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Synthesis and Properties of a Hoechst-Like Minor-Groove Binding Agent Tethered to an Oligodeoxynucleotide

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Abstract—A small Hoechst-like DNA groove-binding fluorophore carrying a terminal bromoacetimido linker has been synthesized. Individual diastereomeric oligodeoxynucleotide dodecamers containing a thiol-based linker attached to an internucleotide phosphoramidate within a sequence of dA–dT residues were each covalently labeled with the new fluorophore. One isomer of the DNA–fluorophore conjugate (Isomer B) hybridizes to the complementary single-stranded target DNA and the presence of the tethered fluorophore results in both increased duplex stability and an enhanced fluorescent signal, presumably due to the fluorophore binding in the dA–dT rich minor groove. Duplex stability is increased by 2–3 °C and the fluorescence quantum yield for the fluorophore increases four-fold. In contrast, the other diastereomer (Isomer A) exhibits reduced helix stability (~2 °C) and only slight changes in fluorescence intensity upon hybridization.

Introduction

The minor groove of the B-form of DNA provides sites for binding by many ligands. It is often the case that such binding occurs preferentially at dA–dT rich sequences, as exemplified by the binding characteristics of the agents Netropsin,[1] Distamycin,[2] CC-1065[3] and Hoechst 33258.[4] Preferential minor groove binding at contiguous dA–dT base pairs is thought to occur due to the absence of N^2-amino groups (present in dG–dC base pairs), which permits the groove-binding agents to penetrate deeply within the groove structure and take advantage of both hydrogen bonding and hydrophobic interactions involving both the 'floor' and the 'walls' of the minorgroove. However, in other cases, the amino groups of dG–dC base pairs can provide important hydrogen bonding contacts,[5] and some groove-binding agents, such as Chromomycin A3,[6] prefer to bind in the minor groove, but at sites rich in dG–dC base pairs.

A groove-binding agent that is also fluorescent could be used in the detection and quantitation of various double-stranded or even multi-stranded forms of nucleic acids providing that the groove-binding agent is specific for the structure of interest. Three well-characterized fluorophores that interact with DNA by binding in the minor groove of the B-form duplex structure include the two Hoechst dyes 33258 and 33342 as well as the diamidinophenylindole derivative DAPI (Fig. 1). Crystal structures of DNA complexes containing Hoechst 33258[4] and DAPI[7] have been reported, confirming the minor groove binding nature of these fluorophores. Binding in the minor groove by DAPI or the Hoechst dyes results in a dramatic enhancement of the fluorescence quantum yield, presumably due to the displacement of water molecules upon binding, both from the minor groove and from the dyes themselves.[8] The binding of Hoechst 33258 to double-stranded DNA has previously been employed in a quantitative manner to automate assays for DNA,[9] to determine cell numbers,[10] to sort chromosomes,[11] and it is sensitive enough to detect one target cell per million in mixed cell populations on a scanning-stage microscope.[12] These highly sensitive applications for minorgroove binding fluorophores, such as Hoechst and DAPI, suggest that an oligonucleotide tethering a Hoechst-like fluorophore could be an effective strategy for the development of a sensitive nucleic acid hybridization diagnostic; one in which the hybridization event generates the requisite binding site (the minor groove) for the fluorophore. These agents are particularly attractive since a dramatic enhancement in fluorescence quantum yield is only observed upon

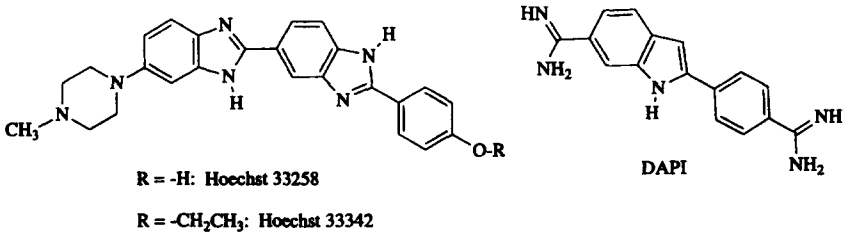


Figure 1. Structures of the groove-binding fluorophores Hoechst 33258, 33342 and DAPI.

binding by the agent in the minor groove;[13] the unbound fluorophore in aqueous solution is only moderately fluorescent.

A variety of fluorophores and other types of conjugates have been tethered to oligodeoxynucleotides in order to provide non-radioactive hybridization probes,[14] or in attempts to enhance the stability and/or efficacy of nucleic acids employed in antisense studies.[15] Various reporter groups, including fluorophores, have been covalently attached to nucleic acids using functional groups present on, or appropriate tethers bound to, either the nucleobases, the carbohydrate residues, or the phosphate backbone, and these approaches have been reviewed in some detail.[16]

We wish to report the synthesis of a simplified Hoechst-like fluorophore, its covalent attachment to an oligonucleotide, and the properties of the complex formed with a complementary target DNA sequence.

Experimental

Materials

Oligodeoxynucleotides were synthesized using 2'-deoxynucleoside phosphoramidites on an Applied Biosystems 381A DNA synthesizer. The four fully protected common 2'-deoxynucleoside phosphoramidites containing aryl- or isobutyrylamides were purchased from Cruachem through Fisher Chemical Co. H-Phosphonate derivatives were obtained from Glen Research (Sterling, VA). The controlled pore glass support containing the 3'-terminal nucleoside was a product of CPG Inc. (Fairfield, NJ). Cystamine dihydrochloride, adamantanecarbonyl chloride, anhydrous carbon tetrachloride, anhydrous pyridine and ammonium hydroxide were all obtained from the Aldrich Chemical Co. (Milwaukee, WI).

Nuclease Pl and snake venom phosphodiesterase were products of Boehringer Mannheim (Indianapolis, IN). Acrylamide and bis-acrylamide were obtained from ICN Biomedicals (Cleveland, OH). Thin layer chromatography was performed on aluminum backed pre-coated silica gel 60 F254 plates purchased from EM Science (Gibbstown, NJ). NMR spectra were obtained at 300 MHz on a Varian XL-300 multinuclear NMR spectrometer.

Methods

Synthesis of the bromoacetylated Hoechst-like derivative. All flash chromatographic separations were performed using a gradient of methanol in dichloromethane. The general synthetic scheme for the synthesis of compound **1** was identical to that published by Hoechst GmbH (Chemical Abstracts*).

*Chemical Abstracts **1969.** 71, 81418.

2-(4-Nitro-1-benzyl)-6-(1-methyl-4-piperazinyl)benzimidazole (2). To 1.0 g of 4-nitrobenzonitrile (6.75 mmol) dissolved in dichloromethane (15 mL) was added 0.43 g of anhydrous methanol and the reaction was placed in an ice/salt bath until a temperature of 0 °C had been reached. The reaction mixture was saturated with anhydrous HCl and then stirred overnight at ambient temperature. The white precipitate was collected, washed thoroughly with dichloromethane and dried to yield 1.3 g (6.0 mmol) (89%) of product 4-nitrobenzoiminomethylester as the hydrochloride salt which was used in the subsequent step without further purification. R_f in dichloromethane:methanol (95:5): 0.45.

To 200 mg of 5-(1-methyl-4-piperizinyl)-1,2-diaminobenzene (**1**)* (0.97 mmol) dissolved in anhydrous methanol (3 mL) was added 314 mg of 4-nitrobenzoiminomethylester (1.45 mmol) followed by 30 μL (0.48 mmol) of glacial acetic acid. The mixture was stirred at 55–60 °C for 3 h. TLC analysis (dichloromethane:methanol, 1:1) indicated the absence of **1** and the presence of a new spot that was fluorescent under long wavelength UV irradiation (366 nm). Solvents were evaporated and the residue was dissolved in water and basified with concentrated ammonium hydroxide. The brown precipitate that formed was collected, dried and purified by column chromatography to yield **2**. Yield: 200 mg (0.59 mmol, 61%). R_f in dichloromethane:methanol (1:1): 0.50. ^{1}H NMR (DMSO-d_6) δ = 2.2 (*s*, 3H, CH_3–N), 3.10 (*br s*, 4H, $-CH_2-$), 3.30 (H_2O + $-CH_2-$), 6.9–7.6 (*m*, 3H, ArH), 8.4 (*s*, 4H, ArH) ppm. UV (water): λ_{max} = 320, 240, 200, λ_{min} = 275, 235 nm. IR (KBr): 3100, 3000, 2800, 2750, 2700, 1612, 1602, 1519, 1490, 1452, 1343 cm^{-1}.

2-(4-Amino-1-phenyl)-6-(1-methyl-4-piperazinyl)benzimidazole (3). To 200 mg of 2-(4-nitro-1-benzyl)-6-(1-methyl-4-piperazinyl)benzimidazole (**2**) (0.59 mmol) in 20 mL of anhydrous methanol was added 70 mg of 10% palladium on carbon and the reaction mixture was hydrogenated at 45 psi for 2 h. TLC analysis (dichloromethane:methanol, 1:1) indicated that the starting nitro compound had disappeared. The catalyst was removed by filtration through Celite, and after thorough washing with methanol the combined filtrate was evaporated to yield **3** which was used in the following reaction without further purification. Yield: 178 mg (0.585 mmol, 99%). R_f in dichloromethane:methanol (1:1): 0.33. ^{1}H NMR (DMSO-d_6) δ = 2.2 (*s*, 3H, CH_3–N), 3.10 (*t*, 4H, $-CH_2-$), 3.40 (H_2O + $-CH_2-$), 5.5 (*s*, 2H, $-NH_2$), 6.60 (*d*, 1H, ArH), 6.80–7.00 (*t*, 3H, ArH), 7.30 (*d*, 1H, ArH), 7.80 (*d*, 2H, ArH) ppm. UV (water): λ_{max} = 360, λ_{min} = 310 nm. IR (KBr): 3439, 3400, 2939, 2884, 2816, 2361, 1613, 1608, 1419, 1476, 1449, 1396, 1385, 1289, 1240, 1185 cm^{-1}.

2-(4-Bromoacetamido-1-benzyl)-6-(1-methyl-4-piperazinyl)benzimidazole (4). To 100 mg of 3 (0.33 mmol) in anhydrous DMF (3 mL) was added 10 mg of *N,N*-dimethylaminopyridine (0.08 mmol) and the reaction was cooled to –70 °C. To the cooled mixture was added

257 mg (0.99 mmol) of bromoacetic anhydride and the reaction mixture stirred for 2 h at –70 °C. TLC analysis (dichloromethane:methanol, 1: 1) after 2 h indicated the absence of starting material. The reaction was quenched with excess methanol and the mixture was reduced to a small volume. The product was precipitated by the addition of diethylether and the resulting solid was purified by flash chromatography on silica gel using a gradient of dichloromethane/methanol to yield compound **4**. Yield: 140 mg (0.32 mmol, 98%). R_f in dichloromethane:methanol (1:1): 0.46. ^{1}H NMR (DMSO-d_6) δ = 2.80 (*s*, 3H, CH_3–N), 3.60 (*t*, 4H, –CH_2–), 3.80 (*t*, 4H, –CH_2–), 4.16 (*s*, 2H, –CH_2–), 7.19 (*d*, 1H, ArH), 7.22 (*dd*, 1H, ArH), 7.60 (*d*, 1H, ArH), 7.80 (*d*, 2H, ArH), 8.20 (*d*, 2H, ArH), 10.90 (*s*, 1H, NH) ppm. UV (water): λ_{max} 268, 321 nm, λ_{min}: 245, 296, 410 nm. IR (KBr): 3403, 3234, 3178, 3114, 3036, 2952, 2847, 2812, 1684, 1627, 1605, 1551 cm^{-1}. Mass spectrum: Calculated for $C_{20}H_{23}N_5OBr$ (M + H$^+$) 428.1086, found 428.1080.

Oligonucleotide synthesis. The oligonucleotides were synthesized on controlled pore glass supports using phosphoramidite chemistry[18] and H-phosphonate chemistry.[19] Introduction of the cystamine tether was accomplished by the following procedure:

The hydrochloride salt of *N*-triphenylacetylcystamine[20] (30 mg, approximately 60 μmol) was dissolved in 1 mL of dichloromethane and extracted with an equivalent volume of 1 M sodium carbonate. The organic phase was evaporated to dryness, co-evaporated from anhydrous pyridine (× 3) and dissolved in freshly distilled anhydrous pyridine/carbon tetrachloride (1:1) to make a solution of the linker at approximately 100 mM.

After introducing the H-phosphonate linkage using standard techniques[19] this linkage was oxidized three times in succession for 30 min each with the *N*-triphenylacetylcystamine solution.[20,21] After the third treatment, the excess material was washed from the column with pyridine/CCl_4 followed by washing the column with acetonitrile. The DNA was elongated further (with phosphoramidite couplings), but the capping steps were eliminated to prevent transacetylation of the *N*-triphenylacetamide with acetic anhydride. After completion of the synthesis, the 5'-terminal DMT group was removed on the column and the sequences were then deprotected under standard conditions (concentrated ammonia for 6 h at 50 °C).

The oligonucleotide tethering the protected cystamine linker contained a trityl group at the terminus of the linker and this group could be used for the isolation of the DNA in much the same manner as a terminal DMT-group is used to retard the chromatographic mobility of the product sequence when employed in standard synthetic/isolation procedures.[22] Placing the trityl group at the stereocenter enhances the resolution of the two diastereomeric sequences. The individual diastereomeric oligonucleotides were separated and isolated using a 9.4 × 250 mm column of ODS-Hypersil at a flow rate of 3.0 mL min^{-1} in 20 mM potassium phosphate (pH 5.5) and a linear gradient of methanol (0–70% over 90 min). After collection, each individual diastereomer was desalted (Sephadex G-10) and lyophilized to dryness.

Conjugation reactions. To a solution of oligodeoxynucleotide (0.2 mM) containing a single phosphoramidate linkage tethering a triphenylmethylacetamido-protected cystamine moiety in 10 mM sodium acetate, pH 4.5 was added sufficient tris(2-carboxyethyl)phosphine TCEP[23] such that the final concentration was 10 mM. After a 15–30 min reaction period at ambient temperature, HPLC analysis (4.6 × 250 mm column of ODS-Hypersil in 20 mM potassium phosphate, pH 5.5 and a gradient of 0–70% methanol over 60 min) revealed the complete absence of starting material (38 min) and the appearance of a new peak containing the free thiol with an elution time near 18 min. The reaction was then rebuffered to pH 8.0 by the addition of 1 M Tris–HCl to a final concentration of 30–50 mM. Alternatively, to a buffered solution (pH 8.0) containing 1 mM of the disulfide-containing oligonucleotide was added DTT to a concentration of 10 mM and the mixture was incubated at ambient temperature for 3 h. To a solution of the free thiol generated by either method, compound **4** (7.25 mM) in dimethylformamide was then added such that the final concentration of **4** was 1.5 mM (20% DMF) and the reaction mixture was heated at 50 °C. When HPLC analysis indicated that the free thiol was consumed, the mixture was diluted with 50% aqueous formamide and the product was isolated by polyacrylamide gel electrophoresis (20% acrylamide, 7 M urea, 1 × TBE buffer). The product band was located by UV shadowing and by its bright fluorescence under long-wavelength UV light (366 nm). The desired band was excised, crushed and soaked in 0.2 M sodium acetate, pH 6.0 at 37 °C overnight. The mixture was centrifuged, the solution decanted and loaded on to a C18 Sep-Pak cartridge, which was then washed with distilled water followed with a gradient of methanol. The methanol was removed and the solution adjusted to the desired concentration (~10 μM). The DNA–**4** conjugate exhibited a UV spectrum characterized by two maxima, one at 260 nm (DNA) and one at 321 nm.

Nucleoside analysis. Nucleoside composition was determined after P1 nuclease/snake venom phosphodiesterase/bacterial alkaline phosphatase hydrolysis: A 20 μL reaction mixture containing 0.5 A_{260} unit of oligomer in 25 mM sodium acetate, pH 5.3 was incubated for 1 h at 37 °C with 2 units of nuclease P1. The reaction mixture was then rebuffered to pH 8 with a solution of 100 mM Tris–HCl, pH 8.0; 10 mM $MgCl_2$ and 3 units of snake venom phosphodiesterase and 2 units of alkaline phosphatase were added and the mixture incubated an additional 2 h at 50 °C. An aliquot containing approximately 0.2 A_{260} units of material was analyzed by HPLC using a 4.6 × 250 mm column of ODS-Hypersil in 20 mM potassium

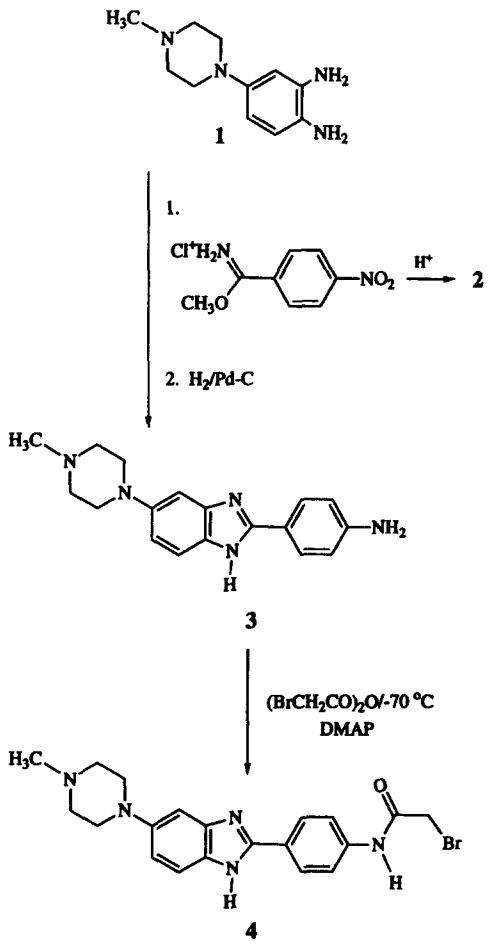
phosphate, pH 5.5 and a gradient of 0–70% methanol (60 min). Under these conditions the following retention times were observed (260 nm): dC: 6.4, dG: 10.4, dT: 11.1, dA: 14.2, d[Tp($NHCH_2CH_2SH$)T]: 29.8 and 30.5, d[Tp($NHCH_2CH_2S$-Fluorophore)]T: 39 and 41 min.

T_m *values.* T_m values were determined in 50 mM Tris–HCl (pH 7.4), 100 mM sodium chloride, 2 mM Na_2EDTA at duplex concentrations in the low micromolar range. Absorbance values were measured with a Perkin–Elmer Lambda 3B UV/Visible spectrophotometer equipped with digital temperature control. The solution temperatures were measured directly with a thermister probe (OMEGA Engineering, Stanford, CT). Absorbance and temperature data were collected after analog to digital conversion (DT-2800, Data Translation, Marlboro, MA) using an IBM-XT computer and the ASYST (version 1.53) scientific software package (MacMillian Software, New York, NY). *T* values were determined from first- and second-order derivatives of the absorbance versus temperature plots.

Fluorescence measurements. Fluorescence spectra were obtained in 50 mM Tris–HCl (pH 7.4), 100 mM sodium chloride, 2 mM Na_2EDTA using a Shimadzu RF5000U fluorescence spectrophotometer containing a Shimadzu DR-15 microprocessor and graphics display terminal. The groove-binding agent was excited at 321 nm and the emission was monitored from 340–600 nm. In a typical experiment, a single-stranded dodecamer (1.2 μM) tethering the groove-binding agent was titrated with a series of aliquots of the complementary strand of DNA. Each aliquot increased the concentration of the complementary strand by approximately 0.2 μM. After each addition the fluorescence spectrum of the solution was collected.

Results

The fluorophore we designed as a minor groove binding agent is a smaller version of the bisbenzimidazole agents typified by the Hoechst fluorophores (see Fig. 1), but only a single benzimidazole ring system is coupled to the terminal aromatic ring. In this respect, the compound is also similar to DAPI (see Fig. 1) which contains a single indole ring system coupled to a terminal amidinophenyl residue. We expected that this simplified ring system would function as a minor groove binding agent and still maintain the desired fluorescence characteristics, but would be easier to prepare than the larger bisbenzimidazole Hoechst derivatives. We also replaced the terminal phenol moiety present in Hoechst 33258 with the corresponding aniline derivative to facilitate the introduction of a bromoacetamido linker to the agent. Synthesis of the fluorophore **4** proceeded by the route[17] used for Hoechst 33258, but the diamino derivative **1** was coupled directly to *p*-nitrophenylimidate prepared from the corresponding nitrile to generate the single benzimidazole system coupled to a nitrophenyl ring (Scheme). The nitro derivative (**2**) was reduced to the corresponding amino compound (**3**), which could then be acetylated with bromoacetic anhydride to yield compound **4**, the Hoechst-like fluorophore containing a bromoacetamido linker (Scheme).



Scheme.

Tethering the fluorophore to the oligodeoxynucleotide probe could be accomplished by a number of procedures.[16] We decided against employing a base residue as the site for attachment since this approach either places the reporter group most commonly in the major groove, or uses a functional group involved in interstrand hydrogen bonding interactions as the site of attachment (or does both). Neither of these characteristics were desirable in the present study. A second possibility would be to use the sugar residue as a site for the incorporation of a suitable linker,[24] but we were concerned that modifications to a sugar residue might alter conformational parameters and adversely affect the hybridization properties to the target sequence. A number of procedures permit the incorporation of reporter groups at one of the nucleic acid termini,[16] but in the present application, this approach would place the agent at one end of the targeted binding site and this location did not seem to be an optimal position to facilitate groove binding.

Instead, we opted to use the internucleotide phosphorus residue as the site for attachment of the groove-binding fluorophore. In this approach, a phosphoramidate internucleotide linkage is introduced into the sequence at a specific position during oligonucleotide assembly.[20a,25] The nitrogen of the amidate tethers an appropriately protected thiol-containing linker.[20,21] After synthesis, deprotection and purification of the adducted sequence, the thiol residue can be unmasked and made available for reaction with the desired thiol-specific agents.[20,21] There are two primary advantages in using a linker tethered to the internucleotide phosphorus: (i) the linker can be placed at any site within the sequence and does not require the presence of a specific nucleoside residue, and (ii) in this location, the reporter group is positioned on the outer surface of the DNA duplex where it is unlikely to interfere with hybridization events. Also, tethering the groove binding agent to one of the internucleotide phosphorus residues positions it on the 'ridge' separating the major and minor grooves, and placement in this position should permit access by the agent to the minor groove binding site. A potential disadvantage with this labeling approach is that a linker attached to the internucleotide phosphorus is present in one of two diastereomeric forms (*Rp* or *Sp*),[20, 21,26] and each isomer may have differing hybridization, groove binding, and fluorescence characteristics. However, such differences can also be advantageous, providing that the diastereomers can be separated and the individual isomers examined and characterized. One diastereomer may provide exceptional binding/fluorescence properties that would otherwise be hidden in a study of isomeric mixtures of DNA conjugates. For example, molecular modeling suggests that one diastereomeric form of the phosphoramidate-tethered complex could direct the groove binding agent more towards the minor groove, while the second isomer may tend to direct the agent away from the minor groove (and/or more toward the major groove).

We synthesized a DNA dodecamer containing an internal sequence of six thymidine residues to provide a dA–dT rich minor groove for binding by the tethered fluorophore. Although a sequence of six base pairs is longer than that typically required for the binding of such minor groove agents, we were unsure how the covalent tethering of the fluorophore to one of the DNA strands would affect its access to, and ultimate positioning within, the minor groove. Therefore, we provided an extended binding site and placed the phosphoramidate linkage tethering the fluorophore at the internucleotide phosphorus residue located between the first two thymidine residues. The thiol linker was incorporated into the sequence as the triphenylacetyl-protected cystamine. After assembly of the dodecamer, the 5'-terminal DMT group was removed and the oligonucleotide was deprotected in the normal fashion, retaining only the triphenylacetyl protecting group at the terminus of the cystamine linker. Placing this hydrophobic moiety directly at the chiral site enhanced the resolution of the two diastereomeric phosphoramidates. The two isomers (Isomer A and Isomer B), each containing a single phosphoramidate stereogenic center could be separated and isolated by reversed-phase HPLC (see Fig. 2). No attempts were made to assign the absolute stereochemistries of the isolated diastereomers.

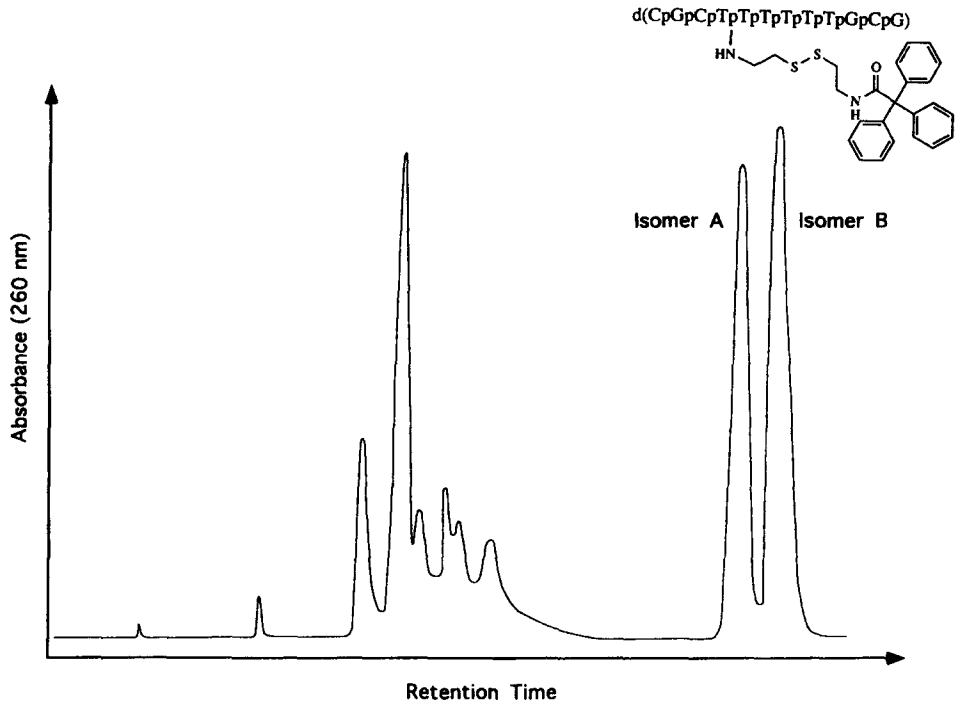
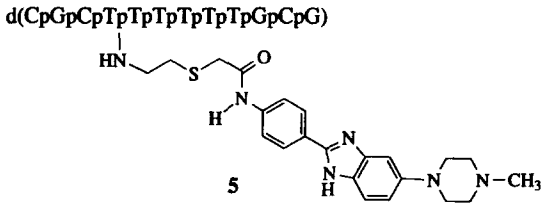


Figure 2. Resolution of the crude mixture of oligodeoxynucleotides obtained after assembly and deprotection of a dodecamer such that a triphenylacetamide residue remained bound to the cystamine linker at the stereogenic site (structure). Earlier peaks represent protection groups and failed sequences.

Each of the two purified diastereomeric dodecamers (Isomer A or Isomer B) were treated briefly with tris(2-carboxyethyl)phosphine (TCEP)[23] or with DTT to cleave the disulfide and unmask the thiol linker. Addition of the groove binding agent **4** then resulted in covalent modification of the free thiol and generation of the conjugates **5** (Isomer A or Isomer B) that could be isolated by HPLC or polyacrylamide gel electrophoresis.



5

The two diastereomeric conjugates (**5**) exhibited HPLC retention times of 47 and 49 min, more than double that of the free thiol (18 min). The UV spectrum of each of the conjugates exhibited two maxima, one at 260 nm, largely the result of the DNA bases, and one at 321 nm, resulting from the tethered groove binding agent. Hydrolysis of a small aliquot of the conjugate by snake venom phosphodiesterase/nuclease P1 and alkaline phosphatase and resolution of the hydrolysate by HPLC resulted in three peaks for the standard nucleosides (dC, dG and dT) at 6.4, 10.4 and 11.1 min, respectively. A fourth peak eluting at 39 min (Isomer A) or 41 min (Isomer B) was identified from its UV characteristics and by coinjection of an authentic standard as the d[Tp(NH–R)T] dimer tethering the groove binding agent.

The effect of both free and covalently attached **4** on the stability of the dodecameric duplex was evaluated by thermal denaturation experiments. A solution of 1.2 μM of the native dodecamer 5'-d(CpGpCpTpTpTpTpTpTp-GpCpG)·d(CpGpCpApApApApApApGpCpG) was treated with varying quantities of **4** and the T_m values determined by UV melting (Table 1). The T_m value for the native dodecamer is 43.8 °C. As the concentration of **4** was increased from 0 to 30 μM the T_m of the duplex rose from 43.4 °C to 46.3 °C. Concentrations higher than 30 μM had no further influence on the T_m value (Table 1). Under the same conditions, addition of 1.2 μM of Hoechst 33258 (see Fig. 1) to the dodecamer results in an increase in T_m value from 43.8 °C to 55 °C (data not shown).

Table 1. T_m Values for a duplex dodecamer in the presence of **4**

4(μM)	Ratio of **4** to DNA 12-mer (1.2 μM)	T_m(°C)
0	0	43.8 ± 0.5
1.2	1	43.5
6.0	5	44.7
12	10	45.4
18	15	46.3
30	25	46.7
120	100	46.2

The T_m values for the two diastereomeric duplex dodecamers tethering the groove binding agent differed by about 4 °C (Table 2). Isomer A of conjugate **5** complexed to its complementary DNA strand exhibited a T_m value of 41.8 °C, suggesting the presence of a duplex structure that was destabilized relative to the unmodified dodecamer by about 2 °C. By comparison, Isomer B of conjugate **5** resulted in stabilization of the duplex by 2–3 °C (Table 2). In fact the T_m value for the duplex containing Isomer B (1.2 μM) was about the same as that obtained for the native duplex in the presence of 15–100 μM of free groove binding agent.

Table 2. T_m Values for a duplex dodecamer 5'-d(CpGpCpApApApApApApGpCpG)·**5**

12-mer duplex tethering **4** (1.2 μM)	T_m (°C)
Isomer A	41.8 ± 0.5
Isomer B	46.0

The fluorescence intensity of the tethered groove binding agent was examined in the presence and absence of the Watson–Crick complementary sequence. We determined the fluorescence spectrum of both diastereomers of **5** and then titrated each of these solutions with the complementary dodecamer 5'-d(CpGpCpApApApApApApGpCpG). The spectra obtained for either isomer of the conjugate **5** did not differ from each other significantly, and one example of the data is illustrated by the spectrum of Figure 3a. Addition of the complementary 12-mer to Isomer A of conjugate **5** resulted in less than a 50% enhancement of the fluorescence signal. In contrast, the addition of one equivalent of the complementary strand to Isomer B of conjugate **5** resulted in nearly a four-fold (> 370%) enhancement in fluorescence for the groove binding agent (Fig. 3b).

Discussion

Compound **4** (see Scheme) represents a derivative structure of a minor groove binding agent that has obvious similarities to the Hoechst fluorophores, or to 4',6-diamidino-2-phenylindole (DAPI) (see Fig. 1). The synthesis of this derivative paralleled that described for Hoechst 33258 or 33324, but was shortened by the incorporation of only a single benzimidazole ring system (see Scheme). The final synthetic step involved the introduction of a bromoacetamido linker to the terminal anilino residue present on the benzimidazole ring system. The truncation of the Hoechst ring system results in a fluorophore that is similar in size to DAPI with one terminus carrying the bromoacetamido alkylating moiety to permit covalent tethering of the fluorophore to the DNA sequence.

Labeling of an internal phosphorus residue places the groove-binding fluorophore on the phosphoribose backbone where it should not interfere with hybridization events. We are unable to demonstrate this lack of interference with the present groove-binding

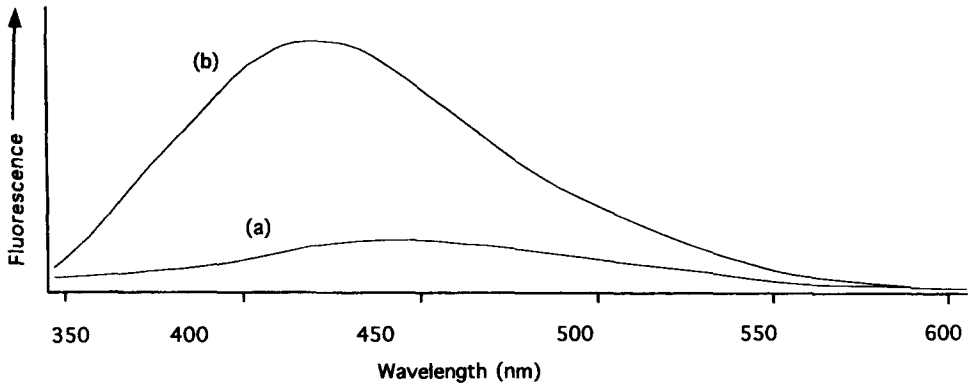


Figure 3. Fluorescence emission spectra (λ_{ex} = 321 nm) for Isomer B (1 μM) of conjugate **5** (a) and Isomer B of conjugate **5** complexed to its Watson–Crick complementary sequence (1.2 μM) (b).

agent since hybridization is followed almost immediately by groove binding. However, we note that either diastereomer of the sequence isolated after solid-phase DNA synthesis (tethering the bulky triphenylacetyl residue, see structure in Fig. 2), complexed to its complementary sequence, results in a T_m value that is essentially identical with that of the native 12-mer. A variety of non-groove binding fluorophores tethered in this manner to a phosphoramidate internucleotide linkage have little effect upon stability of the DNA duplex.[21]

The differences observed, both for duplex stabilization and fluorescence enhancement properties, for Isomer A and Isomer B of conjugate **5** complexed to its Watson–Crick complementary sequence are consistent with differential binding effects as a result of the two diastereomeric locations of the linker tethering the fluorophore to the DNA sequence. Formation of a duplex 12-mer with Isomer A actually results in destabilization of the duplex relative to the native (unmodified) sequence, and little change in fluorescence characteristics is observed. By comparison, the corresponding duplex formed with Isomer B is stabilized by 2–3 °C and results in nearly a 4-fold enhancement in fluorescence. The phosphoribose backbone of a B-form duplex can be viewed in the simplest analysis as a 'ridge' separating the major and minor grooves. A pendant group attached to the phosphoribose backbone, through a phosphoramidate in the *Rp* configuration* will be directed toward the minor groove, while a similar linker in the *Sp* configuration will direct the group away from the minor groove and more towards the major groove. Based upon the initial stability and fluorescence characteristics, Isomer B appears to permit more effective binding in the minor groove by the fluorophore. Thus Isomer B is more likely to contain the fluorophore bound as the *Rp* diastereomer, but conclusive assignment of the two diastereomers must await further studies.

*We should note that because of the vagaries of the Cahn–Ingold–Prelog priorities, the *Rp* phosphoramidate has the same absolute stereochemistry as, for example, the *Sp* phosphorothioate.

The duplex destabilization observed for the complex formed with Isomer A may indicate a non-standard binding mode for this fluorophore. Alternatively, partial binding within the minor groove could result in anomalous deformation of the phosphoribose backbone and an attendant reduction in duplex stability.

Tethered groove-binding fluorophores of the type described in this work have the potential to function as sensitive hybridization probes. The hybridization event generates the DNA duplex with the grooved structure; creation of the ligand binding site 'triggers' the binding event by the fluorophore. Tethering the fluorophore to the hybridization probe raises the effective concentration of the groove-binding agent such that each hybridization event results in a bound agent and the desired fluorescence signal, and this process is now independent of the concentration of any exogenously added groove-binding fluorophore.

The groove structures and their dimensions for duplex nucleic acids (right- or left-handed), triplexes, as well as more complicated structures can vary significantly. For example, DNA–DNA duplexes adopt the B-form structure while RNA–RNA duplexes are found as A-form duplexes. The DNA–RNA heteroduplex, the structure that would result from a hybridization event between a target RNA sequence and DNA probe, may have a structure with some characteristics of both helix forms, although these complexes generally seem to be more A-form in character.[27] The structural details of triplexes, tetraplexes and more heterogeneous complexes are only now emerging, and such details will assist in defining groove structures or other unique binding sites characteristic of specific nucleic acid structures. Although to date, it has not been conclusively shown that specific agents will bind to either the major or minor groove of an A-form RNA–RNA or A-like DNA–RNA helix, triple helix-specific binding by a planar ligand has been demonstrated.[28] With differing groove structures, it should be possible to develop groove-binding agents that recognize specific helix forms, or other complex structures, and permit the detection of various nucleic acid structures/sequences

through hybridization by the requisite complementary sequence tethering an appropriate groove-binding fluorophore.

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