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Synthesis, Binding and Fluorescence Properties of Oligonucleotide Derivatives Having A Dansyl Fluorescence Label Attached to the 2'-Position of A Ribonucleoside

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Abstract: Oligonucleotide derivatives having a dansyl fluorescence label at the 2'-position of a ribonucleoside have been synthesized by using 5'-dimethoxytrityl 2'-(dansylamino)uridine 3'-phosphorobisdiethylamidite. The oligonucleotide containing a dansyl-modified uridine at the 5'-terminal position exhibits normal binding affinity for a complementary DNA segment in an aqueous buffer solution (pH 7.0). A significant increase in fluorescence intensity and blue-shift of the emission maximum were observed for dansyl-modified oligonucleotide upon binding to DNA. The fluorescence increase upon binding of dansyl-modified oligonucleotide to the target DNA was found to be sensitive to base mismatch in the DNA sequence. © 1997 Elsevier Science Ltd.

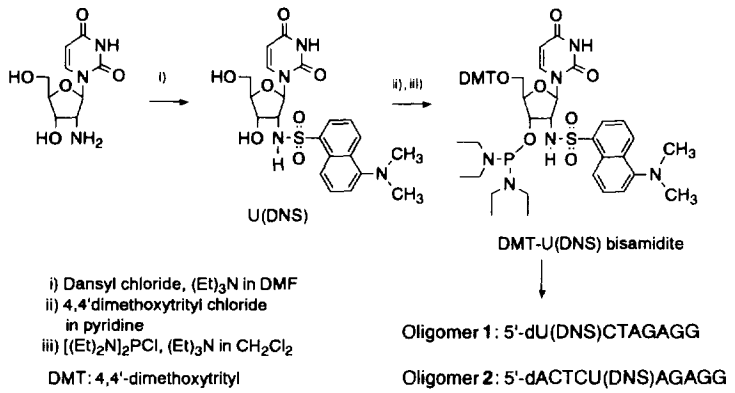
Introduction

DNA sequence determination by hybridization has attracted current interest.[1] This new sequencing method will contribute to the development of rapid and inexpensive techniques for mapping clones, facilitate DNA sequence analysis, and extend the applicability of DNA fingerprinting for diagnostics. The hybridization assay used is performed in a heterogeneous way, where the target DNA is fixed to a solid-support such as nitrocellulose. The procedure of the assay involves several steps, e.g. labeling DNA, hybridization, washing, and detection.

Homogeneous hybridization procedure, on the other hand, has some advantages with respect to hybridization kinetics and unnecessary washing step. It requires specially labeling of oligonucleotide probes in which signal-enhancement on hybridization of probes to complementary DNA sequences is highly desirable. This type of probes basically allows that the assay is done in solution, thus opening the route for easy automation of the assay.

Several approaches have been reported to the synthesis of the fluorescent-labeled oligonucleotides possessing the above properties. One involves the suitable pairs of the labeled oligonucleotides that exhibit the characteristic emission derived from fluorescence resonance energy transfer upon binding to complementary DNA.[2] Other approach has been demonstrated by the oligonucleotide tethered to the ruthenium complex.[3] This approach is based on the intercalation-induced fluorescence enhancement of the designed metal complex. It has recently been shown that fluorescent minor groove binding agent covalently attached to an oligodeoxyribonulceotide exhibits enhanced emission on hybridization to an appropriate sequence of DNA.[4]

We describe here a new approach to the design and synthesis of fluorescence-labeled oligonucleotide probes that exhibit an enhanced emission signal upon binding to specific sequences in nucleic acids. Our design is dependent on the properties of *so called* hydrophobic probes such as 5-dimethylaminonaphthalene-1-



sulfonate (DNS) derivatives. It is well known that placement of DNS derivatives in a hydrophobic environment causes significant increase in quantum yield and blue-shift of the emission.[5] Our recent studies suggested that a polyaromatic fragment atttached via a short linker at the sugar-2' of oligonucleotides is located in between base-pairs of the duplexes formed with complementary DNA segments.[6] It is therefore expected that, upon binding to the complementary DNA segment, a DNS group incorporated into the sugar position of an oligonucleotide would be buried in more hydrophobic interior[7] of the duplex, thereby resulting in fluorescence enhancement. In this paper, we demonstrate that an oligonucleotide possessing a DNS group at the sugar-2' via a linker of an appropriate length satisfies our design criteria.

Results and Discussion

The introduction of a dansyl group into the sugar residue of oligonucleotides was initiated by the synthesis of the dansyl-modified uridine [U(DNS)].[8] U(DNS) was synthesized from the reaction of 2'-amino 2'-deoxyuridine with dansyl chloride [DNS-Cl] in the presence of triethylamine in DMF. This reaction afforded only the desired product, thus indicating that no hydroxyl protection is necessary. U(DNS) was then converted to the 5'-protected 3'-phosphorobisamidite derivative [DMT-U(DNS)] by the reported procedure.[6a] The dansyl-modified oligonucleotides **1** and **2** were synthesized manually by the phosphoramidite and bisamidite methods.[6a] The purification of the oligonucleotides was done by reversed phase HPLC. The base compositions of the purified oligomers were verified by enzymatic digestion analysis.

Figure 1 indicates the UV melting profile for DNA duplex of the oligonucleotide having a dansyl substituent at the 5'-terminal sugar **1**. The melting profile exhibited sigmoidal curve and the midpoint of transition (tm=33.2 °C) similar to the unmodified duplex (tm=31.0 °C). On contrary, the duplex of the oligonucleotide containing a dansyl group at the middle **2** did not show typical sigmoidal transition in the thermal denaturing process. It is therefore suggested that a non-planar bulky substituent such as a dansyl group may be incorporated into the sugar at the terminal fraying end of oligonucleotides in order to retain normal binding affinity for a complementary DNA strand. In contrast, it has been shown that the oligonucleotide derivatives having planar polyaromatic fragments such as anthraquinone and anthracene at any position of 2'-sugar bind to a complementary DNA sequence to form stabilized duplexes.[6]

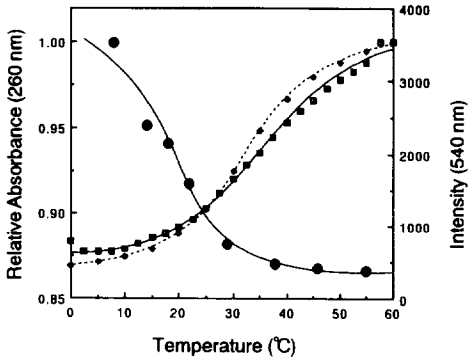


Figure 1. UV (□) and fluorescence (●) melting profiles for duplex of dansyl-modified oligonucleotide **1** with DNA (5'-dCCTCTAGA-GTCGACCT). All measurements were carried out at a total strand concentration of 8 x 10^{-5} M in a phosphate buffer (pH 7.0) containing 1.0 M NaCl. Dashed line indicates UV melting curve for the unmodified duplex.

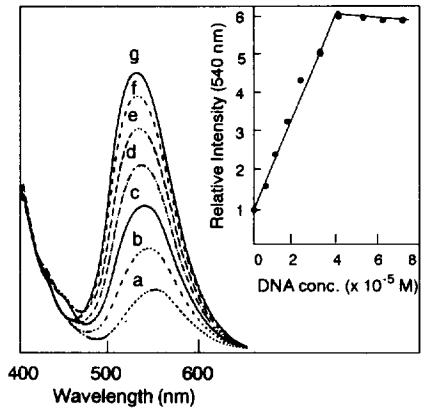


Figure 2. Fluorescence spectra for dansyl-modified oligonucleotide **1** (4 x 10^{-5} M) in the presence of 5'-dCCTCTAGAGTCGACCT (a: none; b: 6.6 x 10^{-6} M; c: 1.3 x 10^{-5} M; d: 2 x 10^{-5} M; e: 2.8 x 10^{-5} M; f: 3.3 x 10^{-5} M; g: 4 x 10^{-5} M). Change in the intensity at 540 nm is shown in the box. Measurements were carried out at 6 °C in a pH 7 phosphate buffer containing 1.0 M NaCl. Excitation wavelength was 330 nm.

We thus focused on investigating the fluorescence properties of the dansyl-oligonucleotide **1**. As shown in Figure 2, at the temperature (6 °C) well below the midpoint of the transition, the fluorescence intensity of the dansyl-labeled oligomer increased proportionally with stepwise addition of DNA. Addition of DNA over equimolar amount caused no further increase in the fluorescence intensity. The fluorescence quantum yield of the dansyl oligonucleotide duplex reached to be 0.134, while the single stranded dansyl oligomer exhibited the emission in a yield of 0.022. As with increase of the fluorescence intensity, blue-shift of the emission maximum by 17 nm was observed. These observations indicated that the attached dansyl group is transferred into a less-polar environment upon forming the duplex. On contrary, it has been shown that oligonucleotides attached to a dansyl group through a relatively long linker at the pyrimidine C-5 exhibit little or no change in fluorescence intensity upon forming duplexes with complementary DNA segments.[9]

The duplex formation-induced increase in fluorescence is further supported by the observation of the temperature-dependent change in the fluorescence intensity of the dansyl-modified duplex. The fluorescence melting profile is seen as a mirror image to that of the UV-melting curve for the dansyl-modified duplex (Figure 1). It was found that the fluorescence melting transition occurs at the lower temperature (tm^f=20.5 °C) than that obtained by the UV-melting profile. This is presumably attributable to the facts that the dansyl fluorophore is located between the base-pair of the terminal fraying end, the terminal base-pair tends to open firstly in the thermal denaturing process[10] and dansyl fluorescence is highly sensitive to a local environment.

Table 1 summerized the fluorescence property and tm values of dansyl-oligonucleotide duplexes formed with DNA sequences containing base-pair mismatches. Introduction of the mismatch base(s) into the target DNA caused relatively small enhancemant in fluorescence upon hybridization of dansyl-modified

Table 1. Fluorescence intensity and tm values for duplexes of dansyl-modified oligonucleotide **1** with DNA having base-pair mismatches.

DNA	Relative Fluorescence Intensity (540 nm)	tm (°C)
No.1: 5'-d*CCTCTAGA*GTCGACCT	6.10	33.2
No.2: 5'-d*CCTCTAG*CGTCGACCT	1.35	25.5
No.3: 5'-d*CCTCTAG*TGTCGACCT	1.42	22.3
No.4: 5'-d*CCTCTAC*AGTCGACCT	3.64	14.5
No.5: 5'-d*CCTCTAT*AGTCGACCT	2.18	18.2
No.6: 5'-d*CCTCTAA*CGTAGACCT	1.73	12.3
No.7: 5'-d*CCTCTAT*GGTAGACCT	1.74	11.6

Fluorescence and tm measuremetnts were carried out for duplexes (8×10^{-5} M) in a pH 7 buffer containing 1.0 M NaCl and 0.01 M sodium phosphate. Relative fluorescence intensities of duplexes were obtained at 6 °C based on the single-stranded dansyl-modified oligonucleotide. The DNA sequence complementary to dansyl-oligonucleotide is shown by italic. The mismatch base is underlined.

oligonucleotide when compared with the duplex of fullmatch DNA (No.1). In the presence of DNA (No. 2, 3, 6 and 7) having the base mismatch facing uracil residue in dansyl-oligonucleotide **1**, little fluorescence enhancement was observed. Introduction of mismatch base at the 3'-side of the uracil residue into the DNA sequences (No. 4 and 5) yielded two to three times larger fluorescence upon binding of dansyl-oligonucleotide **1**. These observations indicate that the base-pairing between the base in dansyl-modified nucleoside and its complement appears to be important to gain significant fluorescence signal on hybridization of the dansyl probe.

In conclusion, a dansyl-oligonucleotide modified at the specified sugar residue exhibits enhanced fluorescence in a sequence-specific manner upon hybridization to DNA. The present research provides a new concept for the design of a fluorescent-labeled oligonucleotide probe which may be particularly useful for homogeneous assay for nucleic acids sequences. A further study to extend the present design concept in the development of practically useful fluorescent probes is now underway in our laboratory.

Experimental Procedure

General Methods. Melting points were uncorrected. ^{1}H NMR was measured on a JEOL-JNM-EX-270 spectrometer using a residual peak at 2.49 ppm of DMSO-d_6 or 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 UV detector at 254 nm, using a reversed phase YMC PAK C_{18} column (6 x 150 mm) or cosmosil C_{18} column(4 x 150 mm). Column chromatography was carried out on Wako silica gel C-200. Ultraviolet (UV) spectra were recorded with a Hitachi U-3200 equipped with a thermoelectrically controlled cell holder (Hitachi SPR-7). Fluorescence spectra were measured on a JASCO FP-777 spectrometer in which temperature of the cell holder was controlled by a ATTO thermostatic circulator.

Materials and Solvents. 2'-Deoxy-2'-aminouridine was synthesized according to the literatures.[11] Bis-(diethylamino)phosphorochloridite was synthesized by the established procedure.[6a] Nuceleoside phosphoramidites and nucleoside-loaded CPG were purchased from ABI. DNA oligomers were synthesized on a ABI model 391 DNA synthesizer. Dansyl chloride (DNS-Cl, Sigma), snake venom phosphodiesterase

(Boehringer), and alkaline phosphatase (Boehringer) were obtained commercially. Dimethylformamide (DMF) was stirred in the presence of CaH_2, distilled under reduced pressure, and stored over CaH_2. Pyridine and triethylamine were dried by refluxing over CaH_2 at least 5 h and then distilled and stored over CaH_2.

Synthesis of 2'-(Dansylamino)-2'-Deoxyuridine [U(DNS)]. The reaction of 2'-amino-2'-deoxyuridine (0.21 g, 0.86 mmol) with DNS-Cl (0.47 g, 1.9 mmol) was carried out in DMF (4 mL) in the presence of triethylamine (0.36 mL) at 40°C for 2h. CH_2Cl_2 (50 mL) was added to the reaction mixture and then extracted with water (30 mL x 3). The combined aqueous layer was evaporated and then applied on a silica gel column eluted by using ethyl acetate-acetone (1:1, v/v). The compound was further purified by recrystallization from acetone to afford 0.31 g (75.2 %). M.p.: 232-233°C, ε(MeOH) at 260 nm and 345 nm: 16.5 x 10^3 and 3.8 x 10^3, ^{1}H NMR (DMSO-d6): δ=2.80 (s, 6H, N(CH_3)), 3.47 (m, 2H, C_5'), 3.84 (m, 1H, C_4'), 3.93 (m, 1H, C_2'), 3.98 (m, 1H, C_3'), 5.10(t, 1H, C_5'OH), 5.18 (d, 1H, uracil C5), 5.79 (d, 1H, C_3'OH), 5.84 (d, $J_{1',2'}$=8.25 Hz, 1H, C_1'), 7.28 (d, 1H, uracil C6), 7.18-8.40 (m, 6H, aromatic), 8.40 (d, 1H, SO_2NH), 11.50 (s, 1H, uracil NH).

Synthesis of 5'-Dimethoxytrityl U(DNS) [DMT-U(DNS)]. U(DNS) (0.31g, 0.65 mmol) was reacted with dimethoxytrityl chloride (0.44g, 1.3 mmol) in dry pyridine (4 mL) at r.t. for 2 h. Ethyl acetate (100 mL) was added to the reaction mixture and the solution was extracted with water (50 mL x 2). The organic layer was dried with Na_2SO_4. The solvent was evaporated to dryness and the residue was dissolved in a small amount of CH_2Cl_2. The solution was applied on a silica gel column (3 x 15 cm) which was eluted with CH_2Cl_2 followed by CH_2Cl_2-methanol (50:1, v/v). The appropriate fractions were collected and the solvent was removed. The residue was dissolved in a small amount of CH_2Cl_2 (5 mL) and the solution was added dropwise to hexane (100 mL). The white solid was corrected to afford 0.37 g (73.1 %) of the desired compound. M.p.: 176-178°C. ^{1}H NMR ($CDCl_3$): δ=2.87 (s, 6H, N(CH_3)2), 3.33 (m, 2H, C_5'), 3.79 (s, 6H, CH_3O-Ar), 3.88 (m, 1H, C_4'), 4.11 (m, 1H, C_2'), 4.23 (dd, 1H, C_3'), 5.02 (d, 1H, uracil C5), 6.00 (d, 1H, C_1'), 8.50 (s, 1H, SO_2NH), 6.81-8.18 (m, total 20H, Ar of trityl and naphtyl and uracil C6). Anal. Calcd for $C_{42}H_{42}N_4O_9S$: H, 5.44; C, 64.78; N, 7.19 Found: H, 5.66; C, 64.36; N, 7.05.

Synthesis, purification and enzymatic digestion of dansyl-oligonucleotides. 5'-dimethoxytrityl U(DNS) phosphorobisdiethylamidite was synthesized by the established procedure.[6a] The synthesis of dansyl-labeled oligonucleotides **1** and **2** was by a syringe technique according to the published protocol.[6a] The coupling efficiency of the bisamidite in each synthesis was estimated by a dimethoxytrityl cation to be 87 %. The purification of oligomers were carried out by a reversed phase HPLC (YMC PAK C_{18}) using a CH_3CN linear gradient (0.5 %/min) starting from 10 % CH_3CN in 0.1 M triethylammonium acetate (pH 7.0) at a flow rate of 1.0 mL/min. The purified oligomers (ca. 0.1 O.D.) were subjected to enzymatic digestion using snake venom phosphodiesterase (0.3 unit/mL) and alkaline phosphatase (100 unit/mL) in 50 mL of Tris-HCl (pH 7.2) at 37°C for 2 h. The reaction mixtures were analyzed by reversed phase HPLC (cosmosil C_{18}); elution with 0.05 M ammonium formate containing a 15 % CH3CN gradient (20 min) at a flow rate of 1.5 mL/min. The expected molar ratios of T, dA, dC, dG and U(DNS) were obtained for oligonuleotides **1** and **2** to be 1:2:1:3:1 and 1:3:2:3:1, respectively.

UV and fluorescence spectroscopic measurements. All solutions for the physical measurements were prepared by using a buffer containing 0.01 M sodium phosphate and 1.0 M NaCl, adjusted to pH 7.0.

Oligomer concentrations were determined based on the measured absorbance at 260 nm at 25°C and the single-strand extinction coefficients calculated based on a nearest-neighbor model.[12]

For UV-melting experiments, the temperature was raised from 0°C to 80°C at a rate of 0.5°C/min. All fluorescence spectra were obtained by excitation at 330 nm. The fluorescence quantum yields were measured using quinine sulfate in 0.1 N sulfuric acid as a standard.

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References

1. Drmanac, D.S.; Strezoska, Z.; Paunesku, T.; Labat, I.; Zeremski, M.; Snoddy, J.; Funkhouser, W.K.; Koop, B.; Hood, L.; Crkvenjakov, R. *Science* **1993**, 260, 1649-1652.
2. a) Cardullo, F.; Agrawal, S.; Flores, C.; Zamecnik, P.C.; Wolf, D.E. *Proc. Natl. Acad. Sci. USA* **1988**, 85, 8790-8794. b) Morrison, L.E.; Halder, T.C.; Stols, T.S. *Anal. Biochem.* **1989**, 183, 231-244. c) Oser, A.; Valet, G. *Angew. Chem. Int. Ed. Engl.* **1990**, 29, 1167-1169. d) Mergny, J.L.; Garestier, T.; Rougee, M.; Alexandre, A.V.; Chassignol, M.; Thoung, N.T.; Helene, C. *Biochemistry* **1994**, 33, 15321-15328.
3. Jenkins, Y.; Barton, J.K. *J. Am. Chem. Soc.* **1992**, 114, 8736-8738.
4. Wiederholt, K.; Rajur, S.; Giulian, Jr., J.; O'Donnell, M.J.; McLaughlin, L.W. *J. Am. Chem. Soc.* **1996**, 118, 7055-7062.
5. a) Chen, R.F.; Kernohan, J.K. *J. Biol. Chem.* **1967**, 242, 5813-5823. b) Li, Y.-H.; Chan, L.-M.; Tyer, L.; Moody, R.T.; Himel, C.M.; Hercules, D.M. *J. Am. Chem. Soc.* **1975**, 97, 3118-3126. c) Hoenes, G.; Hauser, M. Pfleiderer, G. *Photochem. Photobiol.* **1986**, 43, 133-137.
6. a) Yamana, K.; Nishijima, Y.; Ikeda, T.; Gokoda, T.; Ozaki, H.; Nakano, H.; Sangen, O.; Shimidzu, T. *Bioconjugate Chem.* **1990**, 1, 319-324. b) Yamana, K.; Aota, R.; Nakano, H.*Tetrahedron Lett.* **1995**, 36, 8427-8430. c) Yamana, K.; Mitsui, T.; Yoshioka, J.; Isuno, T.; Nakano, H. *Bioconjugate Chem.* **1996**, 7, 715-720.
7. Saenger, W. *Princples of Nucleic Acid Structure*, Springer-Verlag, Berlin, **1984**, pp.368-384.
8. A preliminary study has appeared: Ohashi, Y.; Yamana, K.; Nakano, H.; Sangen, O. *Nucleic Acids Res. Symposium Series* **1991**, No.25, 15-16.
9. a) Allen, D.J.; Darke, P.L.; Benkovic, S.J. *Biochemistry* **1989**, 28, 4601-4607. b) Allen, D.J.; Benkovic, S.J. *ibid.* **1989**, 28, 9586-9593. c) Guest, C.R.; Hochatrasser, R.A.; Dupuy, C.G.; Allen, D.J.; Benkovic, S.J.; Millar, D.P. *ibid.* **1991**, 30, 8759-8770. d) Barawkar, D.A.; Ganesh, K.N. *Nucleic Acids Res.* **1995**, 23, 159-164.
10. Patel, D.J.; Canuel, L.L. *Eur. J. Biochem.* **1979**, 96, 267-276.
11. a) Sowa, T; Tsunoda, K. *Bull. Chem. Soc. Jpn.* **1975**, 48, 505-507. b) Verheyden, J.P.H.; Wagner, D.; Moffatt, J.G. *J. Org. Chem.* **1971**, 36, 250-254.
12. Fasman, G.D., Ed. *Handbook of biochemistry and molecular biology: Nucleic Acids*, 2nd ed., CRC Press, Clevelend OH, **1985**, Vol. 1, p.589.

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