1170–1176 Nucleic Acids Research, 1995, Vol. 23, No. 7

Looped oligonucleotides form stable hybrid complexes with a single-stranded DNA

Elena Azhayeva, Alex Azhayev*, Andrei Guzaev, Jari Hovinen and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20500 Turku, Finland

Received December 23, 1994; Revised and Accepted February 15, 1995

ABSTRACT

Several new branched (1, 2), circular (9) and looped oligonucleotides (14–17) were synthesized. 3′-Deoxypsicothymidine was employed to create the site of branching when required. The circular and looped structures were obtained by oxidative disulfide bond formation between mercaptoalkyl tether groups. All the oligonucleotides prepared contained two T_{11} sequences, and the branched and looped oligomers an additional alternating CT sequence. The melting experiments revealed that the branched oligonucleotides form relatively weak hybrid (double/triple helix) complexes with the single-stranded oligodeoxyribonucleotide, showing a considerable destabilizing effect produced by the structure at the point of branching. The data obtained with looped oligonucleotides demonstrated considerable stabilization of the hybrid (double/triple helix) complexes with the complement. The data reported may be useful in attempting to design new antisense or antigene oligonucleotides capable of forming selective and stable bimolecular hybrid complexes with nucleic acids.

INTRODUCTION

Synthetic oligonucleotides have been widely employed to modulate the biological activity of DNA and RNA (1–5). In the antisense strategy oligonucleotides are used to arrest complementary RNA and thus to inhibit viral RNA replication or to block synthesis of proteins (2). The antigene strategy is in turn based on inhibition of transcription or replication of genes due to the interaction of synthetic oligonucleotides with DNA (3). Recently, bimolecular triple helices have found use in a new strategy for DNA and RNA recognition (6–20). This approach is based on selective and strong complexing of a purine-rich sequence of the target nucleic acid with two pyrimidine-rich fragments of the complementary oligonucleotide. One of the pyrimidine sequences forms Watson–Crick base pairs with the polypurine target, while the other is engaged by Hoogsteen base pairing. Details of nucleic acids recognition by bimolecular triple helix formation have been elucidated by Kool and co-workers (9–18,20). According to their studies, circular oligonucleotides seem to be useful structures for further development of new generation antisense and antigene oligonucleotides. In fact, circular oligonucleotides possess many advantageous characteristics. They bind more tightly and selectively to the complementary DNA single strand than the normal Watson–Crick complement. They also display higher resistance to the action of nucleases (20,21). Nevertheless, these molecules also suffer from some drawbacks. First, circular oligonucleotides bearing two polypyrimidine regions may exhibit specificity for several similar polypurine fragments of the target nucleic acid when used in real biological experiments aimed at suppressing translation, reverse transcription or splicing. Secondly, triplexes formed by circular RNA oligonucleotides when hybridized with RNA may dramatically decrease the catalytic efficiency of RNase H, which plays a very important role in the antisense approach by cleaving the target RNA (23,24). Finally, circular oligonucleotides cannot be easily tethered to various conjugate groups, needed to bring desired biological properties, such as enhanced transport through the cell membrane. We believe that these shortcomings could be overcome by binding a single-stranded RNA or DNA to a looped ribo- or deoxyribonucleotide oligomer that may form hybrid helices, i.e. a triple and double helix simultaneously. This would possibly provide the following advantages. The looped oligonucleotide would be more selective towards the target, since its structure assumes not only strong binding to the polypurine sequence via triple helix formation, but also sequence-specific interaction with the chain fragment adjacent to the polypurine region. Recognition of a particular RNA by the looped oligoribonucleotide would thus lead to the formation of a normal duplex, susceptible to the action of RNase H. Furthermore, the open chain part of the oligomer may be easily derivatized if needed. We now report on the synthesis of new looped oligodeoxyribonucleotides and on their hybridization with a single-stranded DNA fragment.

MATERIALS AND METHODS

Solid supports and monomers for oligonucleotide synthesis

Oligonucleotide synthesis was performed on solid support **5**, bearing a thioester linkage (32), on solid support **12** possessing a disulfide bond (33) or on commercial nucleoside-bound solid supports (Sigma and Cruachem). A 0.2–1 μmol scale was used throughout to assemble the oligomers. 3′-Deoxypsicothymidine phosphoramidites **3** and **13** (25) and non-nucleosidic phosphoroamidite **6** bearing a thioester linkage (32) were synthesized as described earlier.

* To whom correspondence should be addressed

2′-Deoxyadenosine 3′-{*N*-[6-(2-hydroxyacetylamino)-3,4-dithiahexyl]-1- carboxamido-methyl} phosphate (10)

Cystamine (free base, 2.0 g, 13.3 mmol) was dissolved in methyl glycolate (4 ml, 43.2 mmol) and the mixture was kept overnight at room temperature. All volatile materials were removed *in vacuo*. The remaining solid was crystallized from a mixture of ethyl acetate and ethanol to give *N,N′*-bis(hydroxyacetyl)-3,4-dithia-1,6-hexanediamine as a white powder. Melting point 113°C. Found: C, 35.8; H, 6.0; N, 10.6; S, 24.2. $C_8H_{16}N_2O_4S_2$ requires: C, 35.8; H, 6.0; N, 10.4; S, 23.9%. ^{1}H NMR (400 MHz; DMSO-d_6) 7.93 (2 H, t, *J* 6.4), 5.52 (2 H, t, *J* 5.9), 3.80 (4 H, d, *J* 5.9), 3.39 (4 H, dt, *J* 7.3 and 6.4), 2.81 (4 H, t, *J* 7.3). A part of this (1.45 g, 5.4 mmol) was dissolved in dry pyridine (50 ml). 4,4′-Dimethoxytrityl chloride (1.74 g, 5.13 mmol in 20 ml dry pyridine) was then added drop-wise and the mixture was allowed to stir overnight at ambient temperature. The reaction was quenched with ice, the mixture was concentrated, dissolved in methylene chloride, washed with saturated $NaHCO_3$ and dried over Na_2SO_4. Purification on a silica gel column, using a gradient of 0–5% MeOH in CH_2Cl_2 as eluent, gave *N*-(hydroxyacetyl)-*N′*-(4,4′-dimethoxytrityloxyacetyl)-3,4-dithia-1,6-hexanediamine (**30**) as a white foam. ^{1}H NMR (400 MHz; $CDCl_3$): 7.38–6.83 (13 H, m); 4.02 (2 H, s); 3.79 (6 H, s); 3.77 (2 H, s); 3.57 (4 H, m); 2.87 (2 H, t, *J* 6.1); 2.72 (2 H, t, *J* 6.1). Commercial 2′-deoxyadenosine phosphoramidite (Cruachem; 0.5 g, 0.58 mmol) and **30** (120 mg, 0.87 mmol) were dissolved in dry acetonitrile containing ^{1}*H*-tetrazole (3.9 ml, 0.45 M). The mixture was allowed to stir for 30 min at room temperature, after which it was treated with a mixture of iodine:THF:pyridine:water (0.4:90:0.4:9 w/v/v/v, 10 ml). Solvent was evaporated and the residue was dissolved in methylene chloride (20 ml) and extracted with aqueous sodium bisulfite (20 ml). The organic phase was concentrated and the residue was dissolved in methanolic ammonia, kept for 30 h at room temperature and then evaporated. The residue was dissolved in dichloromethane and washed with aqueous KH_2PO_4. The organic phase was concentrated and dissolved in 80% aqueous acetic acid. After 30 min, the solvent was evaporated off, the residue was dissolved in water and extracted with diethyl ether. The aqueous layer was concentrated and the product was purified on a semi-preparative reverse-phase HPLC column (Hypersil ODS, 5 µm, 10 × 250 mm; Shandon) using isocratic elution with 2.5% acetonitrile in water. $\lambda_{max}(H_2O)$ 259 nm, ^{1}H NMR (400 MHz; D_2O): 8.05 (1 H, s); 7.89 (1 H, s); 6.19 (1 H, dd, *J* 6.8 and 6.4); 4.79 (1 H, m); 4.21 (2 H, d, *J* 5.1); 4.15 (1 H, m); 3.86 (2 H, s); 3.66 (2 H, m); 3.34 (2 H, m); 3.25 (2 H, t, *J* 6.1); 2.64 (1 H, *J* 6.4 and 14.1). 2.60 (2 H, t, *J* 6.6); 2.53 (1H, t, *J* 6.6); 2.51 (1H, ddd, *J* 2.5, 6.8 and 14.1). ^{31}P NMR (D_2O): −1.79 p.p.m.

Oligonucleotides

Oligonucleotides reported here were assembled on an Applied Biosystems 392 DNA synthesizer using the solid supports and monomers described above, along with commercial phosphoramidites (Cruachem). Non-nucleosidic monomer **6** and psicothymidine monomers **3** and **13** were introduced as described previously (26,32). Delevulination with 0.5 M hydrazine hydrate in pyridine:acetic acid (4:1 v/v) was carried out for 10 min. Introduction of cystamine residues into assembled oligonucleotides was achieved (32) with 0.5 M cystamine (free base) in water over 6 h. Free mercapto groups were generated by treating the oligonucleotide containing the cystamine residues (14 OD in 1 ml water) with dithiothreitol (3 mg) and triethylamine (30 µl, 0.3 M in ethanol) for 3 h at room temperature. Products were isolated by reverse-phase HPLC and used immediately in the cross-linking experiments. Disulfide bond formation was achieved by diluting the solution of the dithiol (7 OD) obtained from reverse-phase HPLC with freshly degassed hot (90°C) potassium phosphate buffer (50 ml, 0.05 M, pH 7.85) and leaving the solutions to cool to room temperature overnight and then for an additional 36 h at room temperature. All oligonucleotides were purified and desalted as reported earlier in detail (29).

Electrophoresis

PAGE was carried out on 15% gels. The amounts of sample loaded were 0.1–0.2 OD. The oligonucleotide bands were visualized and photographed under UV light (254 nm) using the UV-shadowing technique.

Melting experiments

Melting experiments were performed on a λ 2 UV/VIS spectrophotometer equipped with a PTP-6 temperature programmer, comprising an electronic control unit and a Peltier cell holder (Perkin-Elmer). The temperature was increased at a rate of 1°C/min. The melting curves were recorded at 260 nm in a buffer containing 10 mM Tris–HCl, pH 7.0, 0.1 M NaCl, 10 mM $MgCl_2$. The concentration of pre-annealed oligonucleotides was 0.21 µM each. All melting curves were recorded three times and proved to be well reproducible. Uncertainty in T_m values was estimated as ±0.5°C.

RESULTS AND DISCUSSION

Synthesis

To synthesize the branched (**1**, **2**), circular (**9**) and looped oligonucleotides (**14–17**) we employed several monomeric blocks and solid supports. The structures of these compounds are given in Figure 2.

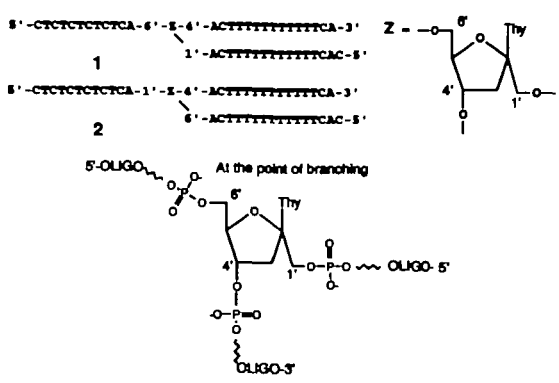
Branched oligonucleotides. We have recently demonstrated the applicability of 3′-deoxy-β-D-psicothymidine (25) in the preparation of short branched oligodeoxyribonucleotides (26). The same approach was now employed to prepare oligonucleotides **1** and **2**, i.e. psicothymidine branched structures containing two T-rich and one normal alternating sequences (Scheme 1). The branched oligomers were synthesized in moderate yield (Table 1) and characterized by denaturing PAGE (Fig. 1) and HPLC (Table 1). When **1** and **2** were digested with a mixture of phosphodiesterases I and II in the presence of alkaline phosphatase, two products in addition to the expected nucleosides appeared. These were tentatively assigned as the psicothymidine-containing trimer A(3′-p-6′)Z(1′-p-3)A and dimer Z(1′-p-3′)A. We have previously made similar observation on the stability of the phosphodiester bonds of psicothymidine against nucleases (26).

Table 1. Overall yield and ion exchange HPLC retention times of the oligonucleotides synthesized

Oligonucleotide	Overall yield (%)	Retention time (min)
1	29	26.2[a]
2	28	27.4[a]
7	45	14.4
8	–	15.1
9	29–33	16.8
18	27	16.3
19	–	17.7
14	19	18.1
20	28	16.3
21	–	17.7
15	22	18.1
22	41	16.4
23	–	17.8
16	34	18.4
24	44	16.4
25	–	17.8
17	38	18.3

Column, Synchropak AX-300, 4.6 × 250 mm, 6.5 μm; buffer A, 0.05 M KH_2PO_4 in 50% aqueous formamide, pH 5.60; buffer B, A + 0.6 M $(NH_4)_2SO_4$; flow rate 1 ml/min; linear gradient of 25–100% B in 30 min.
[a]Linear gradient of 10–70% B in 30 min.

Circular oligonucleotides. Glick *et al.* have shown that RNA and DNA hairpins bearing either a 3-(2-mercaptoethyl)uridine (27) or 5-(ω-mercaptoalkyl)-2′-deoxyuridine (28) unit at both the 5′- and 3′-termini undergo intramolecular disulfide bond formation upon oxidation. The circular 3′,5′-cross-linked oligomers obtained exhibit normal Watson–Crick base pairing and enhanced conformational stability compared with their linear counterparts (27). These observations were utilized in the present work. Linear precursor **7** of the circular oligonucleotide was prepared in 45% yield by the method that we have previously reported for the introduction of various functionalized tether groups at the 3′- and 5′-termini of oligomers (29–32). The approach is based on the use of solid supports and monomeric building blocks that contain an ester (29–31) or thioester (32) bond as part of their structure. Upon completion of the oligonucleotide synthesis this bond is cleaved with a primary amine, which brings the desired functionality. In the present work, cystamine was used to cleave



Scheme 1. Oligonucleotides **1** and **2** and the structure at the point of branching.

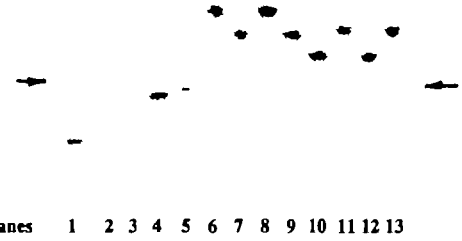


Figure 1. Photograph of 15% denaturing PAGE gel taken under UV light using the UV-shadowing technique. Lane 1, **26**; lane 2, **2**; lane 3, **1**; lane 4, **7**; lane 5, **9**; lane 6, **18**; lane 7, **14**; lane 8, **20**; lane 9, **15**; lane 10, **22**; lane 11, **16**; lane 12, **24**; lane 13, **17**. Arrows indicate the position of the xylene cyanol dye.

the oligomer from solid support **5** and simultaneously the 5′-terminal non-nucleoside unit related to **6** was derivatized. Accordingly, a masked mercapto function was introduced to both termini of the oligonucleotide (Scheme 2). Linear oligonucleotide conjugate **7** was purified by HPLC (29) and characterized by denaturing PAGE (Fig. 1). The disulfide bonds were then reduced with 1,4-dithio-D,L-threitol (DTT) in the presence of traces of triethylamine to obtain **8**. This was isolated by reverse-phase HPLC, heavily diluted with degassed hot potassium phosphate buffer and left to cool down to room temperature. After 48 h circular oligomer **9** was isolated by HPLC (29) in 65% yield. When the same cross-linking reaction was performed in the presence of an equimolar amount of $\mathbf{A_{11}}$, the yield of **9** was increased to 76% (Scheme 2). Presumably the formation of a triple helical structure between **8** and $\mathbf{A_{11}}$ slightly enhanced cross-linking to circular disulfide **9**. Purity of the circular oligonucleotide **9** was checked by denaturing PAGE and HPLC (Table 1). Enzymatic digestion with a mixture of phosphodiesterase I and alkaline phosphatase gave, when analyzed by reverse-phase HPLC, the expected ratio of natural 2′-deoxynucleosides and a modified nucleotide. The modified nucleotide was shown to be **10** by spiking with an authentic sample. This experiment corroborates the structure of **9**, since only the circular oligonucleotide (having a typical migration rate on PAGE) can give rise to **10** upon enzymatic degradation. Consistent with the assumed structure of disulfide **9**, it could be readily converted to **8** by treatment with DTT. Co-injections of the samples withdrawn from the reaction mixtures of DTT with either linear bis-cystamine oligonucleotide **7** or circular disulfide **9** gave single peaks in both ion exchange and reverse-phase HPLC. Dithiol **8**, in turn, could be easily transformed to a bis(pyridyldithio) derivative **11** by treating with 2,2′-dipyridyl disulfide (Scheme 3).

Looped oligonucleotides. The synthetic procedure for the preparation of looped oligomers was essentially a combination of the methods described above. The main difference was the solid support used. Since support **5** proved to be extremely sensitive towards nitrogen nucleophiles and since hydrazinium acetate had to be utilized for cleavage of the levulinyl protection group from the 1′-*O* or 6′-*O* moieties of deoxypsicothymidine, **5** obviously could not be employed. In this particular case the solid support had to fulfil two requirements, to contain a disulfide function needed for the subsequent cross-linking and to stand treatment with hydrazinium acetate. Therefore solid support **12** (33) was

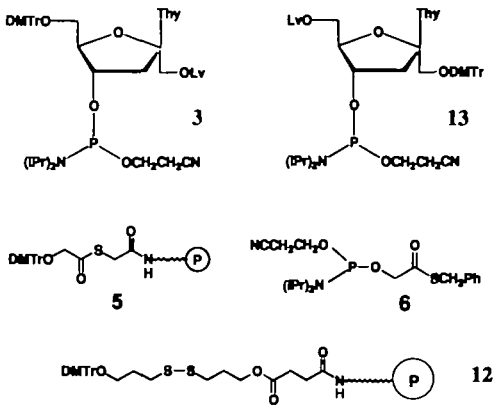
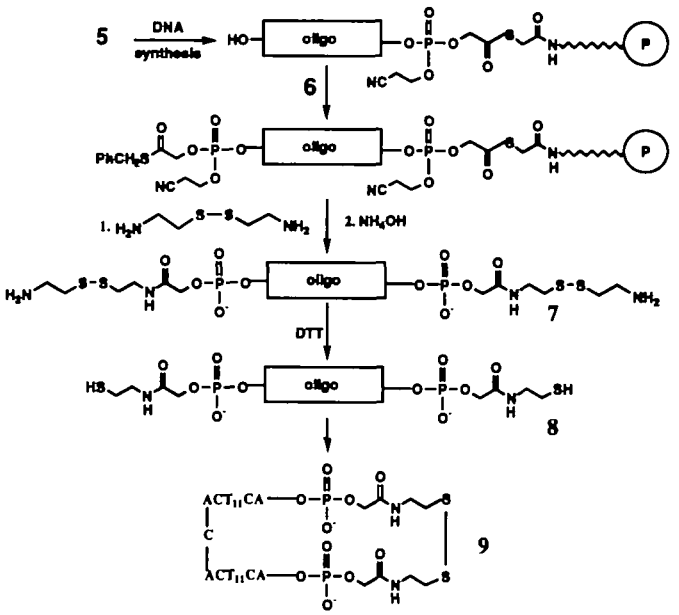
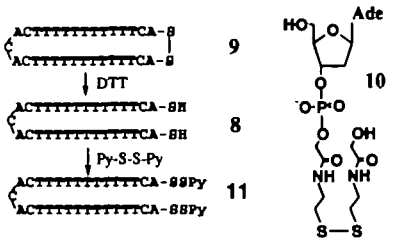


Figure 2. Structures of monomeric blocks 3, 6 and 13 and solid supports 5 and 12.



Scheme 2. Synthetic route for the preparation of the circular oligonucleotide 9.

chosen and two different deoxypsicothymidine phosphoroamidites 3 and 13 (26) were used to create the desired branching points. Looped oligonucleotide **14**, a structural analog of branched oligomer **1**, but cross-linked with a disulfide bond, was prepared as depicted in Scheme 4. Thus the 'normal' strand was assembled on **12** using monomer **3** for subsequent branching and 5′-*O*-benzoylthymidine phosphoroamidite for chain termination. The levulinyl group was then removed and the 1′-*OH* of **Z** was used to assemble the additional branch. Non-nucleosidic thioester monomer **6** was employed to complete the machine-assisted synthesis. The solid support carrying the branched oligonucleotide was then treated with aqueous cystamine to introduce the 5′-disulfide function and then with ammonia to result in complete deprotection. Branched oligonucleotide **18** possessing disulfide tethers was finally isolated in 27% yield, reduced to dithiol **19** and cross-linked to give the desired looped oligonucleotide **14** (Scheme 4). After cross-linking, **14** was isolated by HPLC (29). Looped oligonucleotide **15** (Scheme 4), the analog of **2** cross-



Scheme 3. Structure of the modified nucleotide **10**, obtained upon enzymatic digestion of the circular oligonucleotide **9** and transformations of circular oligonucleotide **9**.

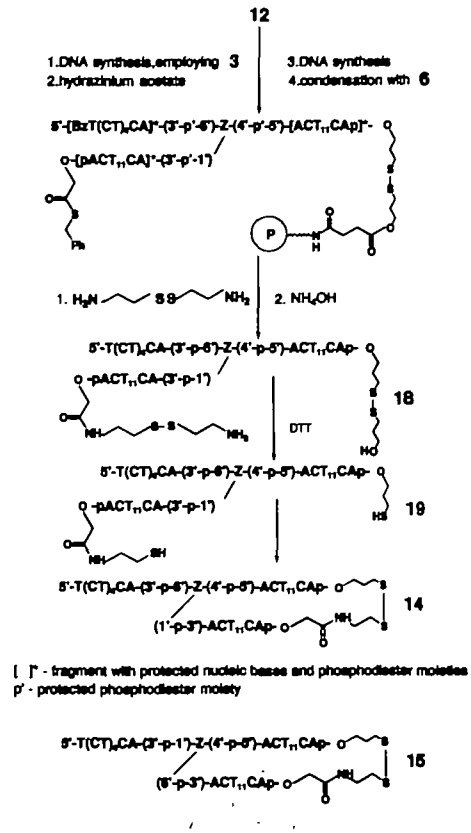
linked with a disulfide bond, was prepared analogously. The only difference was the utilization of monomer **13** for branching (Fig. 2). A slightly modified strategy was applied to prepare looped oligonucleotides **16** and **17**, in which the psicothymidine unit participates in the cross-linking rather than branching. Scheme 5 shows the route to obtain **16**. Accordingly, the entire oligonucleotide chain was assembled on support **12**, introducing **Z** as building block **3** at an appropriate step and terminating the chain with the 5′-*O*-benzoylated thymidine monomer. The 1′-*O*-levulinyl group was then removed and non-nucleosidic monomer **6** was introduced at the final step. The subsequent treatments were similar to those described above for **14** and **15**, resulting in cross-linking of the 3′-terminus to 1′-*O* of **Z**. Looped **17** (Scheme 5) was prepared analogously. The only difference was that monomer **13** was used instead of **3** and therefore the 3′-terminus was cross-linked to 6′-*O* of **Z**. The cyclizations of **14–17** were repeated several times with 70–86% yield, which proved to be independent of the structure of the oligonucleotide precursor or the site of cross-linking. Looped disulfides (**14–17**) were readily converted to dithiols (**19**, **21**, **23** and **25**) with DTT. Co-injections of the samples withdrawn from the reaction mixtures of DTT with either bisdisulfides (**18**, **20**, **22** and **24**) or oligonucleotides **14–17** gave single peaks in ion exchange HPLC.

HPLC

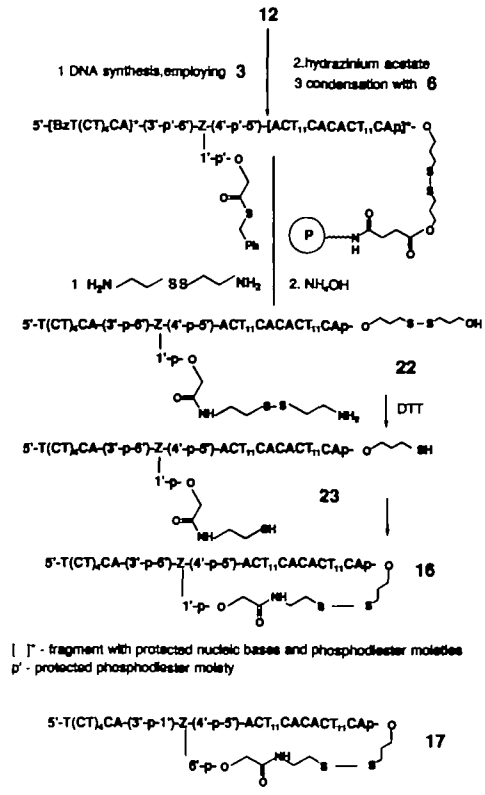
All the oligonucleotides were shown to be homogeneous by both ion exchange and reverse-phase HPLC. The ion exchange HPLC experiments (Table 1) demonstrated that the retention times of the circular (**9**) and looped (**14–17**) oligonucleotides were longer than those of their bis-disulfide precursors (**7**, **18**, **20**, **22** and **24**). Dithiols (**8**, **19**, **21**, **23** and **25**) were retained more efficiently than their bis-disulfide precursors, but less efficiently than the circular or looped oligonucleotides. This observation was utilized in monitoring the cross-linking reactions.

Gel electrophoresis

The 15% denaturing PAGE (Fig. 1) showed reasonable purity for all the oligonucleotides synthesized. It also demonstrated an interesting behavior of modified oligonucleotides which may be regarded as a characteristic feature. Kool *et al.* (13) have noted that the migration rate of circular oligonucleotides is about 0.9 times that of their linear precursors. Our circular oligonucleotide **9**, cross-linked with a disulfide bond, displayed a similar behavior. The branched oligonucleotides **1** and **2**, in turn, migrate unusually slowly. This finding may explain why looped oligonucleotides **16** and **17** migrate more slowly than their linear



Scheme 4. Synthetic route for the preparation of the looped oligonucleotide **14** and structure of the looped oligonucleotide **15**.



Scheme 5. Synthetic route for the preparation of the looped oligonucleotide **16** and structure of the looped oligonucleotide **17**.

disulfide precursors **22** and **24**, while looped oligonucleotides **14** and **15** migrate faster than their branched disulfide precursors, **18** and **20**.

Melting experiments

To elucidate the ability of the branched (**1**, **2**), circular (**9**) and looped oligonucleotides (**14–17**) to hybridize with the complementary sequence, the melting curves for dissociation of the complexes of these oligomers with 5′-A_{11}GTAT$(GA)_5$-3′ (**26**) were determined. The structures of complexes studied are given in Figure 3 and their T_m values in Table 2.

Circular oligonucleotides. The melting curves for the following adducts were measured: initial experiments were performed with the complexes of **9**/A_{11}, **27**/A_{11}, T_{11}/A_{11} and 2 T_{11}/A_{11} (Fig. 4). One can see that while the **27**/A_{11} complex melts at a considerably higher temperature than both T_{11}/A_{11} and 2 T_{11} /A_{11} (47.6°C, 33.2°C and 35°C, respectively, Table 2), consistent with the formation of a bimolecular triple helix, the cross-linking in oligonucleotide **9** additionally increases the melting temperature by 5°C (52.7°C). This observation agrees with the data reported by Kool *et al.* (10) for circular oligonucleotides containing only natural phosphodiester bonds and it demonstrates that cross-linking via a disulfide bond does not dramatically affect the triple helical structure. The melting curve for the complex of **9** with **26** is shown in Figure 4. This complex melts at 52°C, while the complex **9**/A_{11} melts only ~1°C higher, also in accord with the previous data (13).

Table 2. Melting points of the oligonucleotide complexes

Complex	T_m (°C)
T_{11}/A_{11}	33.2
2T_{11}/A_{11}	35.0
27/A_{11}	47.6
9/A_{11}	57.2
1/**26**	28.1, 1st transition
	44.9, 2nd transition
28/**26**	59.1
2/**26**	32.2, 1st transition
	46.0, 2nd transition
29/**26**	59.1
9/**26**	52.0
14/**26**	60.5
15/**26**	60.4
16/**26**	59.4
17/**26**	59.0

Branched oligonucleotides. **1** and **2** display two sites for binding of the single-stranded DNA fragment **26**. With **1**, the $(CT)_5$ site (linked to 6′-*O* of **Z**) may be anti-parallel to the $(GA)_5$ sequence of **26** and hence form a normal Watson–Crick double helix. The other site consists of one T_{11} region (linked to 4′-*O* of **Z**), capable of forming an anti-parallel Watson–Crick type complex with the A_{11} fragment of **26**, and another T_{11} region (linked to 1′-*O* of **Z**), which may form a parallel Hoogsteen type complex with the same

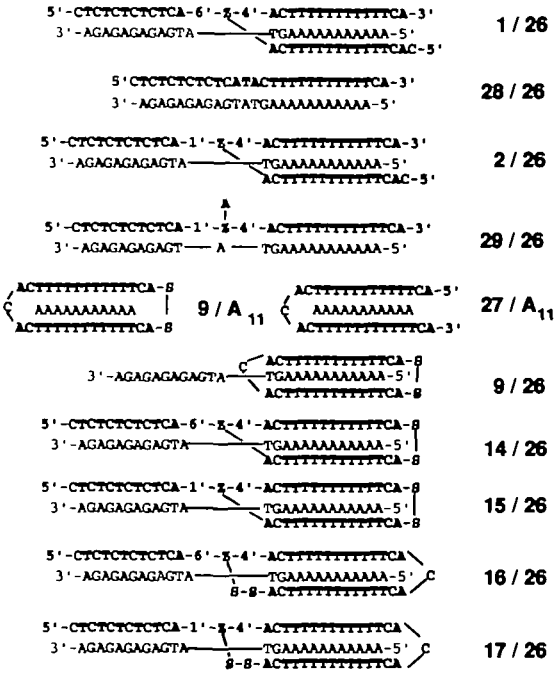


Figure 3. Structures of complexes studied.

A_{11} fragment. Accordingly, the branched oligonucleotide may be expected to form a hybrid complex with **26**, featuring both double and triple helices at the same time. Figure 5 shows the melting curve for this complex (**1/26**) and that for the normal duplex formed by 5′-C(TC)$_5$ATACT$_{11}$CA-3′ (**28**) and **26**. Comparison of these curves reveals that the introduction of an 'additional' branch via 1′-*O* of **Z** in **1**, supposed to provide additional Hoogsteen type base pairing with **26**, leads to rather weak triplex formation. The first transition at 28.1 °C is presumably due to melting of this weak triple helical structure. It is also worth noting that the 'additional' branch considerably destabilizes the Watson–Crick double helix, which now melts at a 14 °C lower temperature than the normal control duplex (second transition at 44.9 °C for **1/26** compared with T_m = 59.2 °C for **28/26**). Branched oligonucleotide **2** has to form a Watson–Crick duplex with the alternating sequence linked via a 3′→1′ phosphodiester bond. In other words, the 'additional' branch is now involved only in Watson–Crick base pairing, while the branch, attached to 6′-*O* of **Z**, containing the T_{11} region and possessing a free 5′-terminus is involved in triple helix formation via Hoogsteen-type base pairing. The melting curve for complex **2/26**, as well as that for the complex of linear reference oligonucleotide **29** with **26**, are also given in Figure 5. The former curve (**2/26**) displays a distinct transition at 32.2 °C, due to melting of the triplex, and a second transition at 46 °C, due to melting of the Watson–Crick-type complex. It is important to note that in this case also the presence of a branch responsible for Hoogsteen base pairing again destabilizes the Watson–Crick complementation. It appears clear that destabilization of the Watson–Crick-type complexes results from the unnatural structure at the site of branching. This structure does not favor triplex formation either. Comparison of the melting curves of **1/26** and **2/26** (Fig. 5) with those of **28/26** and **29/26** reveals that the double helical structures of the former melt at a 10–15 °C lower temperature than the double

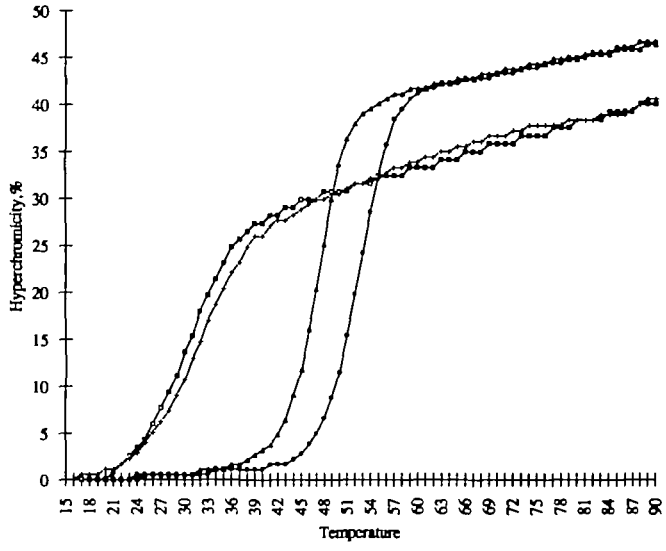


Figure 4. Melting curves for complexes of **9/A$_{11}$** (-○-), **27/A$_{11}$** (-Δ-), **T$_{11}$/ A$_{11}$** (-□-) and **2 T$_{11}$/oligo-A$_{11}$** (-+-).

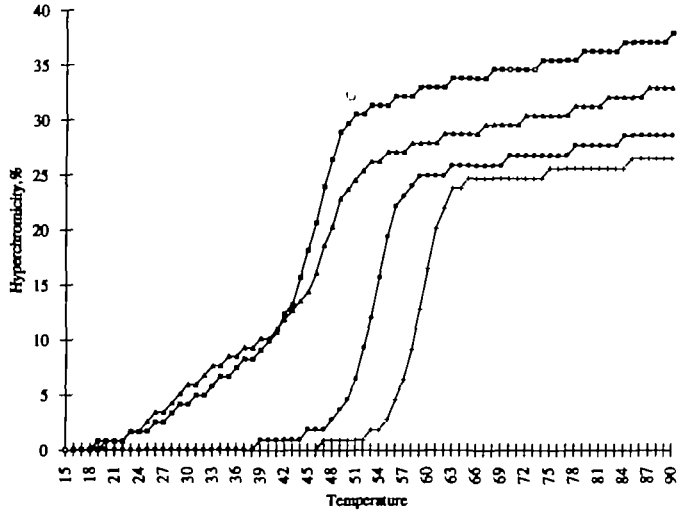


Figure 5. Melting curves for the complexes of branched oligonucleotides **1** (-□-) and **2** (-Δ-) with **26** and control oligonucleotides **28** (-+-) and **29** (-○-) with **26**.

helices of related linear oligomers (T_m of the second transition in curves for branched oligonucleotides compared with the T_m of **28/26** and **29/26**). Comparison with the melting curve of **27/A$_{11}$** (Fig. 4) in turn shows that the triple helices of **1/26** and **2/26** melt at a 15–20 °C lower temperature than **27/A$_{11}$** (T_m of the first transitions in curves for branched oligonucleotides compared with T_m = 47.6 °C for the linear oligonucleotide **27/A$_{11}$** complex in Figure 4). In summary, branched oligonucleotides **1** and **2** appear to form both double and triple helices less efficiently than their linear counterparts.

Looped oligonucleotides. In striking contrast to the branched oligonucleotides, the looped oligomers form stable hybrid complexes. Figure 6 shows the melting curves for the dissociation of complexes of looped oligonucleotides **14** and **15** with the single-stranded **26**. The melting curve for the complex of circular oligonucleotide **9** with linear **26** is included for comparison. These

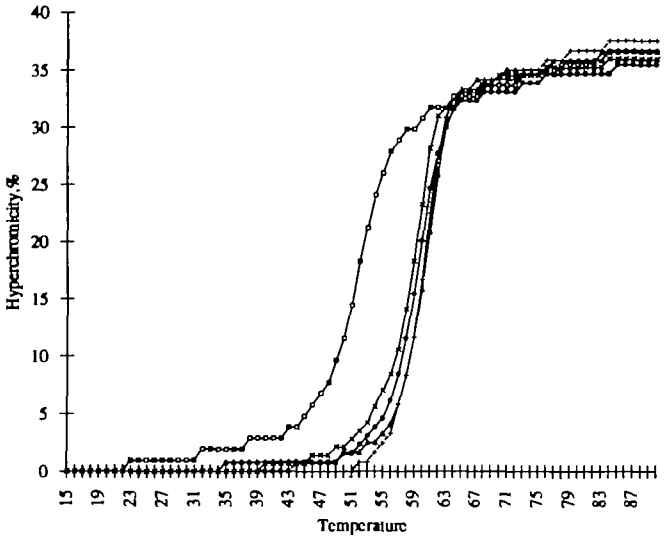


Figure 6. Melting curves for dissociation of complexes of looped oligonucleotides **14** (-Δ-), **15** (-+-), **16** (-○-), **17** (-×-) and the circular oligonucleotide **9** (-□-) with a single-stranded complement **26**.

data allow the following conclusions. Cross-linking of the branched structures to give looped oligonucleotides **14** and **15** indeed results in a considerable stabilization. Only one transition in the melting curves is observed. This fact and a high melting temperature (about 60°C, Table 2) may be taken as an indication of co-operativity in melting of the hybrid (double/triple helix) complexes; stabilization of the triple helical moiety of the hybrid complex, due to loop formation, stabilizes the hybrid structure as a whole. Comparison with the melting curve of a normal Watson–Crick double helix formed between linear **28** and its complement **26** (Fig 5; melting point at 59.1°C, Table 2) suggests that the site of branching in looped oligonucleotides **14** and **15** still somewhat destabilizes the hybrid complex. Nevertheless, its influence appears to be of minor importance compared with the branched oligonucleotides. The melting curves of the complexes of looped oligonucleotides **16** and **17** with single-stranded complement **26** (Fig. 6) demonstrate that the melting curves of all looped oligonucleotides are very similar and the melting points are almost equal (Table 2). Obviously, the position of the cross-link does not significantly affect the stability of the hybrid complexes. This observation may be rather important from the synthetic point of view, since the less branching one performs during oligonucleotide synthesis, the fewer modifications in the target sequence one can expect and the easier the isolation procedures would be.

CONCLUSION

The present work demonstrates the feasibility of a new strategy for DNA recognition, based on high selectivity and stability of bimolecular hybrid complexes. The looped oligonucleotides described undergo selective complexation with purine-rich regions and adjacent polynucleotide sequences at the same time. This feature may be very useful for the design of specific antisense and antigene oligonucleotides.

ACKNOWLEDGEMENTS

Financial support from the Research Council for Natural Sciences, the Academy of Finland, is gratefully acknowledged. The authors wish to thank Dr E. Minyat of Engelhardt Institute of Molecular Biology, Moscow, for valuable discussions during the early stages of this work.

REFERENCES

1 English,U. and Gauss,D. (1991) *Angew. Chem. Internatl Edn English*, **30**, 613–629.
2 Helene,C. and Toulme,J.J. (1990) *Biochem. Biophys. Acta*, **1049**, 99–125.
3 Thuong,N.T. and Helene,C. (1993) *Angew. Chem. Internatl Edn English*, **32**, 666–690.
4 Uhlmann,E. and Peymann,A. (1990) *Chem. Rev.*, **90**, 543–584.
5 Milligan,J.F., Matteucci,M.D. and Martin,J.C (1993) *J. Med. Chem.*, **36**, 1923–1937.
6 Xodo,L.E., Manzini,G. and Quadrifoglio,F. (1990) *Nucleic Acids Res.*, **18**, 3557–3564.
7 Giovannangeli,C., Montenay-Garestier,T., Rougee,M., Chassignol,M., Thuong,N.T. and Helene,C. (1991) *J. Am. Chem. Soc.*, **113**, 7775–7777.
8 Giovannangeli,C., Thuong,N.T. and Helene,C. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 10013–10017.
9 Kool,E.T. (1991) *J. Am. Chem. Soc.*, **113**, 6265–6266.
10 Prakash,G. and Kool,E.T. (1991) *J. Chem. Soc. Chem. Commun.*, 1161–1163.
11 von Squire,R. and Kool,E.T. (1992) *Angew. Chem.*, **104**, 1686–1689.
12 Prakash,G. and Kool,E.T. (1992) *J. Am. Chem. Soc.*, **114**, 3523–3527.
13 D'Souza,D.J. and Kool,E.T. (1992) *J. Biomol. Struct. Dynam.*, **10**, 141–152.
14 Perkins,T.A., Goodman,J.L. and Kool,E.T. (1993) *J. Chem. Soc. Chem. Commun.*, 215–216.
15 Rubin,E., McKee,T.L. and Kool,E.T. (1993) *J. Am. Chem. Soc.*, **115**, 360–361.
16 D'Souza,D.J. and Kool,E.T. (1994) *Bioorg. Med. Chem. Lett.*, **4**, 965–970.
17 Wang,S., Booher,M.A. and Kool,E.T. (1994) *Biochemistry*, **33**, 4639–4644.
18 Booher,M.A., Wang,S. and Kool,E.T. (1994) *Biochemistry*, **33**, 4645–4651.
19 Vasseur,J.-J., Peoch,D., Rayner,B. and Imbach,J.-L. (1991) *Nucleosides Nucleotides*, **19**, 107–117.
20 Wang,S. and Kool,E.T. (1994) *Nucleic Acids Res.*, **22**, 2326–2333.
21 Ashley,G.W. and Kushlan,D.M. (1991) *Biochemistry*, **30**, 2927–2933.
22 Clusel,C., Ugarte,E., Enjolras,N., Vasseur,M. and Blumenfeld,M. (1993) *Nucleic Acids Res.*, **21**, 3405–3411.
23 Moelling,K. (1994) PCT International Application WO 9407367 A1 940414.
24 Volkmann,S., Dannul,J. and Moelling,K. (1993) *Biochimie*, **75**, 71–78.
25 Azhayev,A., Guzaev,A., Hovinen,J., Mattinen,J., Sillanpää,R. and Lönnberg,H. (1994) *Synthesis*, 396–400.
26 Azhayev,A., Guzaev,A., Hovinen,J., Azhayeva,E. and Lönnberg,H. (1993) *Tetrahedron Lett.*, **34**, 6435–6438.
27 Goodwin,J.T. and Glick,G.D. (1994) *Tetrahedron Lett.*, **35**, 1647–1650.
28 Goodwin,J.T. and Glick,G.D. (1993) *Tetrahedron Lett.*, **34**, 5549–5552.
29 Hovinen,J., Guzaev,A., Azhayev,A. and Lönnberg,H. (1994) *Tetrahedron*, **50**, 7203–7218.
30 Hovinen,J., Guzaev,A., Azhayev,A. and Lönnberg,H. (1993) *Tetrahedron Lett.*, **34**, 8169–8172.
31 Hovinen,J., Guzaev,A., Azhayev,A. and Lönnberg,H. (1994) *J. Chem. Soc. Perkin Trans.*, **1**, 2745–2749.
32 Hovinen,J., Guzaev,A., Azhayeva,E., Azhayev,A. and Lönnberg,H. (1995) *J. Org. Chem.*, in press.
33 Guzaev,A.P. (1991) *Nucleic Acids Res. Symp. Ser.*, **24**, 236.