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A New Method for Introduction of a Pyrene Group into a Terminal Position of an Oligonucleotide

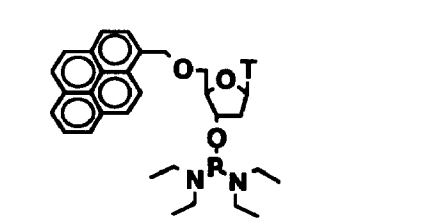
Kazushige Yamana,* Kenji Nunota, Hidehiko Nakano, and Osamu Sangen

Department of Applied Chemistry, Himeji Institute of Technology, 2167 Shosha, Himeji, Hyogo 671-22, Japan

Abstract: *The method for introduction of a pyrenylmethyl group into a 5'- or 3'-terminal hydroxyl function of oligonucleotides has been described. The incorporation of pyrene into the 5'-end was accomplished by preparation of 5'-(1-pyrenylmethyl)thymidine 3'-phosphorobisdiethylamidite which was used for the solid-phase synthesis of 5'-pyrene-modified oligonucleotides. 5'-Dimethoxytrityl 3'-(1-pyrenylmethyl)uridine loaded silica was prepared and used for the solid-phase synthesis of 3'-modified oligonucleotides. The purification of the oligonucleotide-pyrene conjugates thus synthesized was effected by reversed phase HPLC. The fluorescence of these oligonucleotides was not quenched after the hybrid formation with the complementary DNA.*

Fluorescent labeling of oligonucleotides have been attracting current interest in connection with the development of new methods for distinguishing and detecting specific nucleic acid sequences.[1] A pyrene group has been chosen as an attached fragment because of its interesting fluorescent and structural properties, and has been introduced via *a relatively long linker* into oligonucleotides at an internal[2] or a 5'-terminal phosphorus.[3] It has been shown that these pyrene-oligonucleotide conjugates bind to their complementary DNA resulting in considerable quenching of pyrene fluorescence due to its intercalation.[3a] In order to efficiently monitor the hybrid formation with the complementary DNA both in heterogeneous and homogeneous systems, the fluorescent signal should not be weaken after the hybridization. For this purpose, the intercalation of pyrene should be avoided upon forming the complex between pyrene-oligonucleotides and their complementary DNA. We describe here a new chemical method for introduction of pyrene via *a relatively short linker* into a 3'- or 5'-terminal hydroxyl group of oligonucleotides. Spectroscopic studies show that the fluorescence of the attached pyrene is not quenched after the hybrid formation with the complementary DNA. The present pyrene-oligonucleotide derivatives provide promising candidates as new hybridization probes for DNA.

Incorporation of pyrene via a methylene linker into 5'-hydroxyl function of oligonucleotides was initiated by preparation of 5'-(1-pyrenylmethyl)thymidine, 5'-(pyr)T, which was then converted to the phosphorobisdiethylamidite derivative **1**. This reagent was used for the solid-phase synthesis of oligonucleotides with a pyrenylmethyl group. 3'-Dimethoxytritylthymidine (2.0 g, 3.7 mmol) was allowed to react with 1-pyrenylmethyl chloride (1.2 g, 4.5 mmol) in the presence of excess potassium hydroxide (1.5 g) in a mixture of benzene-dioxane (20 mL-10 mL) under refluxed conditions for 2 h. The successive treatment with 80 % acetic acid at r.t. for 15 min gave 5'-(pyr)T (0.55 g, 33 %).[4] 5'-(pyr)T was then phosphitylated with bis(diethylamino)phosphorochloridite in a usual manner,[5] yielding 5'-(pyr)T 3'-phosphorobisdiethylamidite. 5'-(pyr)TGCCAAAAACCTGCC **2** was synthesized manually by coupling (10 min) of 5'-(pyr)T phosphorobisamidite (0.2 M) in the presence of 0.1 M p-nitrophenyltetrazole in CH_3CN with the 5'-OH of the fully protected oligonucleotide (0.2 μmol) bound to CPG.[6] After hydrolysis and successive oxidation in a usual way,[5] deprotection was carried out by treatment with conc.NH_4OH at 55°C for 10 h. The desired



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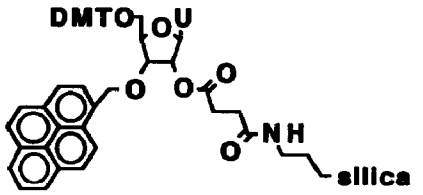

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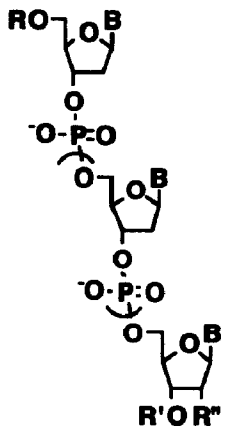

5'-(pyr)TGCCAAAAACCTGCC
2 : R= 1-pyrenylmethyl
R'= R"=H
5'-CCGCGTGGTCGACTU(pyr)
& 5'-CCGCGTGGTCGACU(pyr)
4 & 5 : R'= 1-pyrenylmethyl
R=H, R"=OH

oligonucleotide was purified by reversed phase HPLC (YMC-packed C_{18}, 6 x 150 mm). The elution was carried out by a linear gradient (1%/min) of CH_3CN starting from 10 % CH_3CN in 0.05 M TEAA (pH 7.0) at a flow rate of 1.0 mL/min. With this conditions, the pyrene-modified oligonucleotide (appeared at 27.0 min) could be separated very well from the failed sequence (at 4.5 min).[7] Thus the pure oligomer was isolated in a yield of 5.7 O.D. unit at 260 nm. The base composition and the presence of 5'-(pyr)T in the oligonucleotide **2** were verified by enzymatic digestion analysis[5] (5'-(pyr)T:dC:dG:T:dA=0.93:6.0:1.9:1.0:5.1) and UV-vis spectrum (selected characteristic λmax: 265, 348 nm).

Alternatively, introduction of pyrene into 3'-OH of oligonucleotides was initiated by preparation of 5'-dimethoxytrityl 3'-(1-pyrenylmethyl)uridine bound to silica **3**. 2',5'-Ditrityluridine was derivatized with a pyrenylmethyl group at 3'-OH by essentially same procedure reported for the preparation of 2'-pyrene-modified uridine.[8] By this procedure, 3'-(1-pyrenylmethyl)uridine, U3'-(pyr), was obtained in a yield of 66 %.[9] The nucleoside, U3'-(pyr), was reacted with 4,4'-dimethoxytrityl chloride (DMT-Cl) in dry pyridine at r.t. for 2 h, giving 5'-DMT U3'-(pyr) (62 %).[10] The DMT-nucleoside was covalently attached via 2'-OH to aminopropylsilica in a usual manner,[11] giving the DMT-nucleoside loaded silica **3** (31.6 μmol/g). This silica was applied to DNA synthesizer[6] to prepare the oligonucleotides with pyrene at 3'-terminus. After deprotection by the same way described above, the DMT-oligonucleotides were purified by reversed phase HPLC (YMC-packed C_{18}, 6 x 150 mm).[12] After treatment with 80% acetic acid (r.t., 15 min), the final purification was effected by the same HPLC for the 5'-modified oligonucleotide, yielding 5'-CCGCGTGGTCGACTU(pyr) **4** (R.T.: 22.8 min; 3.7 O.D. unit from 0.2 μmol synthesis scale) and 5'-CCGCGTGGTCGACU(pyr) **5** (R.T.: 22.1 min; 11.2 O.D. unit from 1.0 μmol synthesis scale). The base composition and the presence of U3'-(pyr) in the oligonucleotides **4** and **5** were

verified by enzymatic digestion analysis[13] (dC:dG:T:dA:U3'-(pyr)= 5.1:5.0:3.0:1.0:0.97 for 4; 5.0:4.7:2.0:1.1:1.1 for 5) and UV-vis spectra (selected λmax: 262, 353 nm for 4; 261, 352 nm for 5).

The interaction of the pyrene-oligonucleotides with the DNA oligomer containing complementary sequence was spectrophotometorically investigated in a phosphate buffer (pH 7.0) containing 0.1 M NaCl. The UV-melting curves at 260 nm for the 1:1(mol:mol) mixtures of the pyrene-oligonucleotide (2 or 4) with the complementary DNA (5'-GGCAGGTTTTTGGCAAAGTCGACCACGCGG) are shown in Figure 1. Both profiles show typical sigmoidal curves and exhibit a clear melting transition, giving the tms of 61.2°C (hypochromicity: 12.3 %) for duplex of 2 and of 68.5°C (hypochromicity: 14.5 %) for 4.[14] Figure 2 represents the fluorescence spectra of 2 and 4 in the absence and presence of the DNA 30-mer at 20°C. In each case, the fluorescence due to pyrene was not quenched or rather slightly enhanced upon duplex formation of the pyrene-oligonucleotide with the complementary DNA. The addition of DNA 30-mer (1 molar equiv) to a solution of the pyrene-oligonucleotide at 20°C gave only slight hypochromicity (8.5 % for 2; 2% for 4) at the UV absorption due to pyrene between 300 - 360 nm: red-shift of the absorption maxima was not observed. These fluorescence and absorption spectroscopic studies suggest that, owing to the short linker, the pyrene attached to the oligonucleotide does not intercalate into the base-pairs of the duplex.

Since the attached pyrenes are relatively free from interaction with nucleic acid bases in the template DNA, interesting possibility would involve the pyrene-excimer formation in the ternary complex of 2, 4 (or 5) and the DNA 30-mer. One could see that the pairs of sequences of 2 and 4(or 5) are appropriate for two pyrene to be accessible each other upon forming the complex. Although we observed a clear melting profile (tm: 62.9°C) for the 1:1:1 mixture of 2, 4 and DNA 30-mer, only monomer emission was detected at 20°C in the fluorescence spectrum. We are now exploring new systems for detecting specific DNA by using pyrene-excimer emission as a measurable signal.

In summary, we have developed a new method for incorporation of pyrene through a methylene linker into 5'- or 3'-hydroxyl group of oligonucleotides. The syntheses of these oligomers were easily conducted on DNA synthesizer by using a pyrene-modified nucleoside phosphorobisamidite and a pyrene-modified nucleoside loaded

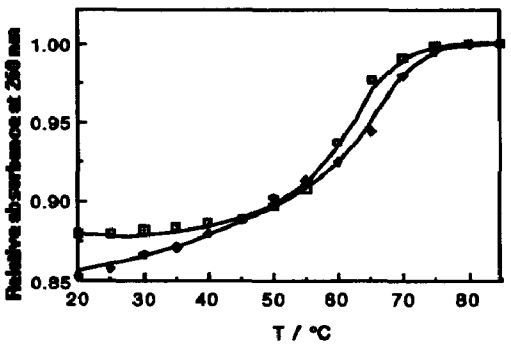

Figure 1. UV-melting curve for the mixture (total strand concentration: 4 x 10^{-5} M) of pyrene-oligonucleotide with DNA 30-mer.

□ : oligomer 2
◆ : oligomer 4

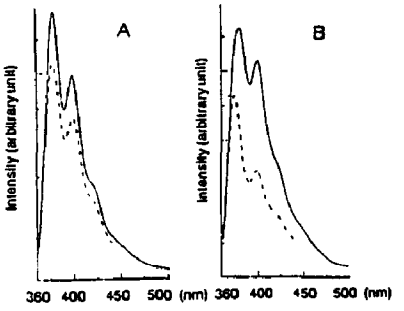

Figure 2. Fluorescence spectra of pyrene-oligonucleotides (2 x in 10^{-5} M) in the absence (dashed line) and presence (solid line) of an equimolar DNA 30-mer. Excitaion at 328 nm.
Panel A: oligomer 2
Panel B: oligomer 4

silica. The isolation of the oligonucleotide derivatives was accomplished by a usual reversed phase HPLC. The properties of the pyrene-oligonucleotides synthesized here would be satisfied in use of these oligomers as probes for specific DNA.

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REFERENCES AND NOTES

1. a) Letsinger, R.L.; Schott, M.E. *J. Am. Chem. Soc.* **1981**, 108, 7394. b) Sun, J-S.; Francois, J-C.; Montenay-Garestier, T.; Saison-Behmoaras, T.; Roig, V.; Thoung, N.T.; Helene, C. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 9198. c) Telser, J.; Cruckshank, K.A.; Morrison, L.E.; Netzel, T.; Chan, C. *J. Am. Chem. Soc.* **1989**, 111, 7226. d) Oser, A.; Valet, G. *Angew. Chem. Int. Ed. Engl.* **1990**, 29, 1167. e) Murakami, A.; Nakaura, Y.; Nakatsuji, Y.; Nagahara, S.; Tran-Cong, Q.; Makino, K. *Nucleic Acids Res.* **1991**, 19, 4097. f) Fideanza, J.A.; Ozaki, H.; Maclaughlin, L.W. *J. Am. Chem. Soc.* **1992**, 114, 5509. g) Jenkins, Y.; Barton, J.K. *J. Am. Chem. Soc.* **1992**, 114, 8736.
2. Yamana, K.; Letsinger, R.L. *Nucleic Acids Res. Symposium Series* **1985**, No.16, 169.
3. a) Mann, J.S.; Shibata, Y.; Meehan, T. *Bioconjugate Chem.* **1992**, 3, 554. b) Korshun, V.A.; Pestov, N.B.; Birikh, K.R.; Berlin, Y.A. *Bioconjugate Chem.* **1992**, 3, 559.
4. 5'-(pyr)T: mp 223-226°C; TLC (silica 60F254, CH_2Cl_2-MeOH 20:1, v/v) Rf 0.23; ^1H NMR (270 MHz, DMSO-d_6) δ 1.21 (d, 3H, CH_3 of thymine) δ 2.08 (dd, 2H, H_2') δ 3.86 (d, 2H, H_5') δ 3.98 (m, 1H, H_4') δ 4.34 (m, 1H, H_3') δ 5.29 (d, 2H, $ArCH_2$) δ 5.37 (d, 1H, 3'-OH) δ 6.19 (t, 2H, H_1') δ 7.44 (s, 1H, thymine H_6) δ 8.06-8.44 (m, total 9H, ArH of pyrene) δ 12.06 (s, 1H, NH of thymine)
5. Yamana, K.; Nishijima, Y.; Ikeda, T.; Gokota, T.; Ozaki, H.; Nakano, H.; Sangen, O.; Shimidzu, T. *Bioconjugate Chem.* **1990**, 1, 319.
6. The chain elongation of fully protected oligonucleotides on a solid support was carried out by a standard phosphoramidite chemistry using DNA synthesizer (Applied Model 394). With a standard protocol, the average coupling efficiency in the synthesis of oligonucleotides was more than 98 %.
7. The coupling efficiency of the bisamidite was roughly estimated to be 85 % based on the HPLC.
8. Yamana, K.; Ohashi, Y.; Nunota, K.; Kitamura, M.; Nakano, H.; Sangen, O.; Shimidzu, T. *Tetrahedron Lett.* **1991**, 32, 6347.
9. U3'-(pyr): mp 178-180°C; TLC (silica 60F254, CH_2Cl_2-MeOH 20:1, v/v) Rf 0.12; ^1H NMR (270 MHz, DMSO-d_6) δ 4.07 (m, 1H, H_4') δ 4.14 (dd, 1H, H_3') δ 4.39 (dd, 1H, H_2') δ 5.37 (d, 2H, $ArCH_2$) δ 5.60 (d, 1H, uracil H_5) δ 5.89 (d, 1H, H_1') δ 7.85, (d, 1H, uracil H_6) δ 8.05- 8.33 (m, total 9H, ArH of pyrene)
10. DMT U3'-(pyr): mp 102-104°C; TLC (silica 60F254, CH_2Cl_2-MeOH 20:1, v/v) Rf 0.35; ^1H NMR (270 MHz, DMSO-d_6) δ 3.14 (m, 2H, H_5') δ 3.61 (s, 6H, CH_3O of DMT) δ 4.15 (m, 1H, H_4') δ 4.24 (dd, 1H, H_3') δ 4.51 (dd, 1H, H_2') δ 5.19 (d, 2H, $ArCH_2$) δ 5.50 (d, 1H, 2'-OH) δ 5.75 (d, 1H, uracil H_5) δ 5.82 (d, 1H, H_1') δ 6.62-7.16 (m, total 13H, ArH of DMT) δ 7.75 (d, 1H, uracil H_6) δ 8.06-8.39 (m, total 9H, ArH of pyrene) δ 11.39 (s, 1H, NH of uracil)
11. LC grade silica (pore size: 300 A; particle size: 60-150 μm) was converted to the aminopropyl derivative and then covalently attached through succinyl linkage to the nucleoside according to the literature procedure; Atkinson, T.; Smith, M. in *Oligonucleotide Synthesis: A Practical Approach* **1984** (M.J. Gait Ed.), 35, IRL press, Oxford, UK.
12. The elution was carried out by a linear gradient of CH_3CN (1%/min) from 20 % CH_3CN in 0.05 M TEAA (pH 7.0) at a flow rate of 1.0 mL/min. With this conditions, the desired DMT-oligonucleotides could be separated very well from the small amount of the failed sequence.
13. Enzymatic digestion was carried out by a solution (100 μL) containing 0.1 O.D. unit of substrate and calf spleen phosphodiesterase (4 unit/mL) and alkaline phosphatase (100 unit/mL) in 50 mM sodium cacodylate buffer (pH 7.0) at 37°C for 2 h.
14. Similarly, tm value for duplex of **5** was estimated to be 65.5°C.

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