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SYNTHESIS OF DNA ANALOGUES WITH NOVEL CARBOXAMIDOMETHYL PHOSPHONAMIDE AND GLYCINAMIDE INTERNUCLEOSIDE LINKAGES

V.A.Efimov*, A.A.Buryakova and O.G.Chakhmakhcheva

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul.Miklukho-Maklaya 16/10, Moscow 117871, Russia

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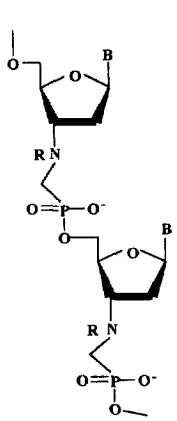
Abstract: Thymidine oligonucleotide analogues with phosphodiester bonds fully substituted by carboxamidomethyl phosphonamide, or glycinamide linkages were synthesized on a solid support, and their hybridization properties toward DNA and RNA targets were determined by T*m* analysis.

Various types of modified oligonucleotides have been developed over the past few years as potential diagnostic probes and therapeutics for the antisense and antigene approach[1]. Among the modifications of phosphodiester bonds in nucleic acids, examples of stretched 5-atom linkage between nucleoside residues have been reported, including non-ionic amide bridges[2], and some such modifications showed interesting hybridization properties and enhanced stability to nuclease digestion[3]. As a part of our investigations on the design and synthesis of modified DNA molecules and mimics[4], we recently reported the chemical synthesis of several DNA analogues containing 5-atom internucleoside linkages with potentially different flexibility and charge representing 3'- and 5'-aminomethyl phosphonate (**1, 2**) as well as 5'-carboxamidomethyl phosphonate (**3**) linkages[5]. In this communication, we report the extension of these studies to homo-thymidine DNA analogues containing 5'-carboxamidomethyl phosphonamide (**4**) and glycinamide (**5**) internucleoside linkages.

Procedures have been developed to obtain corresponding two types of monomer units. Both monomers contain amino and carboxyl functions for chain elongation (via amide bond formation) and a combination of blocking groups compatible with DNA synthesis. The preparation of the monomer (**12**) for the synthesis of



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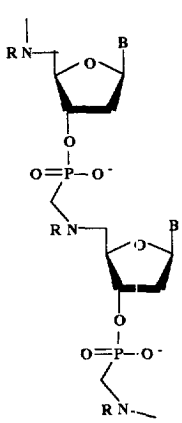

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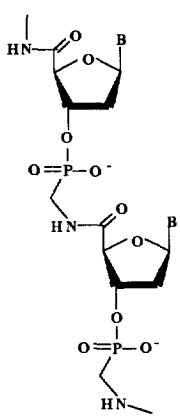

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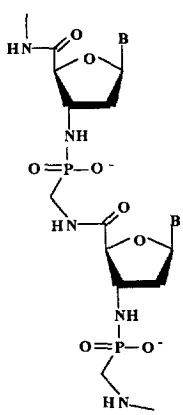

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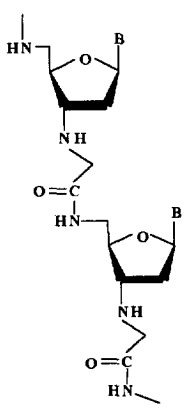

(5)

R = H(a), Acyl (b), or Alkyl (c)

* Fax: +(7095)3365911, E-mail: eva@ibch.siobc.ras.ru

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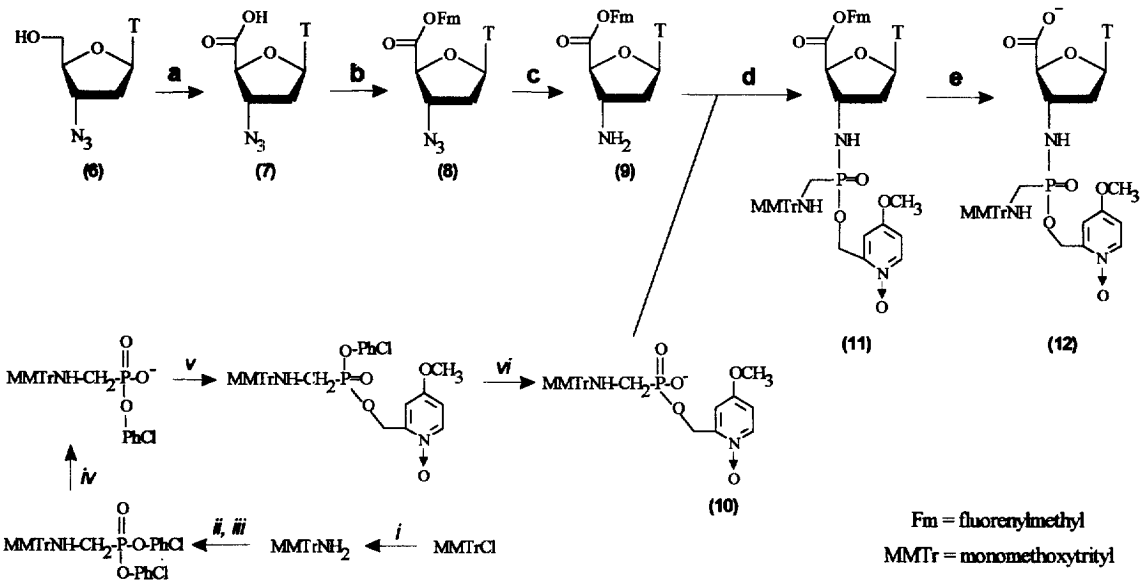


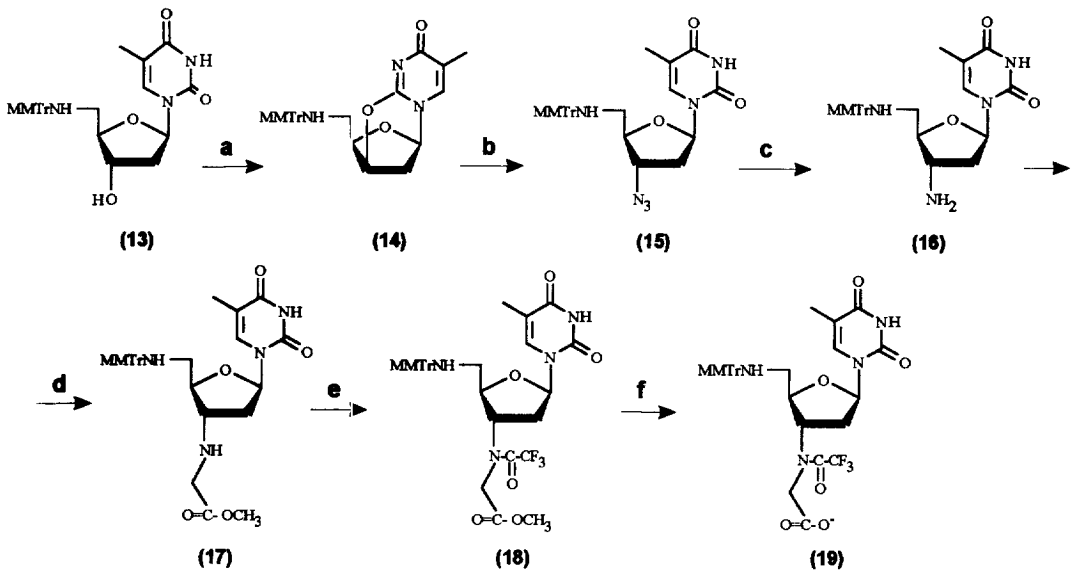
Scheme 1. (**a**) - $RuCl_3 \cdot H_2O$ (cat.), $K_2S_2O_8$ (4.5 eq.), 1 M KOH, 4 h (75%); (**b**) - Fm-OH (1.2 eq.), TPSNT (1.2 eq.) in Py , 30 min (95%); (**c**) - H_2S gas in Py , 1 h (90%); (**d**) - compound (10)[7] (1 eq.), TPSNT (1.1 eq.) in Py, 15 min (85%); (**e**) - DBU (0.5 M) in CH_3CN, 30 min (87%).

oligomers with 5'-carboxamidomethyl phosphonamide linkages of type (**4**) was realised through the reaction sequence shown in **Scheme 1**. As a starting compound, 3'-azido-3'-deoxythymidine was chosen. It was converted into 5'-carboxylic acid derivative (**7**) by mild oxidation as described[6] with subsequent protection of the carboxyl moiety by the fluorenylmethyl blocking group. After the conversion of the 3'-azido function into a 3'-amino group, the N-(4-methoxytrityl)aminomethyl phosphonate residue was introduced by the reaction of the intermediate (**9**) with 1-oxido-4-methoxy-2-picolyl N-(4-methoxytrityl)aminomethyl phosphonate (**10**)[7] in the presence of 1-(2,4,6-triisopropylbenzenesufonyl)-3-nitro-1,2,4-triazole (TPSNT). A phosphonate-protecting 1-oxido-4-methoxy-2-picolyl group was introduced to enhance the rate of the phosphonamide bond formation via intramolecular O-nucleophilic catalysis[8]. The last step was the removal of the fluorenylmethyl protecting group from the carboxyl function by the action of a base.

The synthesis of the monomer (**19**) for construction of a DNA analogue containing glycinamide internucleoside linkages is outlined in **Scheme 2**. It was accomplished starting from 5'-N-(4-methoxytrityl) amino-5'-deoxythymidine (**13**) obtained as previously described[9]. This was then converted to the O^2,3'-cyclonucleoside (**14**) essentially as described by Cosford and Schinazi[10]. Treatment of (**14**) with NaN_3 gave 3'-azido derivative (**15**). The latter was converted to the desired protected monomer (**19**) in 5 stages via the 5'-N-(4-methoxytrityl)- 5',3'-diamino-5',3'-dideoxythymidine (**16**).

The monomers obtained[11] were used for the automated solid phase synthesis of homo-thymidine oligonucleotide analogues of type (**4**) and (**5**) using solid phase protocols summarised in **Table 1**. As a support, we used long chain alkylamine CPG functionalized with 5'-N-(4-methoxytrityl)amino-3'-O-succinyl-5'-deoxythymidine for the synthesis of analogue (**5**) and with 5'-O-succinyl-3'-N-(4-methoxytrityl)amino-3'-deoxythymidine for analogue (**4**). At each step, the carboxylic component was used in 5-fold excess over the

resin capacity. As in the procedure described by Jorba et al. for peptide synthesis[12], the coupling reactions between the carboxyl moiety in solution and the terminal NH_2 -group on the support were performed in the presence of TPSNT as a condensing agent and 1-methylimidazole as a nucleophilic catalyst. An average coupling yield on each elongation step was about 95%, as gauged by spectrophotometrical monitoring the release of monomethoxytrityl cation. The deprotection of the monomethoxytrityl group from the amino function of a growing oligomer chain was achieved by the action of 3% pentafluorophenol in methylene chloride. We have found the latter reagent provides fast removal of the monomethoxytrityl group from the amino function with



Scheme 2. (**a**) - Ph_3P (1.2 eq.), DEAD (1.2 eq.) in CH_3CN, 30 min (85%); (**b**) - NaN_3 (4 eq.) in DMFA, 5 h, 125-130°C (76%); (**c**) - Ph_3P (2 eq.) in Py, 2 h, then NH_4OH, 2 h, 20°C (82%); (**d**) - $BrCH_2COOCH_3$ (1.5 eq.), DiPEA (2 eq.) in CH_3CN, 3 h (88%); (**e**) - $(CF_3CO)_2O$ (4 eq.), TEA (6 eq.) in CH_3CN, 15 min (97%); (**f**) - DBU (0.5 M) in CH_3CN - H_2O (9:1, v/v), 30 min (90%).

Table 1. One elongation cycle for the solid phase synthesis of DNA analogues.

Step	Solvents and reagents*	Time (min)
1. Detritylation	3% Pentafluorophenol in dichloromethane	3.0
2. Wash	0.2 M N,N-Diisopropylethylamine in dichloromethane	0.5
3. Wash	Dichloromethane	1.5
4. Wash	Acetonitrile - pyridine (1:1, v/v)	1.0
5. Coupling**	0.05 M Monomer; 0.06 M TPSNT, 0.15 M 1-methylimidazole in acetonitrile - pyridine (1:1, v/v)	15.0
6. Wash	Acetonitrile - pyridine (1:1, v/v)	0.5
7. Capping	Ac_2O - 1-methylimidazole - pyridine - acetonitrile (1:1:2:6, v/v/v/v)	1.0
8. Wash	Dichloromethane	1.0

* Synthesis of oligomers was performed using Applied Biosystems Synthesizer 381A and 30-40 mg of CPG support containing about 1 μmol of monomethoxytrityl groups.

** Before coupling, carboxylic component was pre-activated by mixing with TPSNT and 1-methylimidazole.

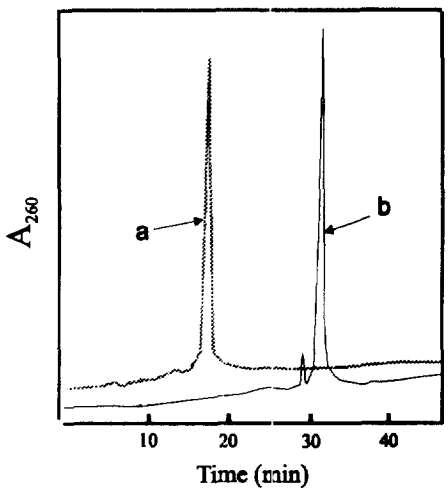


Figure 1. Analysis of purified T_{15} oligomers by FPLC using Pharmacia ProRPC column and a linear gradient of acetonitrile (0 - 30%) in 0.1 M TEAA (pH 7.5): (**a**) - oligomer of type (4) and (**b**) - oligomer of type (5).

minimal influence to the phosphonamide bonds. After completion of the synthesis and removal of the terminal monomethoxytrityl group and prior to the next deprotection steps, free amino groups of the support-bound oligomer were acetylated to suppress possible side reactions, such as cyclization and chain degradation, which can occur under basic conditions, as described for PNAs[13]. In the next step, 1-oxido-4-alkoxy-2-picolyl phosphonate protecting groups were removed from the analogues (**4**) by treatment with triethylammonium thiophenolate[8]. The final deprotection of oligomers and cleavage from the support were effected by ammonolysis (20°C, 5 h). The oligomers were then isolated by ion-exchange chromatography[14] and their purity was confirmed by reversed-phase FPLC analysis (**Fig.1**). The expected molecular weights of the modified oligonucleotides were confirmed by MALDI-TOF MS.

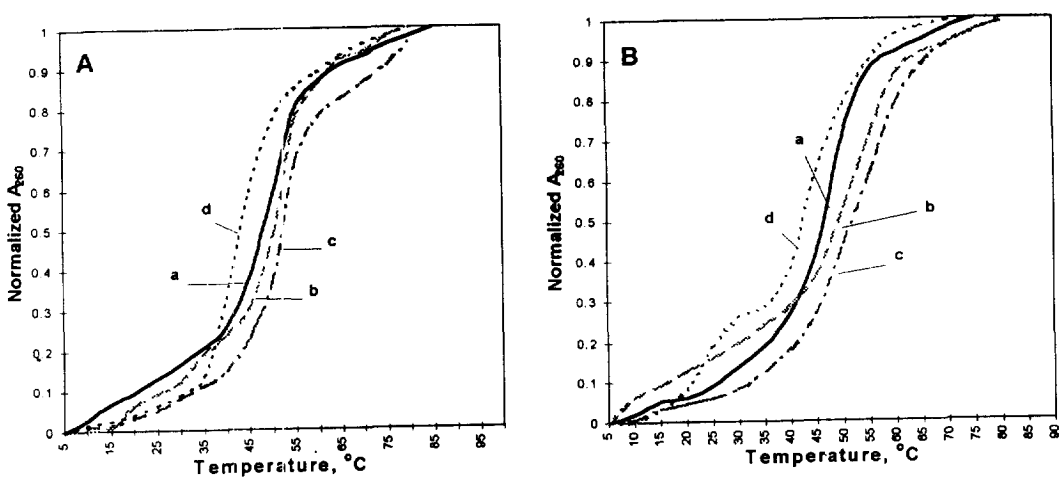


Figure 2. Melting curves of complexes formed by T_{15} oligomers of type **3** (**a**), **4** (**b**) or **5** (**c**) with complementary dA_{15} (**A**) or rA_{15} (**B**) target in 150 mM NaCl /10 mM Tris-HCl (pH 7.5) /5 mM EDTA /10 mM $MgCl_2$ and those formed by non-modified dT_{15} oligonucleotide (**d**).

Preliminary investigations on the properties of modified oligomers revealed that compounds of both types (**4**) and (**5**) are stable to the action of exo- and endonucleases[15]. The hybridization properties of the modified oligomers obtained with complementary dA_{15} or rA_{15} oligonucleotides were determined by thermal denaturation studies[16]. Ultraviolet melting experiments indicate that compounds of type (**4**) hybridized to DNA and RNA with melting temperatures (T*m*) higher than those of complexes formed by oligo-T DNA fragment of the same length, which is similar to the behaviour of modified oligomer of type (**3**) containing 3'-phosphonate ester bonds. However, in contrast to the compound of type (**3**), which is stable in aqueous solutions at pH 2-12, the oligomer (**4**) containing phosphonamide bonds was stable at pH≥7.5, but slowly degraded at pH≤7.0. The molecule of type (**5**) gave more stable complexes with complementary oligo-A fragments than those formed by compound of type (**4**) (ΔTm =2-3°C). Complexes formed by all types of modified oligomers with the RNA target had higher stability than similar complexes formed with the DNA target (ΔTm =2-5°C). Examples of dissociation curves obtained are shown in **Fig. 2**. The experiments on the UV-titration of oligo-A by the oligo-T analogue of type (**4**) clearly show the triplex formation, which is similar to the behaviour of compound (**3**), whereas the titration by the oligomer of type (**5**) revealed that it is able to form duplexes rather than triplexes with complementary oligo-A targets (**Fig. 3**).

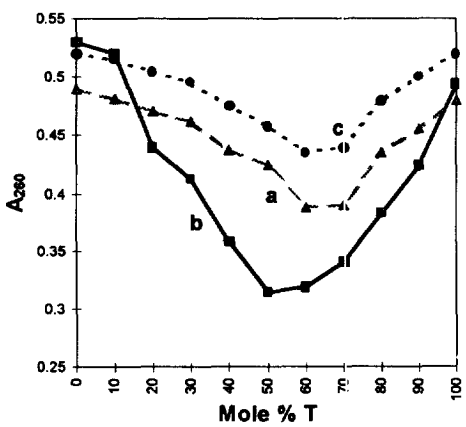


Figure 3. Titration of dA_{15} target with modified T_{15} oligomers of type (4) (**a**); type (5) (**b**), or unmodified dT_{15} (**c**) (Job plot) at 20°C in 0.15 M NaCl / 0.02 M Tris-HCl (pH7.5) / 0.01 M $MgCl_2$ /0.1 mM EDTA.

In conclusion, the synthesis of two types of thymidine monomer units was accomplished. Both were used for the construction of novel oligodeoxyribonucleotide analogues. The first evaluation of properties of the oligomers obtained revealed that these compounds display increased affinity to the complementary DNA and RNA fragments compared to the corresponding natural oligo-dT fragment. Complexes formed by the analogue containing glycinamide internucleoside linkages were the most stable. Also, this paper presents only limited sequence data on the properties of DNA analogues described herein, but they are valid enough for the first analogue evaluation. Further experiments on the examination of the properties of these compounds are currently underway.

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7. The synthesis of compound (**10**) was accomplished as shown in **Scheme 1**. Steps ***i-iii*** were performed essentially as described by Soroka, M. and Zygmunt, J. *Synthesis* **1988**, 370-372: (***i***) reaction with the excess of 10% NH_3 in dioxane - water (3:1, v/v) (97%); (***ii***) action of 38% aqueous formaldehyde (1.2 eq.) in acetonitrile, 30 min; (***iii***) interaction with di(p-Cl-phenyl)phosphite (1.1 eq.) in toluene, 80°C, 15 min (92%). The conversion of di(p-chlorophenyl) ester of N-(4-methoxytrityl)aminomethyl phosphonate to the compound (**10**) (steps ***iv-vi***) was performed with 80% overall yield as it was described earlier for phosphono-PNA analogues (see ref. 4b,c). Analytical data for (**10**): m/z (ES) = 521.5 $(M+H)^+$ ($C_{28}H_{30}N_2O_6P_1$ req. 521.5); ^{1}H NMR ($CDCl_3$) δ = 1.18 (t, 9H, $^+$NHEt$_3$-C*H*$_3$), 2.40 (dd, 2H, N-C*H*$_2$-P), 2.88 (q, 6H, $^+$NHEt$_3$-C*H*$_2$), 3.72 (s, 3H, MMTr-OC*H*$_3$), 3.76 (s, 3H, picolyl-OC*H*$_3$), 5.25 (d, 2H, P-O-C*H*$_2$), 6.65-6.75 (m, 4H, picolyl-*H*-3,-*H*-5 and MMTr-ortho-*H*), 7.05-7.50 (m, 12H, MMTr), 8.05 (d, 1H, picolyl-*H*-6); ^{31}P NMR ($CDCl_3$) δ = 19.2. Chemical shifts are given in p.p.m. relative to tetramethylsilane (^{1}H) or H_3PO_4 (^{31}P).
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11. Relevant analytical data. Compound **12:** m/z (ES) = 758.5 $(M+H)^+$ ($C_{38}H_{41}N_5O_{10}P_1$ req.758.7); ^{1}H NMR ($CDCl_3$) δ = 1.20 (t, 9H, $^+$NHEt$_3$-C*H*$_3$), 1.86 (s, 3H, Thy-C*H*$_3$), 2.15-2.30 (m, 2H, 2'-C-*H*), 2.38 (d, 2H, N-C*H*$_2$-P), 2.95 (q, 6H, $^+$NHEt$_3$-C*H*$_2$), 3.70 (s, 3H, MMTr-OC*H*$_3$), 3.78 (s, 3H, picolyl-OC*H*$_3$), 4.05 (m, 1H, 4'C-*H*), 4.25 (m, 1H, 3'C-*H*), 5.25-5.35 (2xd, rotamers, 2H, P-O-C*H*$_2$), 6.32 (m, 1H, 1'-C-*H*), 6.65-6.80 (m, 4H, picolyl-*H*-3,-*H*-5 and MMTr-ortho-*H*), 7.05-7.50 (m, 13H, MMTr and Thy-*H*-6), 8.07-8.17 (2xd, 1H, picolyl-*H*-6), 8.60 (m, 1H N*H*); ^{31}P NMR ($CDCl_3$) δ = 30.3 and 30.7. Compound **19:** m/z (ES) = 667.4 $(M+H)^+$ ($C_{34}H_{34}N_4O_7F_3$ req.667.7); ^{1}H NMR δ = 1.18 (t, 9H, $^+$NHEt$_3$-C*H*$_3$), 1.85 (s, 3H, Thy-C*H*$_3$), 2.25-2.50 (m, 4H, 2'-C-*H* and 5'-C-*H*), 2.55 (t, 1H, 5'-N*H*), 2.90 (q, 6H, $^+$NHEt$_3$-C*H*$_2$), 3.75 (s, 3H, MMTr-OC*H*$_3$), 4.0 (s, 2H, N-C*H*$_2$-CO), 4.15 (m, 1H, 4'C-*H*), 4.37 (m, 1H, 3'C-*H*), 6.32 (dd, 1H, 1'-C-*H*), 6.80-7.50 (m, 15H, MMTr and Thy-*H*-6).
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14. Anion-exchange separations were performed using Pharmacia Mono-Q column / FPLC system and a linear gradient of NaCl (0 - 1.2 M) in 0.02 M NaOH (pH 12) with a flow rate of 1 ml/min.
15. Snake venom phosphodiesterase and S_1 nuclease assays were performed as described by Jones, G.D.; Lesnik, E.A.; Owens, S.R.; Risen, L.M.; Walker, R.T. *Nucleic Acids Res.* **1996**, *24*, 4117-4123.
16. Melting curves of complexes were measured for 3-5 μM solutions of oligomers using a Gilford 250 UV-VIS spectrophotometer and a Gilford 2527 thermocontroller with a heating rate of 0.5°C/min. Melting temperature was taken to be the temperature of half-dissociation and was obtained from a plot of the derivative of 1/*T* vs absorbance at 260 nm. Molar extinction coefficients: A, 15.4 and T, 8.8 μmol.cm.