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INCORPORATION OF A PYRENE NUCLEOSIDE ANALOGUE INTO SYNTHETIC OLIGODEOXYNUCLEOTIDES USING A NUCLEOSIDE-LIKE SYNTHON

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Abstract: A novel phosphoramidite **4** based on the hydroxyprolinol **1** backbone has been synthesized and used to chemically prepare DNA fragments bearing a pyrene-containing nucleoside analogue.

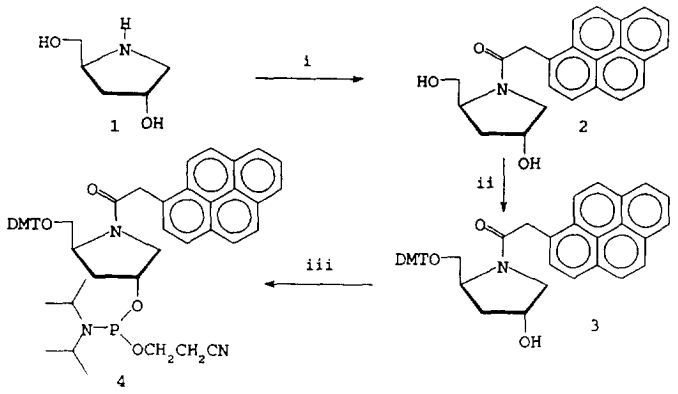
The attachment of fluorescent dyes to oligonucleotides[1] is widely used in the preparation of DNA probes. In this context, the dependence of the emission properties of fluorophores on their covalent attachment to oligonucleotides and the hybridization of such probes to complementary targets[2], and the fluorescence resonance energy transfer (FRET) between different fluorophores linked to DNA[3] are of particular interest.

Our aims are to develop methods of synthesis and to investigate the properties of oligonucleotide conjugates exhibiting fluorescent emission characteristics that are strongly dependent on the microenvironment. A simple model is pyrene, a polycyclic aromatic hydrocarbon, easily capable of being functionalized without significantly altering its fluorescent properties[4]. Remarkable features of pyrene derivatives are: a) intercalation into DNA duplexes[5]; b) formation of an excimer ("excited dimer") resulting in an essential shift of the emission maximum[4,6]; c) a long lifetime of the excited state (up to 0.1 μs) accompanied by a considerable decrease of the fluorescence quantum yield in the presence of quenchers[4].

Various methods for the introduction of a pyrene residue into oligonucleotides include the use of modifying phosphoramidites.[2c,7] Here we describe the synthesis of a novel and convenient reagent, which allows introduction of pyrene residues into oligonucleotides at any predetermined site(s). Such conjugates are suitable for studying pyrene-nucleic bases stacking interactions and fluorescence quenching from non-radiative inactivation or FRET.

As a pseudosugar unit we selected the chiral amidodiol hydroxyprolinol **1** (Scheme), which was easily prepared by the reduction of the minor natural amino acid 4-L-hydroxyproline[8]. Acylation of the compound **1** (as the hydrochloride) at the imino group with an activated (pentafluorophenyl) ester of (1-pyrenyl)acetic acid[9] produced the pyrene nucleoside analogue **2**. In pyridine this reaction occurred very slowly; the addition of a very strong acylation catalyst (e.g. DMAP) accelerated the reaction but also led to non-specific acylation at the

Scheme

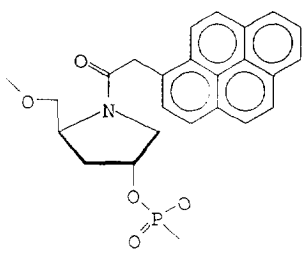


i: Pentafluorophenyl (1-pyrenyl)acetate, Et_3N, pyridine
ii: DMT-Cl, pyridine
iii: $NCCH_2CH_2OP(NPr^i_2)_2$, diisopropylammonium tetrazolide, MeCN

hydroxyl groups. The reagent of choice for this reaction was an equivalent amount of triethylamine which, when added, led to the production of the amidodiol **2** in 81% yield within 1-3 h[10]. Dimethoxytritylation of this diol at the primary OH-group with DMT-Cl in pyridine proceeded with less selectivity than in the case of standard N-protected 2'-deoxynucleosides (bis-DMT-derivative was the major by-product), apparently because of reduced steric hindrances, to afford compound **3** in 63% yield. The phosphitylation of alcohol **3** with bis(diisopropylamino)-2-cyanoethoxyphosphine[11] and diisopropylammonium tetrazolide as a catalyst in acetonitrile gave phosphoramidite **4** (yield 65%), which can be used in automated DNA synthesizers like a 2'-deoxynucleoside phosphoramidite[12] to allow the introduction of the pyrene nucleoside analogue at any position within the synthetic oligonucleotide.

5: T_9PyrT_{10}
6: $T_{10}PyrT_9$
7: A_9PyrA_{10}
8: $A_{10}PyrA_9$
9: $A_{10}Pyr_2A_9$
10: AGCTTGPyrCTGCAGGTCACGGATCCCCGGG

Pyr =



Oligonucleotides **5-10** (all 5'→3') were synthesized as examples[13]. Their fluorescence is well visible in PAG (ca. 0.01 OD_{260} in a 1-cm wide band). The presence of the pyrene residues in the oligonucleotides was

also evidenced by characteristic long-wave absorption peaks in UV spectra of the conjugates (Fig. 1). The complex-forming and fluorescent properties of the conjugates, obtained with the use of phosphoramidite **4**, are under study and will be reported elsewhere.

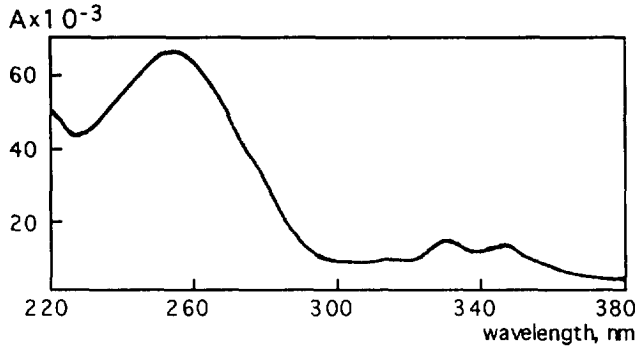


Figure 1. UV spectrum of oligonucleotide **9** in water at pH 7.0.

When this work was in progress, a report appeared on the use of hydroxyprolinol-based phosphoramidites in the preparation of combinatorial libraries of non-nucleotide phosphodiester oligomers[14].

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10. The NMR spectra were acquired on a Bruker AC-500 spectrometer in $CDCl_3$. Analytical TLC was performed on precoated Kieselgel 60 F_{254} aluminium-backed plates (Merck). Procedures were as follows. *(2S,4R)-4-Hydroxy-1[(1-pyrenyl)acetyl]pyrrolidine* **2**: A solution of pentafluorophenyl (1-pyrenyl)acetate (97 mg, 0.23 mmol), L-hydroxyprolinol **1** hydrochloride (36 mg, 0.23 mmol), and Et_3N (32 µL, 0.23 mmol) in dry pyridine (5 mL) was stirred for 2 h, then diluted with CH_2Cl_2 (50 mL), washed with 10% aq. H_2SO_4 (2x100 mL) and 10% $NaHCO_3$ (20 mL), dried (Na_2SO_4), and chromatographed on silica gel using 5% MeOH in CH_2Cl_2 (v/v) as an eluent to give pure **2** (66 mg, 81%) as a white foam, R_f 0.35 (THF). ^1H NMR (δ, ppm): 8.16-7.71 (m, 9H, Ar*H*), 4.35 (m, 1H, OC*H*), 4.16 (m, 2H, ^2J = 16 Hz, ArCH_2), 4.10 (m, 1H, NC*H*), 3.62 (m, 1H, OCH_2), 3.49 (m, 1H, OCH_2), 3.43 (m, 1H, ^2J = 12 Hz, NCH_2), 3.22 (m, 1H, ^2J = 12 Hz, NCH_2), 1.92 (m, 1H, ^2J = 14 Hz, CHCH_2CH), 1.49 (m, 1H, ^2J = 14 Hz, CHCH_2CH). *(2S,4R)-4-Hydroxy-2-[(4,4'-dimethoxytrityl)oxymethyl]-1-[(1-pyrenyl)acetyl]pyrrolidine* **3**: To a stirred and cooled (0°C) solution of **2** (90 mg, 0.25 mmol) in dry pyridine (5 mL) was added in one portion DMT-Cl (85 mg, 0.25 mmol) and stirring was continued for 2 h at 0°C, and then 4 h at ambient temperature. The mixture was diluted with CH_2Cl_2 (50 mL), washed with 10% $NaHCO_3$ (2x20 mL), dried (Na_2SO_4), and chromatographed on silica gel using 1% MeOH and 1% Et_3N in CH_2Cl_2 (v/v) to give **3** (104 mg, 63%) as a yellowish foam, R_f 0.58 (THF). ^1H NMR (δ, ppm): 8.19-6.68 (m, 18H, Ar*H*), 4.53 (m, 1H, OC*H*), 4.33 (s, 2H, ArCH_2), 4.06 (m, 1H, NC*H*), 3.74-3.67 (m, 6H, OCH_3), 3.65 (m, 1H, OCH_2), 3.57 (m, 1H, OCH_2), 3.50 (m, 1H, NCH_2), 3.15 (m, 1H, NCH_2), 2.12 (m, 1H, CHCH_2CH), 1.93 (m, 1H, CHCH_2CH). *(2S,4R)-2-[(4,4'-dimethoxytrityl)oxymethyl]-1-[(1-pyrenyl)acetyl]-4-[(2-cyanoethoxy)-(diisopropylamino)-phosphinyloxy]pyrrolidine* **4**: Compound **3** (120 mg, 0.18 mmol) was coevaporated with dry MeCN (2x10 mL), dissolved in dry MeCN, and then diisopropylammonium tetrazolide (16 mg, 0.09 mmol) and bis(diisopropylamino)-(2-cyanoethoxy)phosphine (86 µL, 0.27 mmol) were added. The reaction mixture was stirred under argon for 2 h, evaporated to dryness and chromatographed on silica gel (CH_2Cl_2-hexane 1:1 (v/v) with 0.5% Et_3N). Pure compound **4** was dissolved in toluene (2 mL) and precipitated in hexane (30 mL) to give **4** in a form suitable for direct use in a synthesizer (101 mg, 65%, yellowish amorphous solid), R_f 0.77 ($CHCl_3$-Et_3N 90:1 (v/v), spots of the diastereomers are not distinguishable). ^1H NMR (δ, ppm): 8.21-6.65 (m, 18H, Ar*H*), 4.61 (m, 1H, OC*H*), 4.48 (m, 1H, NC*H*CH_2), 4.40 (m, 2H, ArCH_2), 3.78-3.69 (m, 6H, OCH_3), 3.62-3.43 (m, 6H, DMTOCH_2, NCH_2 (1H), NC*H*CH_3, POCH_2), 3.13 (m, 1H, NCH_2), 2,52 (t, 2H, J = 7 Hz, NCCH_2), 2.32-2.09 (m, 2H, CHCH_2CH), 1.12 (d, 12H, J = 7 Hz, CHCH_3). ^{31}P NMR, 202.49 MHz, 85% H_3PO_4 as an external standard (δ, ppm): 148.37 and 147.60 (diastereomers).

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12. Oligonucleotide syntheses were performed on an Applied Biosystems 380B DNA synthesizer following manufacturer's instruction. Phosphoramidite **4** was used as a 0.1 M solution in MeCN. The stepwise yields, evaluated by the spectrophotometric monitoring of the dimethoxytrityl release at each cycle of the synthesis, were no different from those obtained using phosphoramidites of the conventional bases.

13. After the chain assembly, the oligonucleotides were cleaved from the support with concentrated ammonia at room temperature using manufacturer's end procedure cycle, deprotected (55°C, 6 h), precipitated with acetone from 1 M $LiClO_4$ and purified by 20% PAGE. The products bands visualized by UV shadowing at 254 nm were cut out, and the oligonucleotides were eluted and desalted on a Sephadex G 25 column.

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