Phenanthridinium as an Artificial DNA Base: Comparison of Two Alternative Acyclic 2′-Deoxyribose Substitutes

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Abstract: (*S*)-1-Amino-2,3-propanediol and (2*S*,3*S*)-2-amino-1,3-butanediol have been used as two different acyclic substitutes for 2′-deoxyriboside in order to synthetically incorporate the phenanthridinium chromophore of ethidium as an artificial DNA base. The comparison of the optical properties of one representative duplex bearing phenanthridinium attached to the two alternative acyclic linkers does not exhibit significant differences.

Key words: DNA, ethidium, fluorescence, oligonucleotide, phenanthridinium

3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide (‘ethidium bromide’) represents a commonly used fluorescent stain for nucleic acids.[1] Bisfunctional intercalators based on ethidium enhance the fluorescence increase upon DNA hybridization.[2] Moreover, the ethidium chromophore represents an interesting photoinducable charge donor if the fluorescence is quenched due to DNA-mediated charge transfer processes.[3–7] Since a detailed photophysics of the binding interactions, the fluorescence and electronic properties of the ethidium intercalation in DNA remain poorly understood,[8] we worked out a DNA phosphoramidite building-block protocol for the site-specific incorporation of the phenanthridinium chromophore of ethidium bromide as an artificial nucleobase into DNA.[9] Using such modified duplexes we were able to study emission and charge transfer in DNA[6] and to detect DNA base mismatches in a homogenous fluorescent assay.[7]

Due to the significant hydrolytic lability in aqueous basic solutions (that are typically used for DNA workup) of the ethidium nucleoside,[10] the 2′-deoxyribofuranoside moiety was replaced initially with (*S*)-2-amino-1,3-propanediol as an acyclic linker system which was tethered to the N-5 position of the phenanthridinium heterocycle **I** (Figure 1). The major difference in this linker in comparison to the 2′-deoxyribofuranoside is the number of carbon atoms between the phosphodiester bridges in the corresponding modified oligonucleotides which has been reduced from three in normal nucleosides to two (in the substitute **I**). Herein, we present the alternative synthesis of phenanthridinium-modified oligonucleotides that have the chromophore connected to D-threoninol **II** (Figure 1) as a linker bearing three carbon atoms. D-Threoninol has been previously used for the incorporation of other chromophores, e.g. acridine,[11] naphthyl red[12] and methyl red,[13] into DNA. Furthermore, we wanted to compare the absorption and fluorescence properties of a **I**-type modified DNA duplex with that of a **II**-type one.

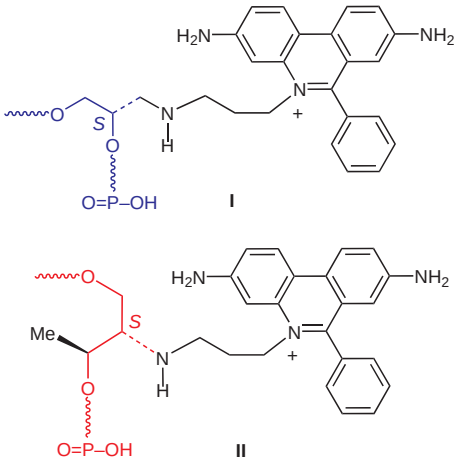

Figure 1 The phenanthridinium nucleoside analogues **I** and **II** bearing two different acyclic linkers as 2′-deoxyriboside substitutes.

(2*S*,3*S*)-2-Amino-1,3-butanediol (D-threoninol, **1**) was first converted into the DMT-protected derivative **4** in three simple steps,[14–16] similar to the preparation of the DMT-protected (*S*)-aminopropanediol linker[17] that we have used for the synthesis of **I**-type modified oligonucleotides.[9] The phenanthridinium **5**[9] was linked to the DMT-protected linker **4** to give **6** under the typical conditions of a nucleophilic substitution in DMF.[18] Subsequently, the allyloxycarbonyl (alloc) protecting groups had to be changed to trifluoroacetyl groups. This procedure is necessary since trifluoroacetyl groups cannot be used for the previous alkylation at N-5 during the preparation of **5**.[9] However, trifluoroacetyl groups are compatible for the DNA phosphoramidite building block chemistry because they can be cleaved under typical DNA workup conditions. Additionally, this protecting group strategy has the advantage that the secondary amino function of the alkyl linker also gets protected. The removal of the two alloc groups of **6** was performed under the previously optimized conditions.[19] After introduction of the trifluoroacetyl groups into **7** yielding **8**,[20] the preparation of the phosphoramidite **9** was finished using standard procedures (Scheme 1).[21]

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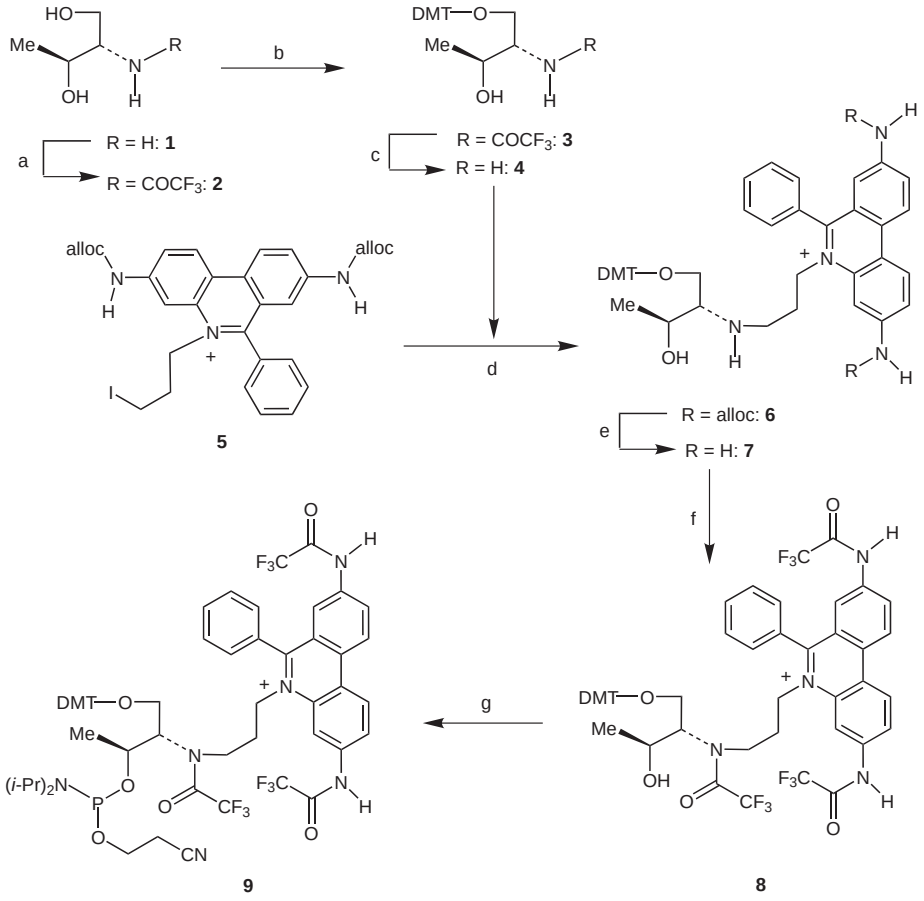

Scheme 1 Synthesis of DNA building block **9**. *Reagents and conditions*: (a) Methyl trifluoroacetate, r.t., 16 h, 89%; (b) 4,4′-dimethoxytrityl chloride (DMT-Cl; 1.6 equiv), pyridine, r.t., 2 d, 65%; (c) MeOH–concd aq NH_3–THF (11:11:5), r.t., 3 d, 86%; (d) **4** (1.2 equiv), *i*-Pr_2NEt (2.0 equiv), DMF, r.t., 5 d, 89%; (e) Bu_3SnH (2.0 equiv), $Pd(PPh_3)_4$ (0.02 equiv), PPh_3 (0.02 equiv), H_2O (0.01 equiv), CH_2Cl_2, r.t., 90 min, quant.; (f) trifluoroacetic anhydride (7.0 equiv), pyridine (16.0 equiv), CH_2Cl_2, 0 °C, 10 min, then r.t., 10 min, 93% (crude); (g) *i*-Pr_2NEt (1.2 equiv), 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (2.0 equiv), CH_2Cl_2 (abs.), r.t., 45 min.

The DNA building block **9** can be used for automated preparation of fluorescent oligonucleotides applying the modified coupling protocol we previously published.[9,22] Representatively, we synthesized two duplexes, **DNA1** and **DNA2**, bearing either the phenanthridinium heterocycle as a **I**-type or **II**-type modification, respectively (Figure 2). The phenanthridinium-modified single-stranded (ss) oligonucleotides **ssDNA1** and **ssDNA2** were quantified by their absorbance at 260 nm[22–24] and identified by mass spectrometry.[25,26] Duplexes were formed by addition of 1.2 equivalents of the unmodified counterstrand to the phenanthridinium-modified oligonucleotide. In contrast to the phenanthridinium chromophore the abasic site analogue **S** was placed to ensure the possibility for complete intercalation of the fluorophore, although previous studies have revealed that the counterbase has no significant influence on the fluorescence properties.

We characterized the duplexes **DNA1** and **DNA2** by UV/Vis absorption, fluorescence and circular dichroism (CD) spectroscopy in order to compare the optical properties of the phenanthridinium chromophore with respect to the **I**-type and **II**-type linkers, respectively. Remarkably, the UV/Vis absorption spectrum of **DNA1** showed a maximum at 533 nm and that of **DNA2** at 530 nm (Figure 3). Both values are very similar and typical for intercalated ethidium.[27] In comparison, the absorption spectrum of 'free' ethidium in aqueous solution had its maximum at ca. 480 nm.[28]

Subsequently, the steady-state fluorescence spectra of both modified DNA duplexes were recorded using an excitation wavelength of 530 nm (Figure 4). The emission of the phenanthridinium chromophore was found in the range between 550 nm and 800 nm, and the emission maxima were very similar, 620 nm (**DNA1**) and 621 nm (**DNA2**). Both values are again typical for intercalated ethidium.[26] The emission maximum of 'free' ethidium in water was found at ca. 635 nm.[28]

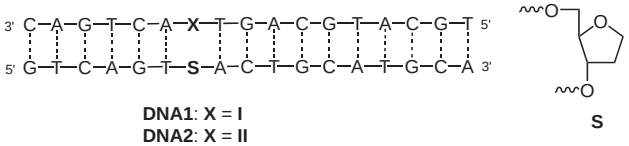

Figure 2 Sequence of duplexes **DNA1** and **DNA2**.

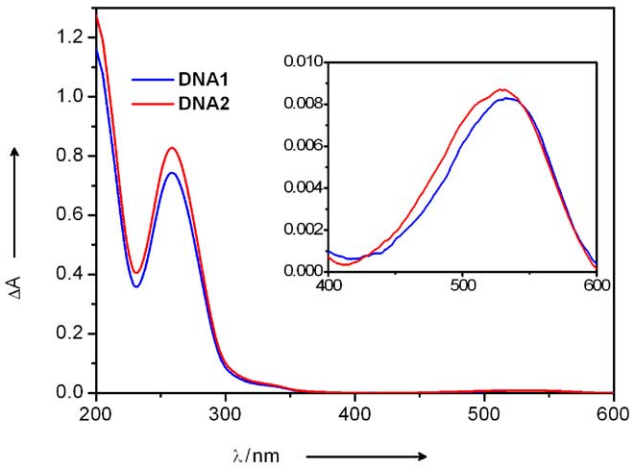

Figure 3 UV–Vis absorption spectra of the phenanthridinium-modified duplexes **DNA1** and **DNA2** (2.5 μM in 10 mM Na-P_i buffer). The inset shows the absorption of the phenanthridinium chromophore between 400 nm and 600 nm.

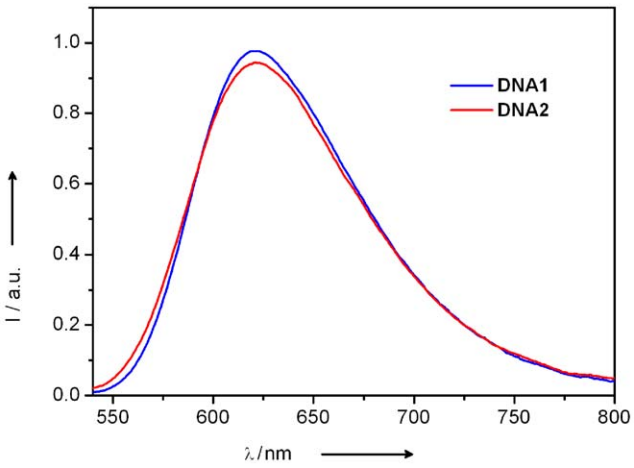

Figure 4 Fluorescence spectra of the phenanthridinium-modified duplexes **DNA1** and **DNA2** (2.5 μM in 10 mM Na-P_i buffer, λ_{exc} = 530 nm).

Finally, both duplexes were characterized by their melting temperatures (T_m) in thermal dehybridization experiments and by CD spectroscopy. Compared to unmodified DNA we observed a significant destabilization of DNA duplexes that had been modified with chromophores and the **I**-type linker.[29] Surprisingly, the T_m value of **DNA2** (62 °C) was slightly lower as compared to that of **DNA1** (66 °C) indicating that the D-threoninol (**II**-type) linker further destabilizes the duplex although it has one carbon atom more (compared to the **I**-type linker). The CD spectra of both duplexes, **DNA1** and **DNA2** (Figure 5), showed a B-like DNA conformation through the signals in the absorption range between 230 nm and 290 nm. In addition to these DNA-typical signals, a broad negative CD band was detected between 300 nm and 400 nm that is typical for intercalated ethidium.[30] CD spectra of ethidium bound to macromolecules in a nonintercalative fashion did not exhibit this negative band.[31]

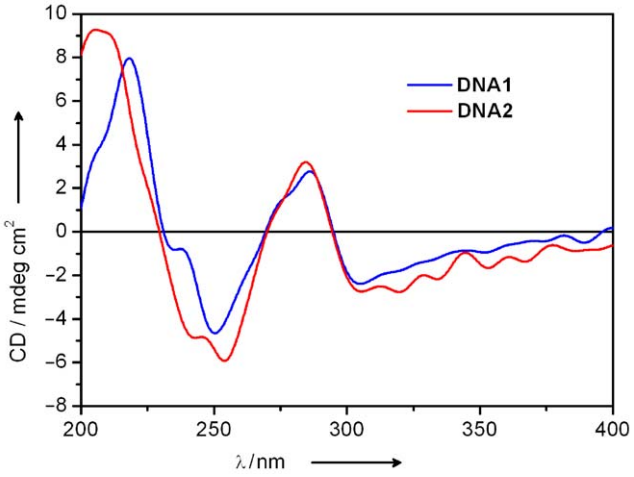

Figure 5 Circular dichroism spectra of the phenanthridinium-modified duplexes **DNA1** and **DNA2** (2.5 μM in 10 mM Na-P_i buffer).

In conclusion, the characterization[32] of both phenanthridinium-containing duplexes (**DNA1** and **DNA2**) by UV–Vis absorption spectroscopy (including the melting behavior), CD spectroscopy, and steady-state fluorescence spectroscopy clearly showed that the chromophore is intercalated within the DNA base stack. The comparison of the optical properties of the two duplexes did not exhibit significant differences; in fact the optical properties were remarkably similar. This shows that the structural difference between the **II**-type (**DNA2**) and the **I**-type linker (**DNA1**) has only a minor influence on the intercalation properties of phenanthridinium as an artificial DNA base.

Acknowledgment

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(14) The product **2** was co-evaporated three times with toluene and dried under high vacuum. Experimental data of **2**: R_f 0.60 (CH_2Cl_2–MeOH, 10:2). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 8.81 (d, *J* = 8.5 Hz, 1 H, NH), 4.70 (m, 2 H, C*H*OH, OH), 3.74 (m, 1 H, C*H*NH), 3.67–3.71 (m, 1 H, OH), 3.53–3.60 (m, 1 H, CH_2OH), 3.46–3.48 (m, 1 H, CH_2OH), 0.85 (d, *J* = 8.3 Hz, 3 H, Me).

(15) The product **3** was purified by flash chromatography (silica gel; CH_2Cl_2, 0.1% pyridine, 0–2% MeOH). Experimental data of **3**: R_f 0.17 (CH_2Cl_2–MeOH, 100:0.5). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.25 (d, *J* = 8.2 Hz, 1 H, NH), 7.21–7.40, 6.86–6.89 (m, 13 H, DMT-H), 4.70 (m, 1 H, C*H*OH), 3.86–3.95 (m, 2 H, OH, NHC*H*), 3.73 (s, 6 H, OMe), 3.14–3.18 (dd, *J* = 3.6, 9.3 Hz, 1 H, CH_2ODMT), 2.94–2.99 (m, 1 H, CH_2ODMT), 0.93 (d, *J* = 6.0 Hz, 3 H, Me). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.9, 156.7, 156.3 (q, $^2J_{CF}$ = 36 Hz), 149.5, 144.8, 136.0, 135.6, 135.4, 129.6, 127.7, 127.5, 126.5, 123.8, 117.9, 114.1 (q, $^1J_{CF}$ = 288 Hz, CF_3), 113.0, 85.1 ($OCPh_3$), 64.7 (CHOH), 62.4 (*C*H_2ODMT), 56.0 (NHCH), 54.9 (OMe), 20.0 (Me). MS (ESI): *m*/*z* (%) = 526.0(8) [M + Na]$^+$, 303.3 (100) [DMT]$^+$, 1028.9 (4) [2 × M + Na]$^+$. $C_{27}H_{28}F_3NO_5$: 503.51.

(16) The product **4** was co-evaporated twice with Et_2O and dried under high vacuum. Experimental data of **4**: R_f 0.24 (CH_2Cl_2–MeOH, 20:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.19–7.41, 6.83–6.92 (m, 13 H, DMT-H), 4.43 (m, 1 H, C*H*OH), 3.73 (s, 6 H, OMe), 3.63 (m, 1 H, OH), 2.99–3.04 (m, 1 H, NH_2C*H*), 2.81–2.85 (m, 1 H, CH_2ODMT), 2.58 (m, 1 H, CH_2ODMT), 0.95 (d, *J* = 6.3 Hz, 3 H, Me). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.9, 145.1, 135.9, 135.8, 129.6, 127.7, 126.4, 113.0, 85.1 ($OCPh_3$), 66.6 (CHOH), 65.1 (*C*H_2ODMT), 56.5 (NH_2CH), 54.9 (OMe), 20.1 (Me). MS (ESI): *m*/*z* (%) = 430.1 (16) [M + Na]$^+$, 303.3 (100) [DMT]$^+$, 815.0 (8) [2 × M + H]$^+$. $C_{25}H_{29}NO_4$: 407.50.

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(18) The product **6** was purified by flash chromatography (silica gel; CH_2Cl_2–MeOH, 100:3, 0.1% pyridine; then CH_2Cl_2–MeOH, 10:3, 0.1% pyridine). Experimental data of **6**: R_f 0.58 (CH_2Cl_2–MeOH, 20:3). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.67 (s, 1 H, NH, 3-alloc), 10.38 (s, 1 H, NH, 8-alloc), 9.08 (d, ^3J = 9.3 Hz, 1 H, H-1), 9.02 (d, 3*J* = 9.1 Hz, 1 H, H-10), 8.57 (s, 1 H, H-4), 8.26 (m, 1 H, H-9), 8.10 (dd, ^3J = 9.1 Hz, ^4J = 1.1 Hz, 1 H, H-2), 7.82–7.71 (m, 6 H, 6-Ph, H-7), 7.39–7.20 (m, 9 H, ArH, DMT-H), 6.88 (m, 4 H, ArH, DMT-H), 5.99 (m, 2 H, CH_2=CH, 3- and 8-alloc), 5.37 (m, 1 H, CH_2=CH, *trans*, 3-alloc), 5.30 (m, 1 H, CH_2=CH, *trans*, 8-alloc), 5.25 (m, 1 H, CH_2=CH, *cis*, 3-alloc), 5.21 (m, 1 H, CH_2=CH, *cis*, 8-alloc), 4.67 (d, ^3J = 5.5 Hz, 2 H, OCH_2, 3-alloc), 4.57 (d, ^3J = 5.5 Hz, 2 H, OCH_2, 8-alloc), 4.73 (m, 1 H, C*H*OH), 4.63 (m, 2 H, H-1′), 3.72 (s, 6 H, OMe), 3.08 (m, 1 H, CH_2ODMT, NHC*H*), 2.80 (m, 2 H, H-3′), 2.58 (m, 1 H, CH_2ODMT), 2.09 (m, 2 H, H-2′), 0.93 (d, *J* = 6.3 Hz, 3 H, Me). MS (ESI): *m*/*z* (%) = 901.4 (100) [M]$^+$, 599.3 (15) [M + H – DMT]$^+$, 303.3 (33) [DMT]$^+$, 468.3 (35). $C_{55}H_{57}N_4O_8^+$: 902.06.

(19) The product **7** was purified by flash chromatography (silica gel; CH_2Cl_2–MeOH, 100:5, 0.1% pyridine; then EtOAc–MeOH–H_2O, 6:2:2, 0.1% pyridine). Experimental data of **7**: R_f 0.60 (EtOAc–MeOH–H_2O, 6:2:2). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.67 (d, ^3J = 9.1 Hz, 1 H, H-1), 8.62 (d, ^3J = 9.3 Hz, 1 H, H-10), 7.67 (m, 5 H, 6-Ph), 7.51 (m, 2 H, H-9, H-4), 7.36–7.20 (m, 10 H, ArH, DMT-H, H-2), 6.86 (m, 4 H, ArH, DMT-H), 6.38 (s, 2 H, 3-NH_2), 6.26 (s, 1 H, H-7), 5.96 (s, 2 H, 8-NH_2), 4.50 (m, 3 H, H-1′, C*H*OH), 3.72 (s, 6 H, OMe), 3.27 (m, 1 H, NH_2C*H*), 3.00 (m, 2 H, H-3′), 2.79 (m, 1 H, CH_2ODMT), 2.63 (m, 1 H, CH_2ODMT), 2.25 (m, 2 H, H-2′), 0.92 (d, *J* = 6.3 Hz, 3 H, Me). MS (ESI): *m*/*z* (%) = 733.4 (100) [M]$^+$, 431.3 (13) [M + H – DMT]$^+$, 303.3 (85) [DMT]$^+$. $C_{47}H_{49}N_4O_4^+$: 733.92.

(20) The product **8** was dried under high vacuum. Due to the high lability of the trifluoroacetyl groups the structure was confirmed only by MS. Experimental data of **8**: MS (ESI): *m*/*z* (%) = 1021.3 (100) [M]$^+$, 303.3 (38) [DMT]$^+$. $C_{53}H_{46}F_9N_4O_7^+$: 1021.94.

(21) The product **9** was dried under high vacuum. Due to the observed high hydrolytic lability the structure was confirmed only by MS. Experimental data of **9**: MS (ESI): *m*/*z* (%) = 1221.4 (100) [M]$^+$, 303.3 (39) [DMT]$^+$. $C_{62}H_{63}F_9N_6O_8P^+$: 1222.16.

(22) An extended coupling time (1 h instead of 1.5 min for standard couplings), a higher phosphoramidite concentration (0.2 M instead of 0.067 M), and three coupling cycles interrupted by washing steps were necessary to achieve nearly quantitative coupling.

(23) For the extinction coefficients of the oligonucleotides at 260 nm, see: Puglisi, J. D.; Tinoco, I. *Meth. Enzymol.* **1989**, *180*, 304.

(24) The extinction coefficients of phenanthridinium at 260 nm is 45.200 $M^{-1}cm^{-1}$.[9]

(25) Experimental data of **ssDNA1**: ε_{260} = 200.700 $M^{-1}cm^{-1}$. MS (MALDI–TOF): *m*/*z* calcd for $C_{181}H_{225}N_{64}O_{98}P_{16}$: 5359; found: 5359.

(26) Experimental data of **ssDNA2**: ε_{260} = 200.700 $M^{-1}cm^{-1}$. MS (MALDI–TOF): *m*/*z* calcd for $C_{182}H_{228}N_{64}O_{98}P_{16}$: 5374; found: 5374.

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(32) UV–VIS spectra and melting temperatures were measured on a Cary 100 (Varian) instrument. Fluorescence spectra were recorded on a Fluoromax-3 (Jobin–Yvon) equipment with a bandpass of 2 nm (excitation and emission) and correction for intensity and for Raman emission from the buffer solution. ESI–MS measurement was performed on a TSQ 7000 (Finnigan) instrument, MALDI–TOF MS on a Bruker Biflex III spectrometer (A = 50 mg/mL 3-hydroxypicolinic acid in MeCN–H_2O, B = 50 mg/mL diammonium citrate, A:B = 9:1 for the matrix formation). C18-RP HPLC columns (300 Å) were from supplied by Supelco. The oligonucleotides were prepared on an Expedite 8909 DNA synthesizer (ABI) using CPG (1 μmol) and chemicals from ABI and Glen Research. The trityl-off oligonucleotides were cleaved and deprotected by treatment with concd NH_4OH at 60 °C for 10 h (unmodified oligonucleotides), for 5.5 h (modified oligonucleotides), dried and purified by HPLC on RP-C5 (300 Å, Supelco) using the following conditions: A = NH_4OAc buffer (50 mM), pH = 6.5; B = MeCN; gradient = 0–15% B (for the unmodified oligonucleotides) and 0–30% B (for the modified oligonucleotides) over 60 min. Duplexes were formed by heating to 90 °C (10 min), followed by slow cooling.