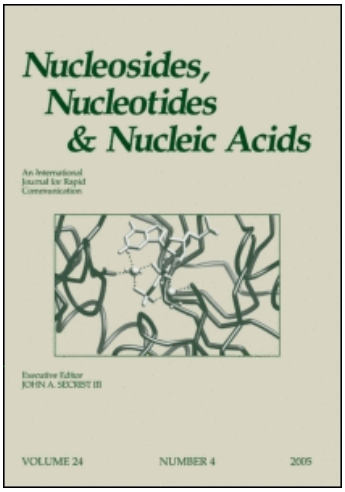

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SYNTHESIS AND BINDING PROPERTIES OF OLIGONUCLEOTIDES CONTAINING AN AZOBENZENE LINKER

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ABSTRACT: Incorporation of an azobenzene-4,4'-diamide group via a linker arm into the 3'-hydroxyl function of one oligonucleotide segment and the 5'-OH of other oligonucleotide has been described. The binding of the oligonucleotides containing the azobenzene linker was investigated by UV melting behaviors. The azobenzene linker has been shown to be useful as an effective bridge for stabilizing hairpin duplex and triplex.

INTRODUCTION

The utility of a non-nucleotide linker for connecting oligonucleotide fragments have been demonstrated in several nucleic acids recognition systems. Two oligonucleotide segments joined by an oligoethylene glycol linker have been shown to exhibit cooperative binding to appropriately positioned sequences in a single stranded RNA, which can, in principle, serve as a structural probe for a natural RNA.[1] When two oligopyrimidines are connected by the linker arm, greatly enhanced stability has been observed in the triple-helix formation with oligonucleotides having appropriate purine-rich sequences.[2] Ribozyme activity can be regulated by the linker length between the substrate and the enzyme oligoribonucleotide segments.[3] The linker containing a rigid aromatic fragment can serve as an effective cap for strongly binding two complementary oligonucleotide strands.[4]

We have designed a new non-nucleotide linker containing an azobenzene fragment which could be used for incorporating into oligonucleotide chains. This type of linker is

This paper is dedicated to the late Professor Tsujiaki Hata.

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attractive since the azobenzene undergoes photochemically-induced *trans-cis* isomerization.[5] Two oligonucleotide strands attached onto the 4,4'-positions of the azobenzene would be reversibly in different proximity imposed by photo-illumination. It is thus expected that the complexes such as duplexes, triplexes, and ribozymes involving azobenzene-linked oligonucleotides would reversibly change their conformation as a result of photo-illumination. This novel light switch should find several interesting biological applications in relation to nucleic acids.[6]

In this paper, we describe the synthesis of oligonucleotide derivatives containing the azobenzene linker[7] and focus on their binding properties.

RESULTS AND DISCUSSION

Synthesis of Oligonucleotides Containing an Azobenzene Linker. The chemical structure of the azobenzene linkers we have synthesized is shown in CHART I. The synthesis of the linkers was initiated by the preparation of 4,4'-azobenzene dicarbonyl chloride **1**. The acid chloride **1** was then converted to the fully protected diol **2** by treatment with an excess of *O-tert*-butyldimethylsilyl-protected aminoalkyl alcohol. Partial deprotection of the silyl group was conducted by use of a limit amount of tetrabutylammonium fluoride (TBAF) in THF. After usual work-up, the monoprotected diol **3** was obtained in a reasonable yield. The alcohol function was protected by a dimethoxytrityl group in the usual way and then the silyl protecting group was removed to afford the alcohol **4**. The remaining hydroxyl group was converted to the phosphoramidite **5** by a standard phosphitilating procedure.

The synthesis of oligonucleotides containing the azobenzene fragment was done by use of a slightly modified standard protocol on a DNA synthesizer in which the azobenzene amidite was use in an excess amount for a longer coupling time. With this protocol, the activated amidite can be coupled into the hydroxyl function of oligonucleotides on a CPG support. TABLE 1 showed the results of the synthesis of each oligomer. After the usual deprotection, the oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis.

The oligonucleotides thus purified were characterized by the chromatographic behaviors, and UV-Vis spectroscopy. TABLE 2 indicates the HPLC and UV data for the oligonucleotides containing the azobenzene linker. The oligonucleotides exhibited single peak in the HPLC conditions, which verified the purity of these oligomers. The purified oligonucleotides exhibited characteristic absorption bands at around 260 nm, 330 nm, and 450 nm. Based on the UV spectrum of compound **3**, the first absorption band is attributable to the nucleic acid bases and the latter two are due to the azobenzene fragment. The observed absorption ratio of 260 to 330 nm for the oligonucleotides appeared to be

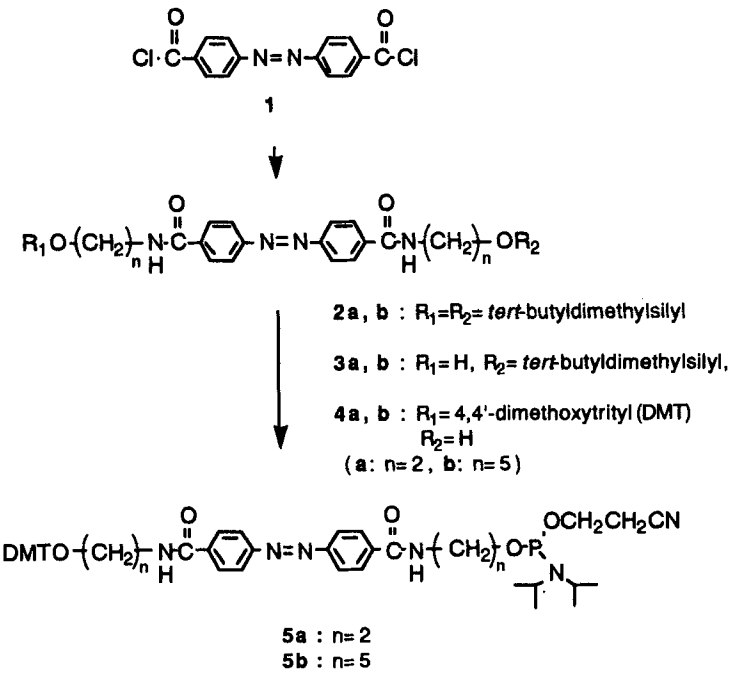

CHART I

TABLE 1. The coupling efficiency of the amidite **5a, b** in the synthesis of oligonucleotides containing an azobenzene linker.

Oligonucleotide sequence	Abbreviation	Coupling efficiency (%)
5'-TTTTTTTT -L2- TTTTTTTT-3'	T8-L2-T8	81.0
5'-TTTTTTTT -L2- AAAAAAAA-3'	T8-L2-A8	86.8
5'-AAAAAAAA -L2- TTTTTTTT-3'	A8-L2-T8	82.5
5'-TTTTCTTTTCCCCCCT -L5- TCCCCCCTTTTCTTTT-3'	16-L5-16	68.8
5'-TTTTCTTTTCCCCCCT -L5- T-3'	16-L5-T	65.1
5'-T -L5- TCCCCCCTTTTCTTTT-3'	T-L5-16	90.3

L2 and L5 indicate the azobenzene units derivatized from amidite 5a and 5b, respectively. The synthesis of oligonucleotides was done on a fully automated procedure by using a phosphoramidite chemistry. The details for the synthesis conditions are described in the text. The coupling efficiency is based on a dimethoxytrityl cation assay.

TABLE 2. HPLC and UV spectral data for oligonucleotides containing an azobenzene linker.

Oligomer	RT1 (min)[a]	RT2 (min)[b]	A330/A260[c]
T8-L2-T8		35.1	0.20 (0.24)[d]
T8-L2-A8		34.7	0.23 (0.17)[d]
A8-L2-T8		34.5	0.21 (0.17)[d]
16-L5-16	21.6	36.8	0.12 (0.13)[d]
16-L5-T	23.4	39.2	0.20 (0.24)[d]
T-L5-16	24.2	41.8	0.20 (0.24)[d]

a) HPLC (YMC-C_{18}, 6 x 150 mm) analysis was done by a linear gradient of CH_3CN (1 %/min) starting from 10 % CH_3CN in 0.1 M triethylammonium acetate (pH 7) at a flow rate of 1 mL/min.
b) HPLC was carried out on the same column above; elution was i) 5 % CH_3CN (5 min), ii) a linear gradient of CH_3CN (5 % to 25 % in 30 min), and then iii) a linear gradient of CH_3CN (25 % to 70 % in 45 min) each at a flow rate of 1 mL/min in 0.1 M triethylammonium acetate (pH 7).
c) UV-VIS spectra were obtained in aqueous non-buffered solutions.
d) Theoretical values for the ratio of A_{330}/A_{260}. Calculations were carried out by using extinction coefficient of 3 at 330 nm and total ε value at 260 nm of T, dC, and dA in the oligomer.

consistent with the calculated value based on the expected molar ratio of the azobenzene to the nucleic acid bases to be present in each synthesized oligomer.

Duplex and Triplex Formation. All the binding studies described here have been carried out without photo-illumination or in the *trans* form of the azobenzene fragment. The binding of A8-L2-T8 and T8-L2-A8 was investigated by their UV melting behaviors in an aqueous buffered solution. FIG. 1 indicated the UV melting curves at 260 nm for these oligonucleotides. While the duplex containing T8 and dA8 exhibited the midpoint of transition (Tm) at 8 °C, both the oligonucleotides, A8-L2-T8 and T8-L2-A8, showed the dramatically high Tm (65 °C). The oligonucleotide solution of A8-L2-T8 at the low concentration (1/10) exhibited the almost identical Tm. On the contrary, the duplex of T8-dA8 was destabilized by lowering its concentration. These observations clearly show that two complementary oligonucleotide strands connected by the azobenzene linker forms intramolecular complex affording the remarkably stable hairpin duplex. Similar phenomena for great stabilization of hairpin duplexes have been demonstrated in the oligonucleotide segments linked by aromatic fragments.[2c,4] The azobenzene fragment provides one of effective bridges for stabilizing hairpin oligonucleotide duplexes, which may be used for generating high-affinity mimics for a nucleic acids binding protein.[8]

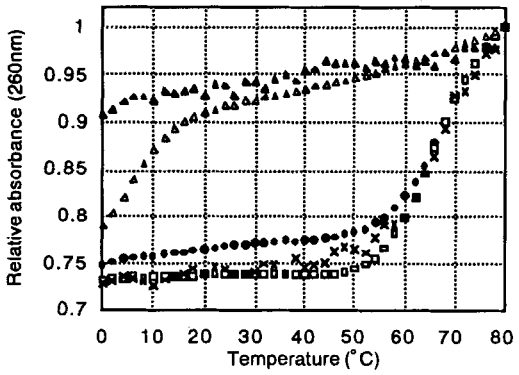

FIG. 1. UV melting curves for hairpin duplexes of oligonucleotides containing an azobenzene linker. All measurements were carried out in a pH 7 phosphate buffer containing 0.1 M NaCl.

- ● : A8-L2-T8 (5 x 10^{-5} M)
- × : A8-L2-T8 (5 x 10^{-6} M)
- □ : T8-L2-A8 (5 x 10^{-5} M)
- △ : T8 + dA8 (5 x 10^{-5} M)
- ▲ : T8 + dA8 (5 x 10^{-6} M)

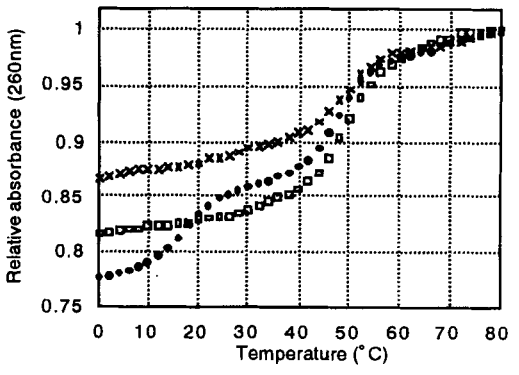

FIG. 2. Triplex formation of 16-L5-16 followed by UV melting behaviors. All measurements were carried out in a pH 7 phosphate buffer containing 0.1 M NaCl at a single strand concentration of 2.5 x 10^{-5} M.

- ● : 16-L5-16 + 29-mer
- × : 29-mer + oligomer I
- □ : 29-mer + oligomer I + oligomer II

29-mer = 5'-CCACTTTTTAAAAGAAAAG-GGGGGACTGG
oligomer I = 5'-TCCCCCCTTTTCTTTT
oligomer II = 5'-TTTTCTTTTCCCCCCT

The binding of 16-L5-16 which contains two oligopyrimidine strands positioned appropriately to form a triple helix with the sequence containing an oligopurine tract (5'-CCACTTTTTAAAAGAAAAGGGGGGACTGG, 29-mer) was investigated by UV melting behaviors in a pH 7 buffered solution. FIG. 2 showed the UV melting profile for the mixture of 16-L5-16 and 29-mer in which two transitions (18 °C and 50 °C) were clearly observed. The UV melting profile for the 1:1 mixture of 29-mer and oligopyrimidine 16-mer I (5'-TCCCCCCTTTTCTTTT) exhibited monophasic transition at 50°C. Therefore the transition at 18°C observed for the 16-L5-16 containing solution is attributable to the triplex to duplex transition. The equimolar mixture of 29-mer, oligopyrimidine 16-mer I and oligopyrimidine II (5'-TTTTCTTTTCCCCCCT) showed only one transition (Tm = 50 °C), indicating that triplex formation is not favorable without linking two oligopyrimidine segments under the neutral conditions. It is therefore concluded that the azobenzene linker for connecting oligonucleotide strands provides a useful tool to stabilizing triplexes under the pH 7 physiological conditions.[2e]

Photoisomerization. FIG. 3a showed that the azobenzene fragment of 16-L5-16 can undergo *trans-cis* isomerization by photo-illumination in the pH 7 phosphate buffer solution. The degree of the isomerization (ca. 25 %) was almost identical to that for the compound **3**, indicating that the oligonucleotide chains incorporated into the azobenzene fragment did not affect the degree of photoisomerization of the azobenzene fragment. However, as shown in FIG. 3b, the oligonucleotide derivatives forming hairpin duplexes appeared to keep its original form even after the photo-illumination. These results may suggest that structural elements or complex formation derived from the oligonucleotide chains attached to the azobenzene unit largely contribute to the photoisomerization reaction. It might be considered that the thermal *cis to trans* isomerization process is much favorable in these cases.

CONCLUSION

We have synthesized oligonucleotides containing an azobenzene fragment as a linker. The synthesis of oligomers have been conducted by a fully automated procedure using an appropriately derivatized azobenzene amidite reagent. The azobenzene linker has been shown to be effective for stabilizing hairpin duplex and triplex structures in a neutral aqueous solution. Considered that the *trans-cis* isomerization of the azobenzene fragment may be affected by complexation of the attached oligonucleotides, it would be possible to choose certain systems to be applicable for light switch to control reversible conformational changes of oligonucleotides in nucleic acids recognition systems.

EXPERIMENTAL PROCEDURE

General Methods. ^{1}H NMR spectra were measured on a JEOL-JNM-EX-270 spectrometer using a residual peak at 7.26 ppm of $CDCl_3$ as an internal standard. High-performance liquid chromatography (HPLC) was performed on a Waters 600E model equipped with a Hitach LC 4200 UV-VIS detector at 260 nm, using a reversed phase YMC-C_{18} column (6 x 150 mm). Column chromatography and thin-layer chromatography (TLC) were carried out on Wako silica C-200 and Merck 60 PF_{254}, respectively. Ultraviolet (UV) spectra were recorded with a Hitachi U-3000 spectrophotometer equipped with a thermoelectrically controlled cell holder (Hitachi SPR-10). Photo-irradiation was carried out by using a high-pressure mercury lamp filtered to transmit light at 313 nm.

Materials and solvent. 4,4'-Azobenzene dicarbonyl chloride was synthesized according to the literature procedure.[9] 2-Cyanoethyl diisopropylchlorophosphoramidite was obtained from Aldrich Chemical Co. Protected deoxyribonucleoside 3'-*O*-(2-cyanoethyl)-*N*, *N'*-diisopropylphosphoramidites and nucleoside-loaded controlled pore

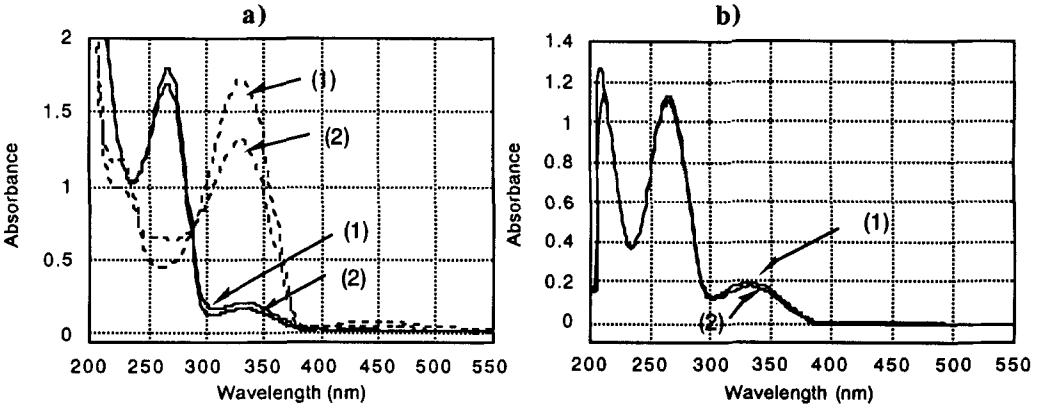

FIG. 3. a) UV-vis spectra for the azobenzene derivative **3b** (dotted line) and 16-L5-16 (straight line) and b) T_8-L2-A_8 before (1) and after (2) irradiation. Irradiation were done at 22 °C for 60 min in a pH 7 phosphate buffer for the oligonucleotides and in CH_3CN for the azobenzene derivative.

glass (CPG) supports were purchased from Cruachem. DNA oligomers were prepared by a standard phosphoramidite chemistry on a Pharmacia LKB Gene Assembler Plus DNA synthesizer. For synthesis of the azobenzene-modified oligonucleotides, the X-bottle was used to supply the azobenzene modified amidite solutions. Dichloromethane, diisopropylethylamine, and acetonitrile were dried by refluxing over CaH_2 for at least 5 h under a nitrogen atmosphere, then distilled and stored over CaH_2 or molecular sieves. Dimethylformamide (DMF) was dried over CaH_2, then distilled under the reduced pressure, and stored over molecular sieves.

Synthesis of *N*-[(2-(Dimethoxytrityl)oxy)-ethyl]-*N'*-(2-Hydroxyethyl) Azobenzene-4,4'-Diamide (4a). 4,4'-Azobenzene dicarbonyl chloride (1.2 g, 3.9 mmol) was allowed to react with *O*-(*tert*-butyldimethylsiloxy)ethanolamine(6.5 g, 30 mmol) in the presence of imidazole (2 g, 30 mmol) in DMF (30 mL) at r.t. overnight. After removal of solvent, the material was participated into CH_2Cl_2 and water. The organic layer was dried over Na_2SO_4 and then the solution was evaporated to a minimum volume. This was applied to a silica gel column which was eluted by CH_2Cl_2-MeOH (50:1, v/v). The appropriate fractions were collected and the solvent was removed to afford the desired compound **2a** [1.7 g, 76 %; m.p.= 203-205°C; Rf = 0.65 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR ($CDCl_3$): δ(ppm) = 0.10 (12H, s, Me_2Si-), 0.92 (18H, s, t-BuSi-), 3.63 (4H, m, CH_2N), 3.83 (4H, t, CH_2O), 6.62 (2H, t, NHCO), 7.91 and 8.12 (total 8H, d, aromatic)]. The compound **2a** (2.0 g, 3.4 mmol) was treated with TBAF (2.6 mmol) in THF (100 mL) at r.t. for 45 min. The solvent was removed and then the

material was dissolved in CH_2Cl_2 (100 mL). The solution was washed with water and then the organic layer was dried over Na_2SO_4. The material was purified by a silica gel column chromatography eluted with CH_2Cl_2-MeOH (9:1, v/v/) to give compound **3a** [0.53 g, 33 %; m.p.= 179-181°C; Rf = 0.43 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR($CDCl_3$): δ(ppm) = 0.10 (6H, s, Me_2Si), 0.92 (9H, s, t-BuSi-), 3.59-3.72 (total 4H, m, CH_2N), 3.87-3.91 (total 4H, m, CH_2OH + CH_2OSi), 6.62 (1H, t, NHCO), 6.70 (1H, t, NHCO), 7.96-8.01 (total 8H, d, aromatic)]. The compound **3a** (1.1 g, 2.5 mmol) was reacted with dimethoxytrityl chloride (DMT-Cl) (1.7 g, 5 mmol) in pyridine (45 mL) at r.t. overnight. After aqueous work-up, the product was purified by a silica gel column chromatography. The purified material (2.0 g, 2.5 mmol) was treated with 4 mmol of TBAF in THF (40 mL) at r.t. for 2 h. After removal of the solvent, the residue was applied to a silica gel column which was eluted with EtOAc-MeOH-Et_3N (93:5:2, v/v). The appropriate fractions were collected and the solvent was removed *in vacuo* to give the desired compound **4a** [0.74 g, 47 % from compound **3a**; m.p.= 161-163°C; Rf 0.41 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR ($CDCl_3$) : δ(ppm) = 3.39 (2H, t, CH_2ODMT), 3.66 (4H, m, CH_2N), 3.78 (6H, s, CH_3O of DMT), 3.87 (2H, t, CH_2OH), 6.51 (1H, t, NHCO), 7.02 (1H, t, NHCO), 6.82, 7.23-7.43 (total 13 H, aromatic of DMT), 7.77-7.90 (total 8H, d, aromatic of azobenzene)].

Synthesis of *N*-[(5-(Dimethoxytrityl)oxy)-pentyl]-*N'*-(5-Hydroxypentyl) Azobenzene-4,4'-Diamide (4b). The synthesis of this compound was carried out by the essentially same procedure described for **4a**. Compound **2b** [54 % from **1**, m.p.= 210-212°C; Rf = 0.78 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR ($CDCl_3$): δ(ppm) = 0.05 (12H, s, Me_2Si-), 0.89 (18H, s, t-BuSi-), 1.46-1.71 (12H, m, -$(CH_2)_3$-), 3.50 (4H, m, CH_2N), 3.64 (4H, t, CH_2O), 6.23 (2H, t, NHCO), 7.92 and 7.96 (total 8H, d, aromatic)]. Compound **3b** [27 % from **2b**; m.p.= 207-209°C; Rf = 0.38 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR($CDCl_3$): δ(ppm) = 0.03 (6H, s, Me_2Si), 0.87 (9H, s, t-BuSi-), 1.47-1.71 (total 12H, m, -$(CH_2)_3$-), 3.44-3.49 (total 4H, m, CH_2N), 3.59-3.66 (total 4H, m, CH_2OH + CH_2OSi), 6.21(2H, t, NHCO), 7.96-8.01 (total 8H, d, aromatic), ε value (330 nm in MeOH) = 33.2×10^3]. Compound **4b** [25 % from **3b**; m.p.= 140-142°C; Rf = 0.29 (CH_2Cl_2-MeOH = 9:1, v/v); ^1H NMR ($CDCl_3$) : δ(ppm) = 1.33-1.72 (total 12H, m, -$(CH_2)_3$-), 3.08 (2H, t, CH_2ODMT), 3.49 (4H, m, CH_2N), 3.67 (2H, t, CH_2OH), 3.77 (6H, s, CH_3O of DMT), 6.34 (1H, t, NHCO), 6.51 (1H, t, NHCO), 6.81, 7.19-7.44 (total 13 H, aromatic of DMT), 7.85-7.89 (total 8H, d, aromatic of azobenzene)].

Synthesis of Oligonucleotides Containing Azobenzene Linker. The azobenzene amidite **5a** and **b** was synthesized by a standard phosphitilation procedure.[10] The synthesis of azobenzene-modified oligonucleotides was accomplished by

phosphoramidite chemistry,[10] beginning with 5'-DMT nucleoside (0.2 μmol) bound to a CPG support. For the coupling of normal deoxyribonucleoside phosphoramidites, the standard protocol (50 μL of 0.1 M amidite and 50 μL of 0.1 M tetrazole in acetonitrile, 2 min) was used. For the coupling of the azobenzene modified amidites 5, 120 μL of the 0.1 M amidite and 120 μL of 0.1 M tetrazole in acetonitrile (10 min) were used. With these conditions, the average coupling efficiency of the normal nucleoside amidites was 99 % in the oligonucleotide synthesis, while the azobenzene amidites coupled in a yield range of 65-90 %. The CPG bound oligonucleotides were treated with concentrated ammonium hydroxide at 55°C for 10 h. Purification of the modified oligonucleotides was performed with denaturing 20 % polyacrylamide gel electrophoresis.

Binding Study. All solutions for UV melting studies were prepared using a buffer containing 10 mM sodium phosphate and 100 mM NaCl, adjusted to pH 7.0. Oligonucleotide concentrations were determined by absorbance at 260 nm and the calculated single-strand extinction coefficient based on a nearest neighbor model.[11] All duplex melting curves by UV spectra were measured with an increase in temperature from 0°C to 80°C at a rate of 0.5°C/min. The solutions were heated to 80°C, kept there for 5 min, and then gradually cooled before melting experiments.

ACKNOWLEDGMENT

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