The Utility of Capped Duplex DNA (σ-DNA) in Triplex Interaction Studies

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Two optimally capped duplex DNA molecules (σ-DNA; see **1** and **4**) were synthesized and their utility demonstrated for triplex investigations with their corresponding homopyrimidine DNA and RNA single strands in the D·(D·D) and R·(D·D) *Hoogsteen* mode. Furthermore, it was established that σ-DNA is an ideal tool to study the pH dependency of triplex formation.

1. Introduction. – Double-stranded DNA is able to align a third strand of DNA or RNA in its major groove to form triple-helix motifs [1][2]. Although restricted to homopurine or homopyrimidine sequences, triple-helix approaches are of interest for the potential control of transcription processes by the *anti* gene approach [3][4]. The triple-helix motif can either be a Pyr·(Pur·Pyr) motif or a Pur·(Pur·Pyr) motif. In the former situation which is more prominent, the pyrimidine strand as the third strand is bound in the major groove of the double-stranded DNA *via Hoogsteen* H-bonds (*Fig. 1*).

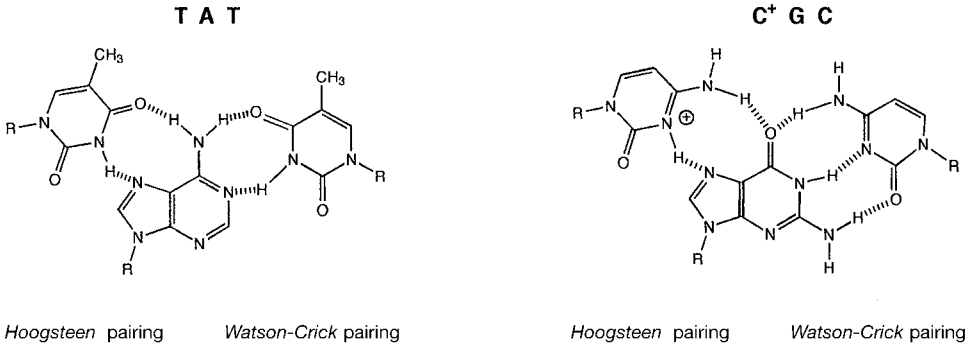

Fig. 1. Hoogsteen *pairing in the Pyr·(Pur·Pyr) mode*

Studies involving double-stranded DNA fragments as targets are limited by the low thermal stability of such duplexes, especially under physiological conditions. Therefore, many investigations have dealt with the design of non-nucleotidylic linker molecules in hairpin structures in order to increase the duplex stability (see, *e.g.*, [5] and ref. cit. therein).

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Recently, we have reported on short double-stranded DNA duplexes in which the terminal bases of the two strands were connected at both ends by non-nucleotidylic linker molecules. This gave a more stable duplex as compared to the corresponding hairpin DNA [6]. We refer to this new structural type as 'sausage'-DNA (σ-DNA) to distinguish it from 'dumbbell' DNA.

2. Results and Discussion. – We aim to demonstrate the utility of σ-DNA to study triplex interactions. This is especially attractive since the *Watson-Crick* melting of σ-DNA is so high that no interference with the *Hoogsteen* melting of the third strand can be expected. Therefore, clear-cut results during investigation of the triplex melting can be anticipated.

The sequences **1**–**11** used in the present study are shown in *Fig. 2*. The homopyrimidine sequences were selected in such a way that they consist mostly of alternating T-C pairs. This avoids complications which could arise from longer C stretches due to the formation of i-DNA [7][8] motifs or hemiprotonated $CH^+ \cdot C$ base-pair duplexes [9][10]. The chosen homopurine sequence is part of the gag initiation site of HIV.

σ-DNA **1** and σ-DNA **4** (*Fig. 2*) differ with respect to the linker unit (*Fig. 3*). Linker 1 (L_1) of **1** consists of two propane-1,3-diol units interspaced by three phosphate groups. According to molecular-modeling studies, this linker approximately spans the distance of a G·C or a A·T pair at the end of a duplex. The linker can be easily incorporated as a building block during the synthesis of the linear precursor of the cyclized, final product [6].

In **4**, we have inserted linker 2 (L_2), a hexaethylene-glycol spacer containing two phosphate groups at its termini. This linker provides added flexibility as compared to linker 1 and has also been used in hairpin oligomers [11]. It can be introduced in synthesis as the phosphoramidite building block of hexaethylene glycol.

The synthesis of the σ-DNA molecules **1** and **4** was performed *via* chemical ligation of the corresponding 3′-phosphorylated unprotected DNA fragments employing *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC) as outlined in *Fig. 4*. The desired alignment, resulting in the close proximity between the phosphate group and the OH function to form the phosphodiester bond, is achieved *via* backfolding of both ends of the linear precursors [6]. A very high conversion of the linear precursor to the circular σ-DNA was achieved (*Fig. 4*).

The corresponding hairpin sequences **2** and **3** equipped with L_1, and the backfolding duplexes **5** and **6** equipped with L_2, were also synthesized for comparison. The target sequences for triplex binding are represented by the homopyrimidine oligodeoxynucleotide **7** and the corresponding ribo sequence **8**. In addition, we have prepared sequences **9**–**11** to estimate the values of duplex meltings.

In a first set of experiments, the melting temperatures were determined at two different pH values, *i.e.*, at pH 5.5 and 7.0. The buffer systems NaOAc/AcOH and Na_2PIPES (= piperazine-1,4-diethanesulfonic acid sodium salt) were chosen as their pH values are temperature-independent. Results are summarized in *Tables 1* and *2*. The melting temperatures for the duplexes composed of the single strands **7** and **11** revealed a pH dependency. Furthermore, the melting temperature at pH 7 was higher than that at pH 5.5 (50.3 *vs.* 39.2°). The corresponding heteroduplex resulting from the

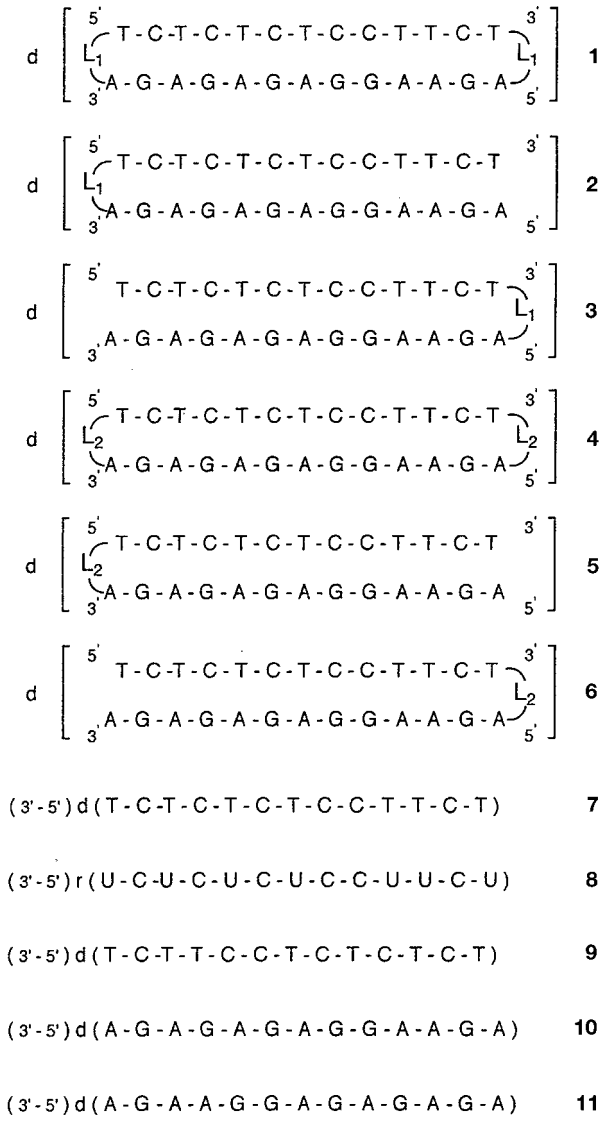


Fig. 2. *DNA and RNA sequences used in the melting experiments*

ribosequence **8** and the oligodeoxynucleotide sequence **11** showed a lower melting temperature than the corresponding DNA duplex. However, they showed the same trend with respect to pH dependency. Compared to these duplexes, all the hairpin sequences showed an increased melting temperature which can be attributed to entropic reasons. In the hairpin sequences **2** and **3**, duplex melting showed a dependency on the side at which the linker was attached, and the effect was independent of the pH value. In the hairpins **5** and **6** such a behavior was not observed.

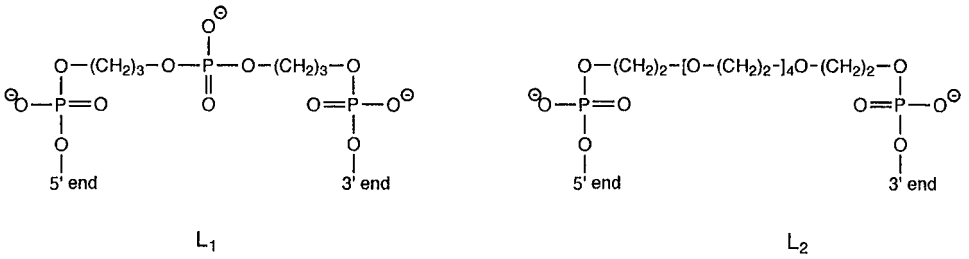
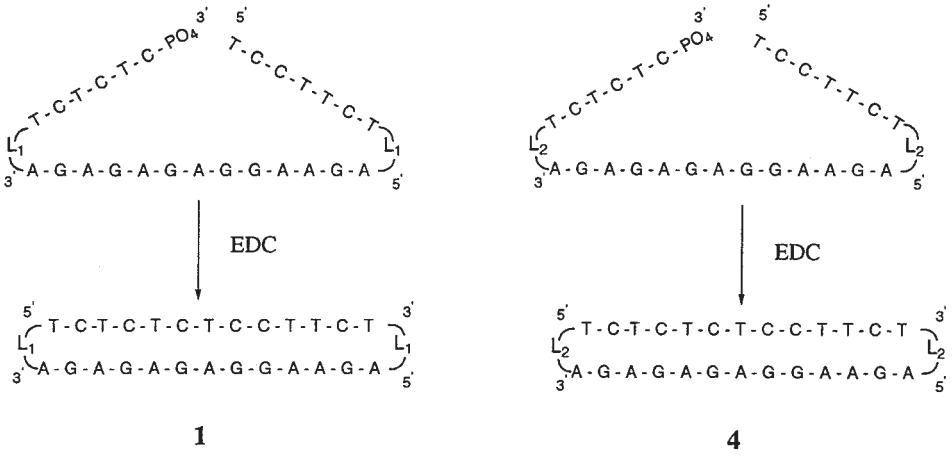


Fig. 3. *Structures of the linker molecules L_1 and L_2*



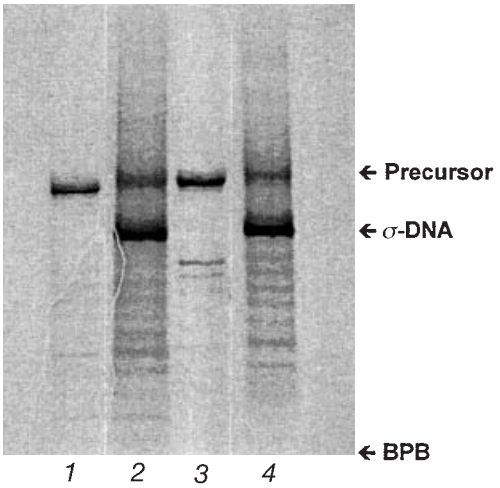


Fig. 4. *Cyclization strategy for the synthesis of the σ-DNA molecules* **1** *and* **4** *and PAGE* (polyacrylamide-gel electrophoresis) *of the crude reaction mixtures. Lane 1:* crude linear precursor for **1**; *Lane 2:* cyclization reaction leading to **1**; *Lane 3:* crude linear precursor for **4**; *Lane 4:* cyclization reaction leading to **4**.

Table 1. *Results of Duplex and Triplex Melting Experiments at pH 5.5*[a])

σ-DNA or hairpin	Duplex	**7** as 3rd strand		Melting of **7**·**11**[b])	Gain[c])	**8** as 3rd strand		Melting of **8**·**11**[b])	Gain[c])
		Melt 1	*Melt 2*			*Melt 1*	*Melt 2*		
1	82.4	42.2	82.2	39.2	3	56.0	82.3	30.7	25
2	66.0	43.5	66.3	39.2	4	58.3	66.6	30.7	28
3	70.4	43.9	70.2	39.2	5	56.5	70.2	30.7	26
4	85.1	46.8	85.4	39.2	8	58.9	85.4	30.7	28
5	70.5	46.4	70.6	39.2	7	57.7	70.6	30.7	27
6	71.7	46.2	71.5	39.2	7	59.8	71.5	30.7	29

[a]) Conditions: 1 μmol of DNA (RNA), 100 mM NaOAc/AcOH (pH 5.5), 1 mM EDTA; all melting values in °C. [b]) *Watson-Crick* melting of **7**·**11** or **8**·**11**, resp. [c]) Gain = gain of *Hoogsteen* melting with respect to the corresponding duplex melting.

Table 2. *Results of Duplex and Triplex Melting Experiments at pH 7*[a])

σ-DNA or hairpin	Duplex	**7** as 3rd strand		Melting of **7**·**11**[b])	**8** as 3rd Strand		Melting of **7**·**8**[b])
		Melt 1	*Melt 2*		*Melt 1*	*Melt 2*	
1	82.7	–	82.5	50.3	–	82.5	44.1
2	72.9	–	73.4	50.3	–	75.9	44.1
3	74.7	–	74.8	50.3	–	74.8	44.1
4	85.6	–	85.6	50.3	–	85.9	44.1
5	75.9	–	75.9	50.3	–	75.9	44.1
6	76.2	–	76.2	50.3	–	76.3	44.1

[a]) Conditions: 1 μmol of DNA (RNA), 10 mM Na_2PIPES (pH 7.0), 1 mM EDTA; all melting values in °C. [b]) *Watson-Crick* melting of **7**·**11** or **8**·**11**, resp.

As to be expected, the melting temperature of the σ-DNA molecules **1** and **4** was higher at both pH values as compared to the corresponding hairpin sequences. A slightly higher melting point was found for σ-DNA **4** as compared to σ-DNA **1**. As the DNA sequence for both compounds is the same, this difference must be attributed to the presence of the different linkers. Although optimal by molecular modeling, L_1 is probably too short to allow for optimal base pairing of the adjacent A·T pair. It is known from X-ray studies that the cross-strand distance of two phosphates in opposite strands in B-DNA varies between 16.7 and 19.1 Å. L_1 spans a maximal distance of 15.6 Å. The lack of optimal base pairing of the base pair adjacent to the linker has recently also been confirmed by NMR studies of a hairpin sequence carrying L_1 [12]. L_2 spans a distance of 18.5 Å, and this distance is almost ideal to bridge terminal base pairs without causing conformational strains in B-DNA. L_2 is still short enough to restrict effectively the conformational flexibility of the two connected strands [12]. Nevertheless, both σ-DNA molecules **1** and **4**, involving only 13 base pairs, showed melting temperatures above 80° and are, therefore, ideal tools to study triplex interactions since the *Watson-Crick* melting does not interfere with the *Hoogsteen* melting of the third strand.

Unlike for hairpin sequences, only slightly higher melting points were observed for **1** and **4** at pH 7 compared to pH 5.5. Hairpin sequences appear to be more influenced by different pH values or salt conditions. With respect to triplex melting, the *Hoogsteen*

binding requires the protonation of C (see *Fig. 1*). At pH 7.0 this cannot be achieved and, therefore, no triplex melt was observed at this pH (*Table 2*). At pH 5.5, there was no difference for the melting temperature of the *Hoogsteen* pairing of the oligodeoxynucleotide **7**, irrespective if the σ-DNA molecules or the corresponding hairpin sequences provided the major groove for triplex binding. Generally, in the case of sequences **4**–**6** with **7** as the third strand, melting occurred at *ca.* 3° higher as compared to the sequences **1**–**3** with the same third strand. This indicates that L_2 allows for an optimal triplex arrangement also involving the base pair adjacent to the linker whereas this may be hampered in the case of linker L_1.

With the oligoribonucleotide **8** as the third strand, *Hoogsteen* melting was generally higher as compared to the corresponding oligodeoxyribonucleotide **7**. This can be explained by the higher stability of $R\cdot(D\cdot D)$ triplexes as compared to $D\cdot(D\cdot D)$ triplexes [13][14]. As in previous experiments, the same triplex melting was obtained regardless of whether the hairpin sequences or the corresponding σ-DNA were employed. In this respect, the σ-DNA offered no advantage as compared to hairpin structures. Representative melting curves of the σ-DNA molecules **1** and **4** with the corresponding homopyrimidine sequence are shown in *Figs. 5* and *6*. Triplex melting at pH 5.5 with σ-DNA and the deoxyoligomer **7** was several degrees higher than the melting of the duplex composed of the corresponding single strands **7** and **11**. This difference was even more pronounced when the ribo sequence **8** was used as the third strand (see *Table 1*).

In a second set of experiments, we found that σ-DNA can also be used to study the pH dependency of triplex formation. As already indicated, triplex formation requires protonation of cytosines in the third strand to achieve *Hoogsteen* binding. The p*K* value of cytosine is 4.6 but is shifted to higher values whenever cytosine is involved in *Hoogsteen* binding. Thus, protonated cytosine in the third strand of a triplex was observed even at neutral pH [15]. The pH dependency of triplex formation when using

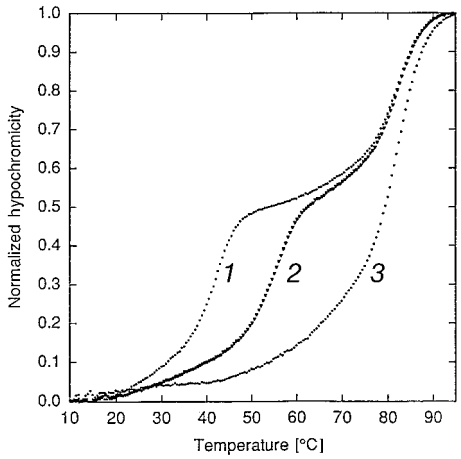


Fig. 5. *Melting-point experiments with σ-DNA* **1**. *Curve 1:* with DNA **7** as third strand; *Curve 2:* with RNA **8** as third strand; *Curve 3:* melting of σ-DNA **1**. Conditions: 1 μM DNA (RNA); 100 mM NaOAc/AcOH (pH 5.5); 1 mM EDTA.

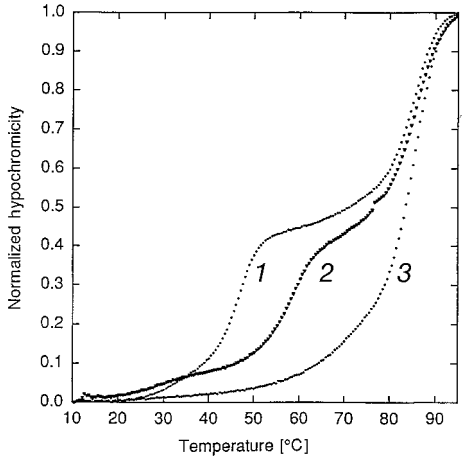


Fig. 6. *Melting-point experiments with σ-DNA* **4**. *Curve 1:* with DNA **7** as third strand; *Curve 2:* with RNA **8** as third strand; *Curve 3:* melting of σ-DNA **4**. Conditions: 1 μM DNA (RNA); 100 mM NaOAc/AcOH (pH 5.5); 1 mM EDTA.

σ-DNA is shown in *Fig. 7* where σ-DNA **4** and the homopyrimidine DNA sequence **7** were used for the evaluation. At pH 5.0, the *Hoogsteen* melting was 54° and decreased slowly with increasing pH. No *Hoogsteen* melting could be detected at pH 7. At pH values where triplex formation is obtained, a hysteresis was observed indicating the slow formation of the triplex. Triplex stability was higher with the homopyrimidine RNA sequence **8** as the *Hoogsteen* strand at all investigated pH values when compared to DNA as the third strand. However, this also decreased with increasing pH (*Fig. 8*). Surprisingly, the melting temperature for the third strand at pH 7 was still 17°. This suggests that σ-DNA may be useful in antisense approaches for binding of RNA at neutral pH.

3. Conclusion and Outlook. – Short DNA duplexes can be stabilized by incorporating non-nucleotidylic linker molecules at both ends of the duplex [6]. Such σ-DNA molecules can be efficiently prepared by incorporating the linker units in standard solid-phase DNA synthesis protocols to yield linear precursor molecules which can be further cyclized by chemical ligations. Thus, we synthesized two σ-DNAs in which the same DNA duplex was stabilized by two different linker molecules. The melting point data showed that linker 2 (L_2) when attached to both ends of the duplex resulted in a higher stability as compared to linker 1 (L_1). L_1 was probably too short as to allow efficient *Watson-Crick* hybridization of the base pair adjacent to the linker.

We demonstrated the application of σ-DNA for triplex investigations in a Pyr·(Pur·Pyr) mode with the corresponding homopyrimidine DNA or RNA sequence at pH 5.5 and 7. The homopyrimidine RNA was more tightly bound as compared to the corresponding homopyrimidine DNA sequence. The pH dependency of the melting behavior of σ-DNA **4** in the presence of the homopyrimidine DNA or RNA sequence indicated that with RNA, a triplex melting can still be observed at neutral pH. In

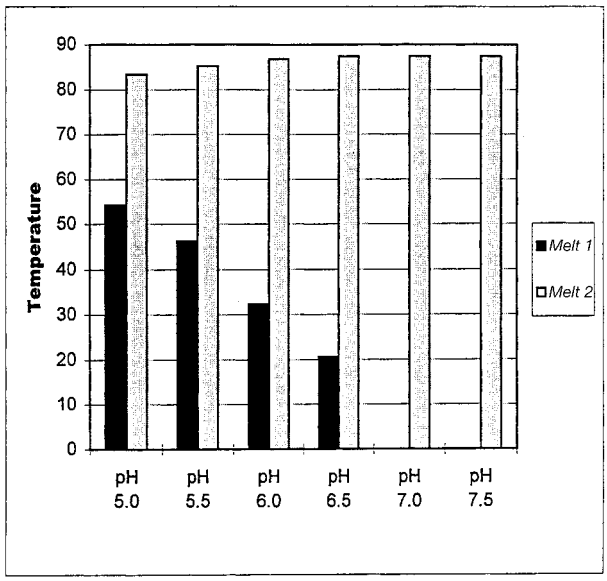


Fig. 7. *pH Dependency of the melting point of the combination of σ-DNA* **4** *and DNA* **7** *as third strand. Melt 1: Hoogsteen* melting point; *Melt 2: Watson-Crick* melting point. Conditions: 0.7 μM DNA, 100 mM NaOAc/AcOH (pH 5.0–7.5); 1 mM EDTA.

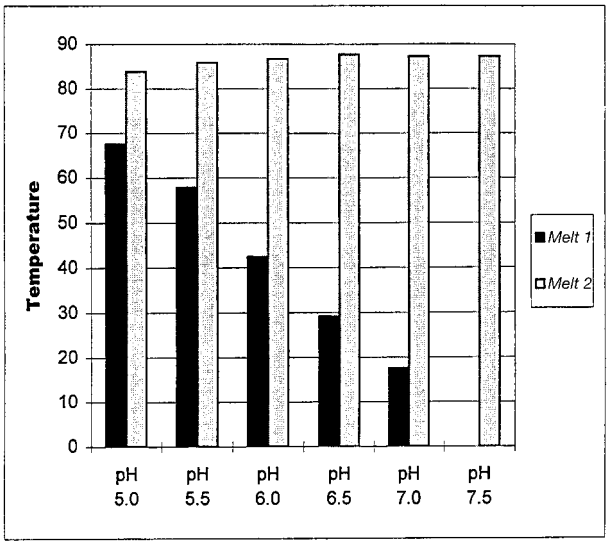


Fig. 8. *pH Dependency of the melting point of the combination of σ-DNA* **4** *and RNA* **8** *as third strand. Melt 1: Hoogsteen* melting point; *Melt 2: Watson-Crick* melting point. Conditions: 0.7 μM DNA, 100 mM NaOAc/AcOH (pH 5.0–7.5); 1 mM EDTA.

addition, the circular DNA was resistent to exonucleases. Thus, it may be possible to inhibit translation processes with σ-DNA molecules. This would be an alternative to the possible application of σ-DNA to inhibit the expression of genes by binding transcription factors.

Experimental Part

1. *General*. All solvents were of the highest purity available. The 1,4-dithioerythritol (= *erythro*-1,4-dimercaptobutane-2,3-diol; DTE), *N*-[3-(dimethylamino)propyl]-*N*-ethyl carbodiimide hydrochloride (= ECD), undecane, and *Stains-all* were all from *Fluka*, tetrabutylammonium fluoride trihydrate from *Aldrich*, and *NAP 10 Sephadex* columns from *Pharmacia*. All DNA and RNA syntheses were performed on a *394-ABI* synthesizer, the different solns., supports, and amidites for DNA synthesis were from *ABI*. RNA building blocks were from *Milligen*. The 30% sat. NH_3/EtOH soln. was prepared by slowly bubbling NH_3 gas through 30 ml of ice-cooled EtOH (*Aldrich*; H_2O max. 5 ppm) for 2 h. All DNA and RNA fragments were purified by prep. PAGE (polyacrylamide-gel electrophoresis) and assessed for purity by anal. PAGE after staining with *Stains-all*. Short column chromatography (CC) [16]: silica gel *60* (0.063–0.040 mm, *Merck*). UV-Absorption melting curves: *Cary 3* UV photometer.

2. *DNA Fragments* **7** *and* **9**–**11** were prepared on controlled-pore glass (CPG; *Sigma*) as solid support [17] applying a 10-fold excess of 2-cyanoethyl phosphoramidites [18] and a 130-fold excess of 1*H*-tetrazole. The workup was done using our standard technology [19].

3. O,O′-*(3,6,9,12,15-Pentaoxaheptadecane-1,17-diyl) Tetrahydrogen Phosphate* (L_2) prepared by modifying a published procedure [20]. Hexaethylene glycol (= 3,6,9,12,15-pentaoxaheptadecone-1,17-diol) was first reacted with $(MeO)_2$TrCl to yield 17-[(4,4′-dimethoxytrityl)oxy]-3,6,9,12,15-pentaoxaheptadecan-1-ol. Subsequent phosphinylation was performed with 2-cyanoethyl tetraisopropylphosphorodiamidine with diisopropylammonium tetrazolide activation [21]. Yield after CC: 77%. ^{31}P-NMR ($CDCl_3$; rel. to H_3PO_4): 148.5.

4. *Fragments* **2**, **3**, **5**, *and* **6** were prepared starting with 1 μmol of CPG support. Whenever linker unit L_1 had to be introduced, two successive couplings using a 10-fold excess of the appropriate building block were carried out applying standard cycles [6]. For the introduction of L_2, one coupling with a 10-fold excess of the corresponding building block was performed.

5. *σ-DNAs* **1** *and* **4** were synthesized in two steps. Firstly the 3′-phosphorylated precursors were prepared, and in a second step, these were cyclized to yield **1** and **4**. For the synthesis of the 3′-phosphates we used a disulfide linker as described in [22] but employing a sarcosine-modified CPG for the attachment of the linker unit. Preparation of the linear fragments was performed starting with 1 μmol of the modified support. When the linker had to be introduced, coupling with the appropriate phosphoramidite was carried out applying a standard cycle. After complete assembly, the $(MeO)_2$Tr group was removed. Further deprotection and release as 3′-phosphate was by treatment with 1 ml of 0.2M DTE in conc. NH_3 soln. for 2 h at 70°. After evaporation of the soln. on a *Speed-vac* concentrator, the fragments were precipitated after addition of 100 μl of H_2O, 200 μl of dioxane, and 600 μl of THF. Part of the crude material was analyzed by anal. PAGE to assess the performance of the synthesis (*Fig. 4*).

The cyclization reactions of the linear 3′-phosphorylated fragments with EDC to yield the σ-DNAs **1** and **4** were performed in 1 ml of 50 mM MES (morpholine-4-ethane sulfonic acid) pH 6.0, 20 mM $MgCl_2$, and 400 mM EDC containing 200 mmol (80 *OD*) of the crude linear precursor as described in [6]. After 5 h, the crude mixture was desalted on a *NAP-10* column, purified by prep. PAGE, and assessed for purity by anal. PAGE after staining with *Stains-all*. MALDI-TOF MS confirmed the correct molecular mass of the fragment.

6. *RNA Fragment* **8** was synthesized on a 1-μmol scale using per coupling a 15-fold excess of 2′-*O*-[(*tert*-butyl)dimethylsilyl]-protected phosphoramidite [23] and 150-fold excess of 1*H*-tetrazole with a coupling time of 15 min and the standard RNA cycle [24]. Workup was performed under sterile conditions. The support was treated with 1 ml of sat. NH_3/EtOH soln. at 70° for 3 h. The NH_3 soln., including 1 ml of washing soln. of the support with EtOH/H_2O 6 : 4, was evaporated on a *Speed-vac* concentrator. After addition of 0.5 ml of fresh 1M Bu_4NF/THF, the pellet was vortexed for 16 h at r.t. until a soln. was obtained. Then, 0.4 ml of 0.5M NH_4OAc (pH 7.0) was added, the soln. evaporated to 300 μl and desalted by filtration over *NAP 10*. The fragment was purified by prep. PAGE (20%) under denaturing and sterile conditions [24].

7. *Melting Curves*. The UV absorbance was measured at 260 nm and 95° and the following extinction coefficients were employed. A, 15000; C, 7500; G, 12500; T, 8500 [25]. Each fragment (1 nmol) was dissolved in 1 ml of 100 mM NaOAc (pH 5.5) and 1 mM EDTA. After transfer into the cuvettes, the solns. were degassed by

sonications at 90°. A layer of 0.1 ml of undecane was added, and the cuvettes were closed by a stopper. UV-Absorption melting curves were measured against cuvettes containing the buffer alone. The heating program was from 10 to 95° with a 0.5° increase per min and from 95 to 10° with 0.5° per min. Each melt was performed at least twice, and the given melting temp. is the average of the two measurements. Only the melting temp. from the heating run was used for the calculation. Melting temp. were calculated from the first derivative of the raw data. The buffers used were 100 mM NaOAc (pH 5,5)/1 mM EDTA or 10 mM Na_2PIPES (pH 7.5)/10 mM $MgCl_2$/100 mM NaCl. The buffers for the measurements of the m.p.s at different pH values were 1 mM EDTA/100 mM NaOAc (pH 7.5) adjusted to the corresponding pH with AcOH. The buffers were analyzed at 90° for their pH thermal stability, and no pH shift was observed.

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