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SYNTHESIS OF OLIGONUCLEOTIDE DERIVATIVES CONTAINING PYRENE LABELED GLYCEROL LINKERS : ENHANCED EXCIMER FLUORESCENCE ON BINDING TO A COMPLEMENTARY DNA SEQUENCE

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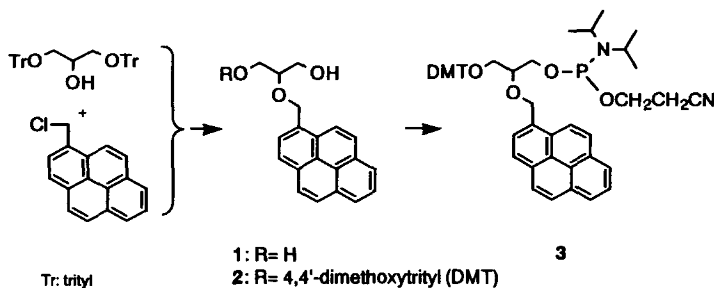
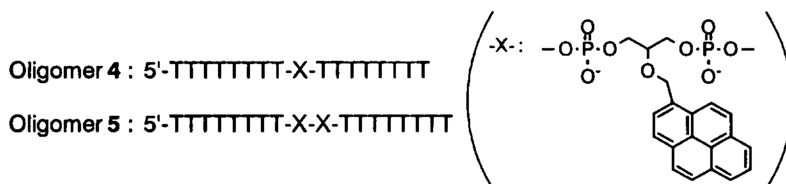
Abstract : The synthesis of a pyrene labeled glycerol linker for multiple internal labeling of oligonucleotides has been described. The oligonucleotide containing two successively incorporated pyrenyl residues exhibits enhanced excimer fluorescence upon hybridization to the complementary DNA segment. © 1997 Elsevier Science Ltd.

The design and synthesis of fluorescence-labeled oligonucleotides that exhibit significantly enhanced emission on hybridization to complementary nucleic acid sequences have been the subject of intense research. The oligonucleotide derivatives having such properties would be useful in a homogeneous hybridization assay for DNA sequences, leading to the route for easy automation of the assay. Several approaches to the synthesis of the oligonucleotide probes which display significant emission upon binding to DNA sequences. 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.[1] Other approach has been demonstrated by the oligonucleotide tethered to the ruthenium complex.[2] This approach is based on the intercalation-induced fluorescence enhancement of the designed metal complex. It has been shown that the fluorescent minor groove binding agent covalently attached to an oligodeoxyribonulceotide exhibits enhanced emission on hybridization to an appropriate sequence of DNA.[3]

We have been interested in pyrene as an attached fluorophore and developed the method for its introduction into the 2'-O-position of oligonucleotides.[4] It was shown that a pyrene group attached via the methylene linker into the specified 2'-hydroxyl function of oligopyrimidines exhibits a significantly enhanced signal derived from monomer emission upon hybridization to poly (rA).[5] Recently, this interesting fluorescence has also been observed for the pyrene-modified oligonucleotides containing all four bases.[6]





Other possibility of pyrene as an attached fragment involves the ability to form bimolecular complexes called excimers, consisting of one ground state and one excited state species, which are themselves fluorescent in the visible region (500 nm).[7] The recent report has demonstrated the utility of pyrene excimer as a signal for detection of hybrid formation of two oligonucleotides suitably labeled at the terminal positions with the complementary DNA.[8] It has also been shown that oligonucleotides attached through a terminal end to an appropriate moiety containing multiple pyrene residues exhibit significant excimer fluorescence.[9]

We describe here the synthesis of a new pyrene-modified fragment that is suitable for multiple internal labeling of oligodeoxyribonucleotides. The design of the present pyrene-modified fragment is based on the considerations that a glycerol unit covalently attached to a pyrenylmethyl substituent at the secondary hydroxyl function may be used as a linker group for multiple incorporation internally into DNA segments and that a glycerol-based linker may be a good spacer maintaining three-carbon internucleotide distance between the phosphates of oligonucleotides.[10] Therefore, our pyrene-labeled glycerol can be regarded as an acyclic counterpart of a pyrene-modified C-nucleoside that has been reported very recently.[11] In this report, we demonstrate that an oligothymidine possessing internally two pyrene labeled glycerols retains its normal binding affinity for the complementary DNA and exhibits a significantly enhanced excimer emission upon hybridization.

1,3-O-Ditrityl glycerol was allowed to react with 1-pyrenylmethyl chloride under reflux conditions in the presence of KOH in benzene-dioxane for 2.5 h. The resulting material was treated with 0.2 N HCl in THF at r.t. overnight, affording 2-O-(1-pyrenylmethyl)glycerol **1**[12] (44 % yield from the protected glycerol). One of the hydroxyl group of **1** was then protected with a dimethoxytrityl group. The reaction of **1** (0.7 g, 2.3 mmol) with 4,4'-dimethoxytrityl chloride (0.66 g, 2.0 mmol) in pyridine at r.t. for 2.5 h followed by a silica gel column chromatography gave the protected pyrene-labeled glycerol **2**[13] in a yield of 40 %. The remaining hydroxyl function of the compound **2** was converted to phosphoramidite **3** by the usual procedure.[14] Oligothymidines containing one or two pyrene labeled glycerols **4** and **5** were then synthesized by a slightly modified phosphoramidite protocol on a DNA synthesizer.[15] Purification of the oligomers was done with a usual denaturing polyacrylamide gel electrophoresis.[15] The TOF-mass analysis for these oligomers gave satisfactory results.[16]

The binding of oligomers **4** and **5** to their complements[17] was investigated by the UV melting behaviors in a pH 7 buffer solution containing 0.1 M NaCl and 0.01 M sodium phosphate. The UV melting profile at 260 nm for the solutions containing the oligomer **4** and dA_{17} exhibited sigmoidal curve whose shape is similar to the unmodified oligonucleotide duplex, T_{17}-dA_{17}. Although the oligomer **4** does not have full-match bases to its complement, dA_{17}, the midpoint of the melting transition (tm = 38°C) was found to be slightly higher than that (tm = 35°C) of T_{17} - dA_{17}. It was indeed shown that the duplex of dA_{17} formed with 5'-T_8AT_8 which

contained one base mismatch had considerably lower tm (22°C). The melting profile for the duplex of oligonucleotide **5** with dA_{18} also exhibited sigmoidal curve whose shape is similar to the unmodified oligonucleotide duplex, T_{18}-dA_{18}. Although the oligomer **5** contains two base mismatches, the tm value (34°C) for the modified duplex exhibited slightly lower than that (37°C) of the corresponding unmodified duplex.

The fluorescence spectra for oligomer **4** and **5** before and after hybrid formation with their complementary DNA segments were shown in Figure 1. A significant increase in the pyrene monomer fluorescence of **4** was observed upon hybridization to its complementary strand, dA_{17}. No other structureless band in the fluorescence spectrum for **4** was observed. The oligonucleotide containing two pyrenyl residues **5** in which the two chromophores are separated by 11 atoms exhibited a dual fluorescence. One is a structured part around 400 nm similar to the emission of the oligomer **4** and the other is a structureless band shifted to longer wavelength around 500 nm. The fluorescence excitation spectra scanned on the structures and the structureless bands are identical, thus indicating that the structured and the structureless band are ascribable to the monomer fluorescence and the intramolecular excimer emission, respectively. In the single stranded state, the excimer-to-monomer fluorescence intensity ratio was 0.72. Upon hybridization to the complementary DNA strand, dA_{18}, the excimer fluorescence was significantly enhanced, resulting in the excimer-to-monomer fluorescence intensity ratio of 2.56.

The binding of oligomer **4** or 5'-X-T_8 (X indicates the pyrenyl labeled linker) with dA_8 resulted in formation the relatively unstable duplex (tm = 12°C) in which the fluorescence enhancement did not occur upon hybrid formation. Similarly the hybridization of **5** or 5'-XX-T_8 with dA_8 did not yield both the monomer and the excimer emission enhancement. These observations clearly indicate that the observed fluorescence enhancement is derived from the duplexes with internal labeling by the pyrenyl linker(s). The mechanistic details accounting for the present results should await further experiments involving fluorescence lifetime measurements.

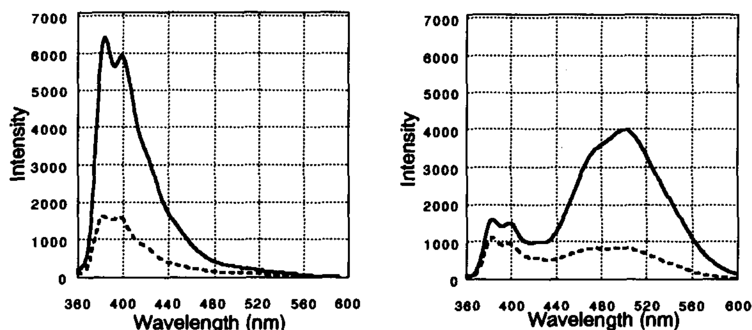


Figure 1. Fluorescence spectra for duplexes of oligomer **4** (Left Panel) and **5** (Right Panel) in the presence (——) and absence (- - -) of complementary DNA (dA_{17} for **4**, dA_{18} for **5**). The measurements were carried out at 5°C in a pH 7 buffer containing 0.1 M NaCl and 0.01 M sodium phosphate. Excitation wavelength was 338 nm.

We have synthesized the pyrene labeled glycerol linker which can be used for incorporation into the internal position(s) of oligodeoxyribonucleotides. Important findings of this research are that the oligonucleotides with two successively incorporated pyrenyl residues exhibited enhanced excimer fluorescence upon hybrid formation with the complementary DNA segment. Further study to elucidate the mechanism of the observed fluorescence and to evaluate the potential of the pyrenyl linker in development of new fluorescent DNA probes are now in progress.

Acknowledgment

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12. Compound **1**. M.p.: 183-185°C; Rf(Merck 60PF, ethylacetate-hexane=1:3, v/v): 0.45; 270 MHz ^1H NMR(DMSO-d6): δ= 3.49-3.62 (5H, m, $-CH_2CHCH_2-$), 4.63 (2H, t, OH), 5.52 (2H, s, $ArCH_2O$), 8.04-8.47 (9H, m, aromatic of pyrene).
13. Compound **2**. M.p.: 71-73°C; Rf(Merck 60PF, CH_2Cl_2-MeOH:50:1, v/v): 0.43; 270 MHz ^1H NMR(DMSO-d6): δ= 3.10 (2H, d, $DMTOCH_2-$), 3.47-3.60(2H, m, $-CH_2OH$), 3.64 (6H, s, CH_3O), 3.79 (1H, m, CH), 4.70 (1H, t, OH), 5.34 (2H, d, Jgem= 11.88 Hz, $ArCH_2O-$), 7.05-7.32 (13H, m, aromatic of DMT), 8.03-8.50 (9H, m, aromatic of pyrene).
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16. The TOF Mass spectra were obtained on a Bruker ReflexII model using a negative linear mode. Oligomer **1** : 5172.0 (calcd.5172.5), oligomer **2** : 5540.0 (calcd.5540.8).
17. All the duplexes studied here consist of 1:1 molar ratio of oligonucleotide strands at a total concentration of 5×10^{-5} M.

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