINO-2-DEOXY-β-D-ARABINOFURANOSYL)ADENINE

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ABSTRACT: The synthesis of modified oligodeoxyribonucleotides# containing 2'-amino-2'-deoxyarabinoadenosine residues (aA^n) was carried out by means of the standard phosphoramidite chemistry. A high reactivity of such compounds to electrophilic reagents was shown. The cross-link formation between 2'-amino group of aA^n and carboxyl function introduced into complementary strands occurs with 55% yield. The aA^n residues was shown to induce the increased resistance of modified oligomers towards the enzymatic cleavage and provide the insignificant destabilization of DNA duplexes.

INTRODUCTION

The development of chemical synthesis of nucleic acids have given the opportunity to apply oligonucleotides as available tools for the study of cellular activity, as well as for sense and antisense biotechnology. In these applications modified oligonucleotides containing active functional groups have been used most frequently. Therefore due to the significance of oligonucleotides

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This publication is dedicated to Professor Tsujiaki Hata, who made a valuable contribution to the chemistry of nucleosides and nucleotides.

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Abbreviations used: Prefix "d" (deoxy) is omitted; aA^n, 2'-amino-2'-deoxyarabino-adenosine - 9-(2-amino-2-deoxy-β-D-arabinofuranosyl)adenine; U^n - 2'-amino-2'-deoxyuridine; aA - arabinoadenosine; aC - arabinocytidine.

and their wide acceptance in numerous molecular biology investigations it may be of interest to synthesize new compounds with predetermined chemical and physical chemical properties.

Of particular importance are oligomers which contain aliphatic amino groups since they prove to be useful for the further incorporation of reporter groups into DNA[1,2] and production of DNA duplexes with covalently linked strands.[3,4]

It was reported earlier that introduction of 2'-amino-2'-deoxyribonucleoside residues can increase the resistance of oligonucleotides to enzymatic degradation,[5] but affects its ability to hybridize to complementary nucleic acids.[6,7] The primary aliphatic amino group attached to oligonucleotides was shown to be highly reactive towards electrophilic reagents. According to our recent studies,[7] the introduction of 2'-amino-2'-deoxyribopyrimidine residues into oligonucleotides resulted in reduction of thermal stability of the DNA duplexes formed by these oligomers and complementary DNA targets. In this paper we describe the synthesis of oligodeoxyribonucleotides containing 9-(2-amino-2-deoxy-β-D-arabino-furanosyl)adenine residues (aA^n), *i.e.* units with reversed configuration at C2'-atom. The reactivity of aA^n containing oligonucleotides to electrophilic reagents, their ability to form duplexes with complementary DNAs and hydrolytic properties towards snake venom phosphodiesterase were also examined. Hopefully, comparison of hybridization properties of oligonucleotides containing aA^n and U^n containing oligomers would elucidate the influence of sugar configuration on the thermal stability of DNA duplexes formed by either oligomer and complementary DNAs.

RESULTS AND DISCUSSION

The synthesis of oligodeoxyribonucleotides containing 2'-amino-2'-deoxyarabinonucleoside residues requires an adequate method for preparation of modified monomer synthon ready to be introduced into oligonucleotides during automatic phosphoramidite procedure.

The synthesis of the 3'-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite of the 9-(5-*O*-dimethoxytrityl-2-trifluoroacetamido-2-deoxy-β-D-arabino-furanosyl)-N^6-benzoyladenine was performed from adenosine as follows (SCHEME 1).

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 380B automatic synthesizer following standard procedures. When modified phosphoramidite synthon **10** was used, standard procedures for synthesis with normal ones had to be altered by extending the coupling time to 10 min. Deprotection and isolation of oligonucleotide products were performed according to Kuznetsova *et al.*[7]

We obtained four modified oligonucleotides **I** - **IV**. Their sequences are given below:

5' - TTT**aA^n**TT - 3' **I**

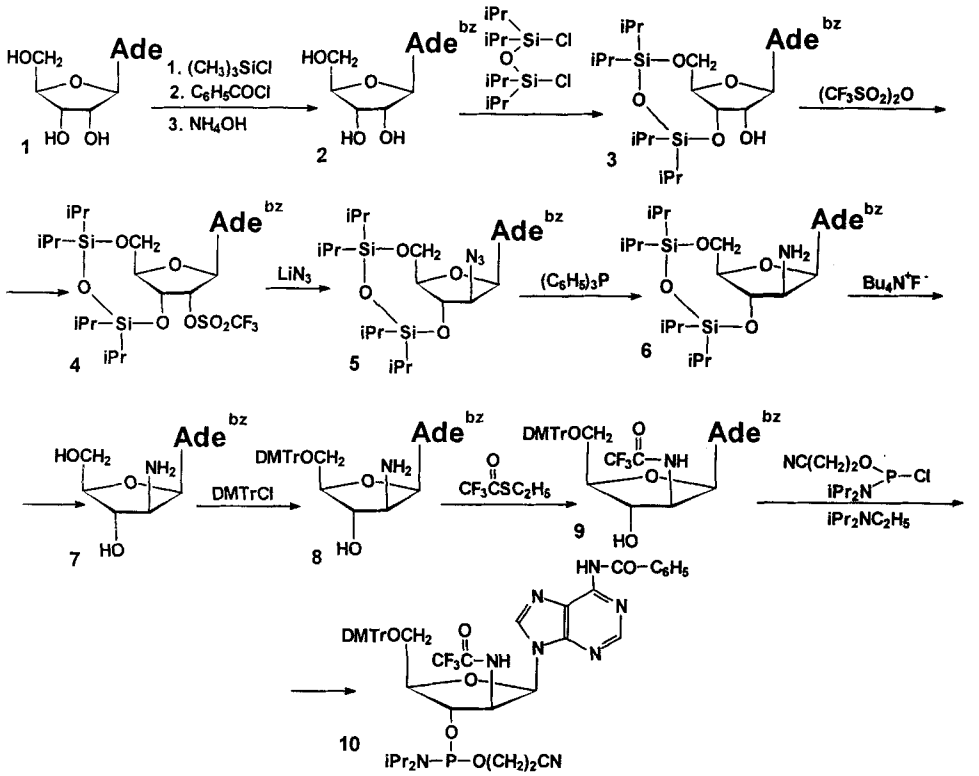
5' - CTAC**aA^n**C**aA^n**CGC - 3' **II**

5' - GCCA**aA^n**TCGGAAAGTCCCCTCTACCG - 3' **III**

5' - TGCG**aA^n**G**aA^n**GTAGG -3' **IV**

Oligonucleotide **I** was used as a model in perfecting and developing techniques for synthesis, isolation and analysis of modified oligonucleotides. Oligonucleotides **II**, **III**, **IV** were used to study stability of such compounds against degradation by nucleases, to explore reactivity of the 2'-amino group and to determine the effect of aA^n on the ability of oligomers to form duplexes with complementary DNA targets, respectively. Besides two dinucleoside phosphates **aA^n**pT, Tp**aA^n** and dinucleotide Tp**aA^n**p were synthesized to analyze their stacking behaviour.

The nucleoside composition of the modified oligonucleotides was analyzed by reversed phase HPLC after complete digestion with a mixture of snake venom phosphodiesterase and alkaline phosphatase. HPLC analysis of oligonucleotide **III** hydrolysis products indicates that there are five peaks among which four ones correspond to natural nucleosides and the fifth one - 2'-amino-2'-deoxyarabinoadenosine. This was confirmed by comparison of their



SCHEME 1

retention times with those of synthetic standards. HPLC analysis of the hydrolysis products also demonstrated the correct base ratios.

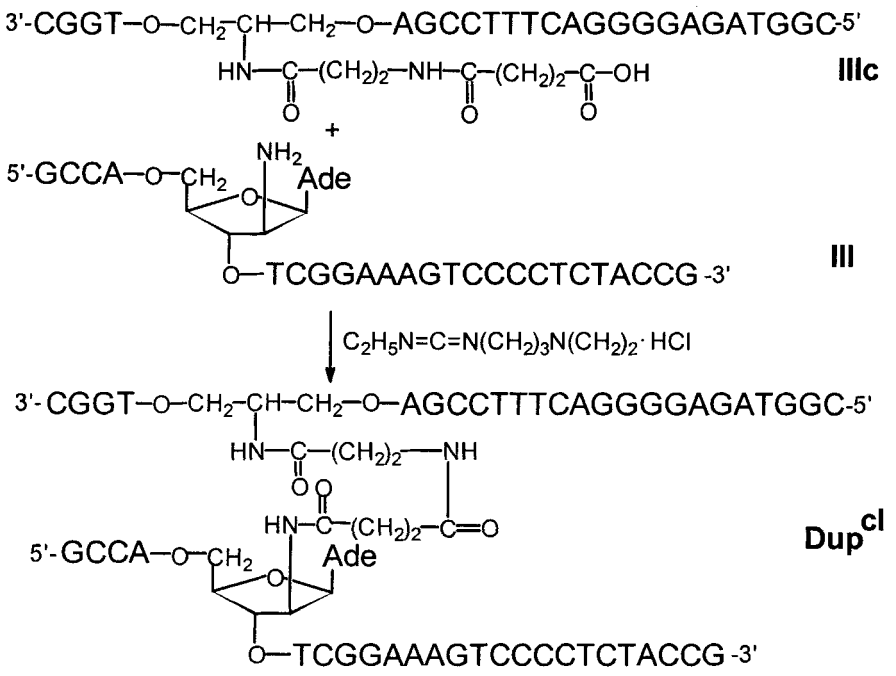
The primary amino group fitted into 2'-position of arabinosyl residue has been shown to have high reactivity to electrophilic reagents. We carried out the reaction between oligonucleotide **I** and acetic anhydride, as well as the reaction between oligonucleotide **III** and succinic anhydride followed by the reversed phase HPLC analysis (in ion-pair mode) of the reaction products. It should be noted that the acylation of amino group proceeds completely and the retention time of the modified oligonucleotides with acylated amino group was greater than that of corresponding oligonucleotides with free amino group. So, the reversal of configuration at C2'-atom does not affect reactivity of incorporated amino group.

The high reactivity of aliphatic amino group of aA^n towards electrophilic compounds enabled the interstrand reaction of oligonucleotide **III** and complementary DNA **IIIc** carrying the carboxyl function located on the non-nucleoside insert to be carried out under conditions of DNA duplex stability (SCHEME 2).

The cross-linking reaction conditions were similar to those described earlier.[3,4] The yield of DNA duplex with covalently linked strands was 55% (FIG. 1).

The thermal stability of native (A) and modified (B) - (D) DNA duplexes were studied (TABLE 1). It turned out that the introduction of two aA^n residues in one strand (duplex (B)) results in insignificant (4 °C) reduction in its melting temperature (Tm) as compared to parent duplex (A). In contrast, the two U^n residues in the complementary strand lowered the Tm of the double helix (C) by 18 °C. The similar reduction (19 °C) of Tm was observed for duplex (D) containing both types of modifications - two aA^n *vs* two U^n residues. Thus, the strongest destabilizing effect is associated with the presence of modified $aA^n \cdot U^n$ base pairs, or the U^n residues. In contrast, the aA^n residues have a weak influence on the helix stability. This data could be important for design of antisense oligonucleotides containing sugar modified residues.

In our opinion destabilizing action of these modifications can not be related to the furanose conformation of U^n or aA^n residues. It was shown that furanose in U^n adopts C2'-endo puckering mode[8] which is typical for B-DNA. As for nucleoside residues with reversed configuration at the C2'-atom, arabinonucleotides is known to have a clear preference for particular sugar conformation depending on the position of the phosphate group: C3'-endo (N-type) for paA, and C2'-endo (S-type) for aAp.[9] This dependence is maintained at the oligomer level. A study of the trimer aApaApaA allowed it to be described as a S-S-N conformer.[9] This findings and data on *Eco*RI cleavage of DNA duplexes containing aA in the cleavage site[10] allow to suppose that within DNA duplex the arabinonucleoside residues and their 2'-deoxy-



SCHEME 2

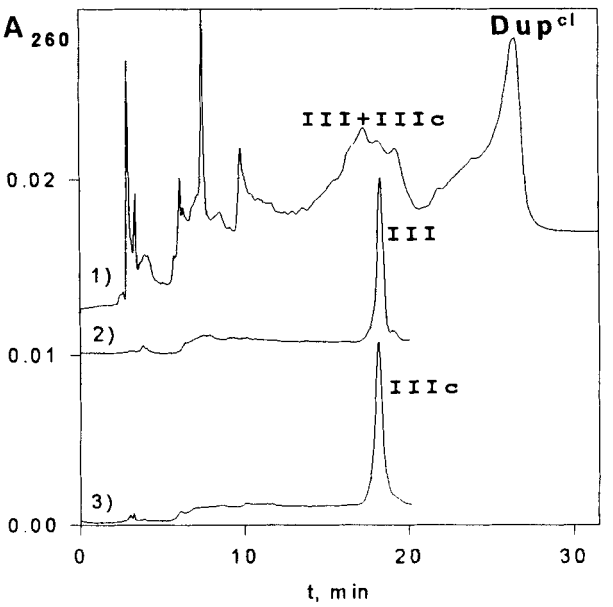


Fig. 1. HPLC analysis of the reaction mixture resulted from the cross-linking reaction between DNA duplex strands (1), initial oligodeoxyribonucleotides (2), (3). Conditions see Exp. Section.

TABLE 1

Abbreviations		DNA duplex	Tm, °C (±1)
A	**VI** **VII**	(5') CTACTCTCGC (3') GGATGAGAGCGT	48
B	**VI** **IV**	(5') CTAC T C T CGC (3') GGATG**aA**^{n}G**aA**^{n}GCGT	44
C	**V** **VII**	(5') CTAC**U**^{n}C**U**^{n}CGC (3') GGATGA GA GCGT	30
D	**V** **IV**	(5') CTAC **U**^{n}C **U**^{n}CGC (3') GGATG**aA**^{n}G**aA**^{n}GCGT	29

2'-amino derivatives would also be in C2'-endo conformation. Thus, this factor can not be responsible for duplex destabilization.

In case of U^{n} containing double helix reduced Tm may at least partially be associated with formation of ionic bonds between protonated amino group and negatively charged phosphodiester backbone[11,12] which could contribute to local conformational changes. However, this effect is not very essential at pH 7.25 (experimental conditions) because the pK_a of the 2'-amino group in U^{n}pU is 6.0.[12] It seems most likely that the reason of U^{n} destabilizing effect is connected with its unusual stacking behaviour. For example, almost complete loss of stacking interactions was observed for dinucleoside phosphate containing U^{n} as a 5'-terminal residue.[7]

To analyze the factors affecting thermal stability of DNA duplexes containing aA^{n}, we have studied the intramolecular stacking properties of a set of modified dinucleoside phosphates and dinucleotide - **aA**^{n}pT, Tp**aA**^{n}, Tp**aA**^{n}p, along with natural ones - TpA and ApT. FIG. 2a,b show the CD spectra of isomeric dimers and monomeric components aA^{n} and pT. It is seen that the CD of Tp**aA**^{n} exhibits the characteristic exiton type spectrum similar to that

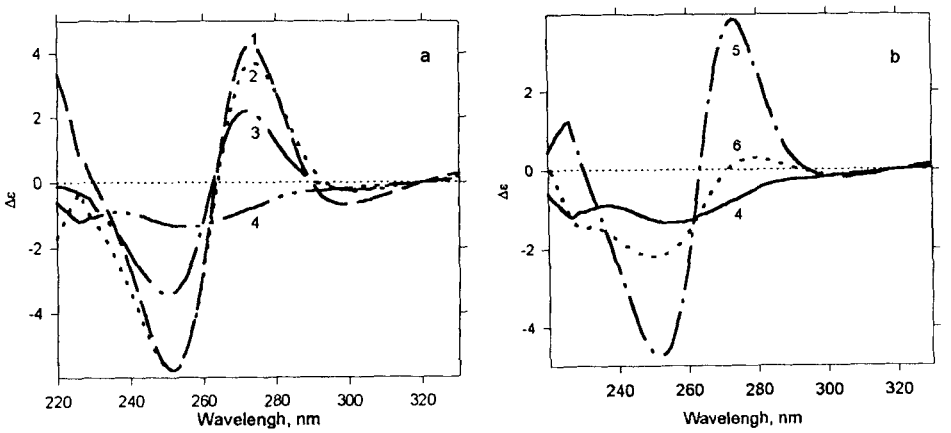


FIG. 2 a,b. CD spectra of Tp**aA**^{n} (1), Tp**aA**^{n}p (2), TpA (3), the contribution of the monomers pT and aA^{n} (4), ApT (5), **aA**^{n}pT (6). All spectra were measured in SSC-buffer, pH 7.25.

of non-modified TpA; the spectrum representing the monomer contributions is quite different (FIG. 2a). These data indicate the stacked state of this dimer. It is interesting that the presence of a phosphate group at the 3'-end of modified dinucleoside phosphate does not induce significant changes in CD spectrum. It means that even conversion of sugar puckering mode from C3'-endo (-aA^{n}) to C2'-endo (-aA^{n}p) (see above) does not effect the stacking properties of Tp**aA**^{n}(p).

In contrast, the CD spectrum of **aA**^{n}pT is similar to that of the corresponding pT and aA^{n} monomers (FIG. 2b). In other words, the bases are predominantly unstacked in **aA**^{n}pT. The same difference in behaviour is observed for different isomeric pairs of modified dinucleoside phosphates: ApaC and aCpA, UpaC and aCpU,[13] aUpdA and ApaU,[14] **U**^{n}pT and Tp**U**^{n}.[7] These data indicate that independing on nucleotide composition and type of sugar modification - arabino derivative, 2'-amino-2'-deoxyribo derivatives or 2'-amino-2'-deoxyarabino derivatives, the dramatical conformational changes are observed only for dimers carrying modified residues on 5'-end. Obviously,

the direction (vector) of stacking property changes induced by sugar modified residues effects always from 5'- to 3'-end.

Summarizing the data discussed above we can conclude that the trend of aA^n and U^n residues to destroy the stacking interactions with downstream partner could explain their duplex destabilizing ability. The different influence of aA^n and U^n on helix stability is not completely clear. We can suppose the non-equivalent geometry of aA^n or U^n containing base pairs with nearest neighbours.

The data concerning the stability of the oligonucleotides with incorporated sugar modified units to nucleases currently gain additional importance due to the search for the nuclease insensitive oligonucleotide probes. That is why we have compared hydrolytic stability to snake venom phosphodiesterase of oligonucleotide **II** containing aA^n residue in the oligomer chain and its native analogue. The enzyme digestion products were analyzed by the reversed phase HPLC in equidistant ion-pair mode which would be helpful in evaluating the length of oligonucleotide.[15] FIG. 3 shows the representative chromatography separation profiles of the enzymatic hydrolysis of both oligonucleotides. The native oligonucleotide was found to be completely hydrolyzed (FIG. 3a) under the chosen conditions (see Experimental section). In contrast, oligonucleotide containing two modified units was not completely cleaved after 60 min of incubation with snake venom phosphodiesterase under the same conditions (FIG. 3b). HPLC analysis enabled the presence of 7-mer - product of partial hydrolysis containing modified units at the 3'-end to be observed. Its further enzymatic hydrolysis proceeded very slowly. Experiment described here demonstrates that 2'-amino-2'-deoxyarabinoadenosine residues incorporated into oligonucleotide chain causes significant difficulty of the enzyme degradation of oligonucleotides.

CONCLUSION

The increasing demands for selectively modified DNA fragments provide impetus to the development of efficient techniques for their synthesis. For this

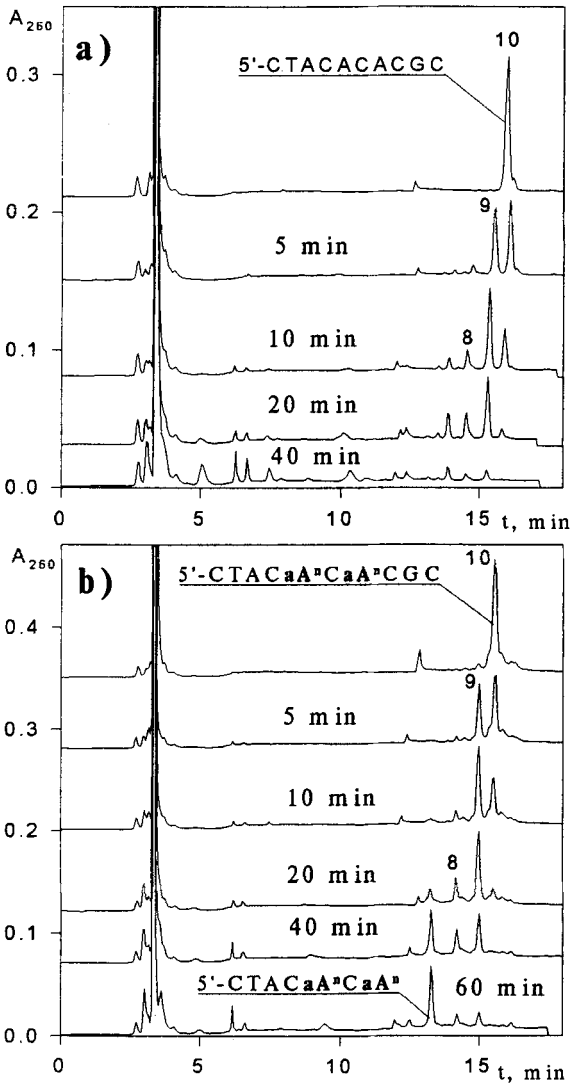


FIG. 3 a,b. HPLC analysis of crude product mixture resulting from enzymatic cleavage by snake venom phosphodiesterase of modified oligonucleotide **II** and its native analogue. Figures above peaks correspond to length of oligonucleotides. Conditions see Exp. Section.

reason, our efforts in the present work were directed towards the synthesis of the new type of sugar modified oligodeoxyribonucleotides containing 9-(2-amino-2-deoxy-β-D-arabinofuranosyl)adenine residues at predetermined positions. Oligonucleotides under investigation demonstrated high reactivity to electrophilic reagents, increased resistance to enzymatic degradation and ability to form a double helix with complementary nucleic acids, the destabilizing effect of aA^n residue being much less then previously described U^n residues.

This research is of great interest in determining possible areas of application of oligonucleotide carrying such modifications. In particular, in our recent study we developed an efficient method to synthesize DNA duplexes with covalently connected strands.[3,4] The covalent linkage was organized between 2'-amino-2'-deoxyuridine residue introduced into one strand of DNA duplex and the carboxyl group located on the non-nucleoside insert in the complementary strand using a water-soluble carbodiimide as a condensing agent. This combination of interacting units provided the double destabilizing action: due to modifications introduced and the loss of hydrogen bonds at abasic site of non-nucleoside insert. However, the base pairing could be retained at cross-linking point. For instance, coupling reaction can be carried out between 2'-amino group of aA^n residue introduced into the one strand of DNA duplex and the carboxyl group incorporated into other strand by the acylation of U^n with anhydride of dicarbonyc acid. This study is being continued.

EXPERIMENTAL SECTION

Materials. 5'-*O*-(4,4'-Dimethoxytrityl)-*N*-acyl-2'-deoxynucleoside-3'-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites and controlled pore glass supports derivatized with protected deoxynucleosides were obtained from Applied Biosystems. Snake venom phosphodiesterase, alkaline phosphatase, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane were purchased from Sigma. β-Cyanoethyl-*N*,*N*-diisopropylamidochlorophosphite, trifluoromethanesulfonic anhydride were obtained from Aldrich. 1-Ethyl-3-(3'-dimethylamino-

propyl)carbodiimide hydrochloride and triphenylphosphine from Merck were used.

General Methods. TLC was performed on Kieselgel 60 F_{254} plates (Merck) using one of the following solvent systems: **a**) $CHCl_3$/EtOH 9:1 (v/v); **b**) $CHCl_3$/EtOAc 8:2 (v/v); **c**) $CHCl_3$/EtOH 95:5 (v/v); **d**) CH_2Cl_2/Et_3N 99:1 (v/v). Column chromatography was performed on Silikagel L40/100 (Chemapol) and Kieselgel 60 (Merck) using one of the following solvent systems: $CHCl_3$/EtOAc 95:5 (v/v) - (A); $CHCl_3$/MeOH 95:5 (v/v) - (B).

NMR spectra were registrated on a spectrometer VXR-400 (Varian). IR spectra were recorded on a UR-20 spectrometer (Germany). UV spectra and thermal melting profiles were monitored with a 150-20 Spectrophotometer (Hitachi) equipped with a thermoelectrically-controlled cell holder. The melting behavior of the DNA duplexes was determined in the SSC-buffer (0.15 M NaCl, 0.015 sodium citrate, pH 7.25). Tm was determined as the maximum of the differentials (dA/dT *versus* T) of profiles. Molar extinction coefficients of aA^n and U^n were taken equal to that of corresponding natural nucleosides. CD spectra were recorded by a Jobin Yvon dichrograph (Mark V). Samples in SSC-buffer were analyzed at room temperature. Digitized data obtained every 1 nm were corrected for baseline and smoothed by least-squares polynomial fit up to the third order. ***N*⁶-Benzoyladenosine 2** and **5',3'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-*N*⁶-benzoyladenosine 3** were prepared according to van Boom and Wreesmann.[16] Overall yield of **3**: 60%. R_f 0.5 (system **a**).

5',3'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-trifluoromethanesulfonyl-*N*⁶-benzoyladenosine 4 was synthesized according to the method described by Herdewijn.[17] Compound **3** (3.5 g, 5.8 mmol) was dried by co-evaporation with dry pyridine. Then a solution of trifluoromethanesulfonic anhydride (1.9 ml, 11.5 mmol) in dry CH_2Cl_2 (9 ml) was added dropwise at -10 °C to a stirred mixture of **3** and dry pyridine (12 ml) in dry CH_2Cl_2 (96 ml). The mixture was stirred for 40 min at 10 °C, then

temperature was slowly raised to 20 °C. After stirring for 2 h at 20 °C, the reaction mixture was quenched with 10 ml H_2O and then dissolved in 100 ml $CHCl_3$. The solution was washed three times with 50 ml portions of H_2O and then the organic layer was dried over Na_2SO_4. The material was purified by silicagel column chromatography eluting with solvent system (A) to give 3.2 g (75%) **4**; R_f 0.5 (system **b**). ^{1}H-NMR ($CDCl_3$, δ): 6.21 (s, 1H, H-1'), 5.80 (wide d, 1H, H-2', $J_{2',3'}$ 4.7 Hz), 5.25 (dd, 1H, H-3', $J_{3',4'}$ 9.28 Hz), 4.15 (m, 1H, H-4'), 4.08 (dd, 1H, H-5'a, $J_{5'a,5'b}$ 13.18 Hz, $J_{4',5'a}$ 2.68 Hz), 4.20 (dd, 1H, H-5'b, $J_{4',5'b}$ 1.70 Hz).

9-(5,3-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2-azido-2-deoxy-β-D-arabinofuranosyl)-N^6-benzoyladenine 5. Compound **4** (3.2 g, 4.3 mmol) dried by co-evaporation with dry pyridine was dissolved in a mixture of DMF and dioxane (1:1) (86 ml). LiN_3 (2 g, 41 mmol) was added to the mixture under stirring. TLC (system **b**) showed completion of the reaction after 4 h. The reaction mixture was concentrated *in vacuo* to a thick oil, which was dissolved in 100 ml $CHCl_3$. The solution was washed three times with 50 ml portions of H_2O and the organic layer was dried over Na_2SO_4. The material was purified by silicagel column chromatography eluting with solvent system (B) to give 2.4 g (88%) **5**; R_f 0.23 (system **b**). IR (KBr, ν, cm^{-1}): 2120 (N_3), 1666, 1616 (C=O). ^{1}H-NMR ($CDCl_3$, δ): 6.52 (d, 1H, H-1', $J_{1',2'}$ 6.72 Hz), 4.50 (dd, 1H, H-2', $J_{2',3'}$ 8.56 Hz), 4.63 (t, 1H, H-3', $J_{3',4'}$ 8.45 Hz), 3.95 (m, 1H, H-4'), 4.07 (dd, 1H, H-5'a, $J_{5'a,5'b}$ 13.13 Hz, $J_{4',5'a}$ 2.98 Hz), 4.21 (dd, 1H, H-5'b, $J_{4',5'b}$ 3.06 Hz).

9-(5,3-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2-amino-2-deoxy-β-D-arabinofuranosyl)-N^6-benzoyladenine 6 was synthesized according to the method described by Staudinger and Meyer.[18] 9-(5,3-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2-azido-2-deoxy-β-D-arabinofuranosyl)-N^6-benzoyladenine **5** (2.4 g, 3.8 mmol) dried by co-evaporation with dry pyridine was dissolved in THF (50 ml). Ph_3P (2.5 g, 9.5 mmol) was added with stirring and the reaction mixture was stirred for 2 h. Then H_2O (3 ml) was added and the resulting mixture was stirred at 50 °C.

TLC (system **b**) showed completion of the reaction after 10 h. The reaction mixture was concentrated *in vacuo*, dissolved in 100 ml EtOAc and treated as described above. The material was purified by silicagel column chromatography eluting with solvent system (B) to give 1.2 g (50%) **6**; R_f 0.28 (system **c**).

Deprotection of the 5',3'-hydroxyl groups of **6** was carried out according to Sproat and Lamond.[19] Tritylation of **7** was carried out according method described by Jones.[20] Overall yield of **8**: 68%. R_f 0.4 (system **c**). UV (EtOH, nm): λ_{max} 279, λ_{min} 256. ^{1}H-NMR ($CDCl_3$, δ): 6.33 (d, 1H, H-1', $J_{1',2'}$ 6.54 Hz), 3.74 (m, 1H, H-2'), 4.36 (t, 1H, H-3', $J_{2',3'}$ 7.69 Hz, $J_{3',4'}$ 7.69 Hz), 4.02 (m, 1H, H-4'), 3.43 (dd, 1H, H-5'a, $J_{5'a,5'b}$ 10.65 Hz, $J_{4',5'a}$ 4.48 Hz), 3.52 (dd, 1H, H-5'b, $J_{4',5'b}$ 3.47 Hz).

9-(5,3-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2-trifluoro-acetamido-2-deoxy-β-D-arabinofuranosyl)-N^6-benzoyladenine 9 was prepared from **8** according to literature procedure.[7,21] Yield of **9**: 95%. R_f 0.5 (system **c**). ^{1}H-NMR ($CDCl_3$, δ): 6.48 (d, 1H, H-1', $J_{1',2'}$ 6.60 Hz), 4.80 (m, 1H, H-2'), 5.10 (t, 1H, H-3', $J_{2',3'}$ 7.69 Hz, $J_{3',4'}$ 7.69 Hz), 4.18 (m, 1H, H-4'), 3.48 (m, 2H, H-5'a, H-5'b), 8.72 (d, 1H, NH-2', $J_{NH\text{-}2',H\text{-}2'}$ 5.1 Hz).

Phosphitylation of **9** was carried out according to Barone *et al.*[22] Yield of **10**: 95%. R_f 0.53 (system **d**). ^{31}P-NMR ($CDCl_3$, δ): 149.86, 150.18.

Oligonucleotide Synthesis was performed on an Applied Biosystems 380B DNA synthesizer by using standard protocol. When modified phosphoramidites are used, a longer coupling time (10 min) was employed. The oligonucleotides were normally synthesized in 0.2 μmol scale in the "Trityl-On" mode. Standard deprotection conditions (30% NH_4OH, 55 °C, 16 h) were employed.

Oligonucleotide **IIIc** containing non-nucleoside insert was prepared as described by Antsypovich *et al.*[3] as well as oligonucleotide **V** containing 2'-amino-2'-deoxyuridine was prepared as described earlier.[7]

Analysis of reaction mixtures and isolation of oligonucleotides containing dimethoxytrityl group were done by reversed phase HPLC as described earlier.[7] The nucleoside composition of the modified oligonucleotides were analyzed as described in literature.[7]

Reactions with acetic anhydride and succinic anhydride for oligonucleotide **I** and **III** were performed as described earlier.[3]

The Cross-linking Reaction was carried out in 0.05 M MES-buffer (pH 5.5) with 0.02 M $MgCl_2$. 0.1 A_{260} units both oligonucleotides were dissolved in 37 μl buffer, the mixture was heated to 95 °C, then cooled slowly to 0 °C and 37 μl 0.4 M 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride in the same buffer was added. After 72 h the reaction mixture was analyzed and the final product was isolated by means of the reversed phase HPLC in ion-pair mode as described earlier.[7,15]

Enzymatic Hydrolysis of oligonucleotide II by snake venom phosphodiesterase. 0.2 A_{260} of oligodeoxyribonucleotide material and 1.0 A_{260} of uridine as internal standard were dissolved in 18 μl of 0.2M Tris-HCl buffer (pH 8.5, 0.04M $MgCl_2 \cdot 6H_2O$). 2 μl of snake venom phosphodiesterase (1×10^{-4} U/ml) were added. The reaction mixtures were incubated at 37 °C. At predetermined times, aliquots (4 μl) were withdrawn from the reaction mixture and the reaction was terminated by freezing in the liquid nitrogen. The products of enzymatic hydrolysis were analyzed using reversed phase HPLC in equidistant ion-pair mode on Waters chromatograph (Millipore) according to literature.[7,15]

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