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Synthesis and hybridization of dodecadeoxyribonucleotides containing a fluorescent pyridopyrimidine deoxynucleoside

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ABSTRACT

Partially self-complementary dodecadeoxyribonucleotides containing a fluorescent nucleoside, 3-β-D-2'-deoxyribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine (pyridopyrimidine deoxynucleoside, dF) were synthesized by the phosphotriester solid-phase method. A dodecanucleotide d(GGGAAFGTTCCC) pairing the analog and guanine at the centre of the chain showed a higher melting temperature than the corresponding G-C paired duplex. A similar comparison between A-T and A-F suggested that weaker hydrogen bonds exist when adenine and pyridopyrimidine residues are paired.

INTRODUCTION

3-β-D-2'-Deoxyribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine (1)(pyridopyrimidine deoxynucleoside or dF)[1] and its ribo counter part[2] are fluorescent nucleosides. This analogue is considered to form hydrogen bonds with guanine and adenine as illustrated in Fig 1. To investigate the hybridizing properties of pyrido-pyrimidine, partially self-complementary dodecadeoxyribo-nucleotides containing the analog (F), d(GGGAAFXTTCCC) (X=T,C,A,G) have been synthesized. The corresponding natural fragments containing d(CG) and d(TA) have been prepared for comparison. The sequences of dodecamers are shown in Fig. 2. The thermal stabilities, CD spectra and fluorescent properties of these oligonucleotides are also reported in the present paper.

MATERIALS AND METHODS

General methods Ultraviolet spectra were recorded with a Shimadzu-240 spectrophotometer. UV temperature profiles were recorded with a Beckman DU-8B spectrophotometer. CD spectra and

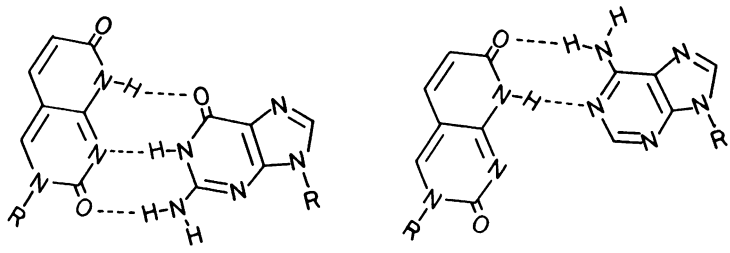

Figure 1. Possible hydrogen bonds between pyridopyrimidine and guanine or adenine.

fluorescent spectra were measured with a JASCO 500A spectrophotometer and a Hitachi MPF-A spectrophotometer (1 cm light-path). H'-NMR spectra were determined with a JASCO FX-100 spectrometer, and are reported as ppm downfield shifts from an internal standard of TMS. HPLC was performed using a Gilson apparatus with an anion-exchange support (TSK gel DEAE-2SW). The reverse-phase HPLC was performed on a support (MS pack C-18 silica, 5μ). Silica gel (Wacogel C-200) was used for column chromatography, silica gel (Kiesel gel 60 F_{254}, Merck) for TLC and silanized silica gel (Kiesel gel 60 F_{254}, Merck) for reverse-phase TLC. A C-18 silica gel (35-100 , Waters) was used for reverse-phase low pressure chromatography. Mobility shift analysis was performed as described previously.[7]

5'-O-Dimethoxytrityl-3-β-D-2'-deoxyribofuranosyl-2,7-dioxopyrido-[2,3-d]pyrimidine (2)

The nucleoside(1, 2.80 g, 10.0 mmol) was dried by evaporation from pyridine and resuspended in pyridine (60 ml). Dimethoxytrityl chloride (3.81 g, 11.2 mmol) was added and the mixture was stirred for 3 hr at room temperature. The reaction was terminated by the addition of water and the product extracted into chloroform (100 ml). The organic layer was washed 3 times with water (150 ml), dried over sodium sulfate, concentrated and finally coevaporated with toluene. The residue was subjected to silica gel chromatography and the product was precipitated with n-hexane from its concentrated solution in chloroform. The yield was 5.24 g, 90 % UV : λmax(MeOH) 250 nm, 330 nm. 'H-NMR ($CDCl_3$), (ppm): 9.89 (brs, 1 H, N-H); 8.93 (S, 1 H, H-4); 7.3-6.8 (m,

13 H, aromatic protons); 6.32 (t, 1 H, H-1'); 6.10 (d, J = 9.8 Hz, 1 H, vinylic proton); 5.95 (d, J = 9.8 Hz, 1 H , vinylic proton); 3.76 (S, 6 H, $-OCH_3$). Anal. Calcd for $C_{33}H_{31}O_7N_3 \cdot 1/2H_2O$: C, 67.11; H, 5.46; N. 7.11. Found: C, 67.35; H, 5.25; N, 6.93.

The phosphodiester (3)

The 5'-protected nucleoside (2, 1.69 g, 2.91 mmol) was dried by evaporation from pyridine and dissolved in dioxane (5 ml). A solution of 2-Chlorophenyl phosphoroditriazolide (3.30 mmol in 10 ml of dioxane) was added and the mixture was incubated at room temperature for 30 min. The reaction was stopped by the addition of a mixture of 0.1 M triethylammonium bicarbonate (30 ml) and pyridine (10 ml). The product was extracted into chloroform (3 x 50 ml), washed with water (3 x 100 ml) and dried over sodium sulfate. The solvent was evaporated and the residue was precipitated with hexane-ether (6 : 4) containing 1 % triethylamine. The yield was 2.01 g, 85 %.

The dinucleotide (5a)

The 3'-phosphorylated nucleosides (4) and dinucleotides containing the major nucleosides were prepared as previously described (3). Dinucleotides containing the analog (5) were synthesized using nucleotide (3). For example, 5a was synthesized by the following procedure: 3 (432 mg, 0.53 mmol) and 4a (200 mg, 0.41 mmol) were dried by evaporation from pyridine. The mixture was treated with MSNT (249 mg, 0.84 mmol) in pyridine (10 ml) at room temperature for 25 min. Completion of the reaction was confirmed by TLC and the reaction was terminated by the addition of water (1 ml). The mixture was concentrated, dissolved in chloroform, washed with water (3 x 15 ml) and dried over sodium sulfate. Volatile materials were removed by evaporation and the residue was dried by coevaporation from toluene. The product was isolated by chromatography on silica gel and precipitated with hexane from solution in chloroform. The yield was 414 mg, 84 %.

Removal of the cyanoethyl group

The compound 5a (400 mg, 0.33 mmol) was dissolved in a solution of pyridine-triethylamine (3 : 1, v/v, 8 ml) and water (2 ml) was added to the mixture. After 10 min the solvents were removed by

evaporation. The product was dissolved in chloroform and precipitated with hexane-ether (6 : 4, v/v containing 1 % triethylamine). The yield was 398 mg, 96 %.

Polymer support synthesis of the dodecamers

A typical synthesis used 5 μmol of nucleoside resin (0.1 mmol/g). Usual condensation employed ca. 25 μmol of protected dinucleotides and MSNT (100 μmol). Overall yields of dodecamers varied 10 to 21 % and an average yield of each condensation was 91-96 %.

RESULTS

Synthesis of dodecanucleotides

The dodecanucleotides (I-VI) (Fig. 2) were synthesized by the phosphotriester solid-phase method using preformed dinucleotide blocks previously.[3] The dinucleotides containing pyridopyrimidine deoxynucleotide were prepared as shown in Fig 3. The 5'-dimethoxytritylated nucleoside (2), was phosphorylated with 2-chlorophenyl phosphoroditriazolide[5] and condensed with the 5'-hydroxy component (4) using mesitylenesulfonyl nitrotriazolide (MSNT).[6] The reaction conditions and yield of dinucleotides (5) are summarized in Table I. The protected dodecamers were released from 1 % cross-linked polystyrene by treatment with concentrated ammonia at room temperature. It was found that the pyridopyrimidine ring in oligonucleotides was decomposed in the presence of 1,1,3,3-tetramethyl-guanidine which was used for deblocking.[6] To avoid strong alkaline conditions aryl esters of the internucleotide phosphates were cleaved by prolonged ammonia treatment at room temperature (24 hr). After removal of the N-acyl protecting group with ammonia at 50 °C, the dimethoxy-

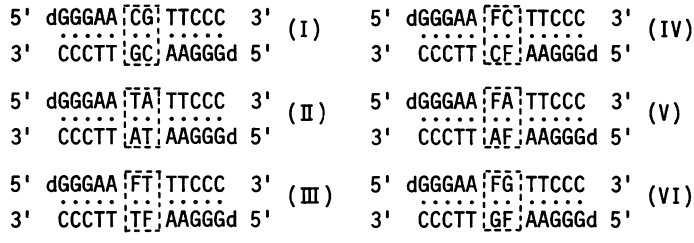

Figure 2. Sequences of dodecanucleotide I-VI

tritylated oligonucleotides were fractionated by reverse-phase column chromatography. The dimethoxytrityl group was removed with 80 % acetic acid and the product was purified by reverse-phase high pressure liquid chromatography (HPLC). The purity and sequence of the product were analyzed by anion-exchange HPLC (Fig. 4) and a mobility shift analysis.[7] The pyridopyrimidine nucleotide was found to be cleaved by snake venom phosphodiesterase. (Fig. 5)

Properties of dodecanucleotides

Ultra violet (UV) spectra of the dodecamer containing FT

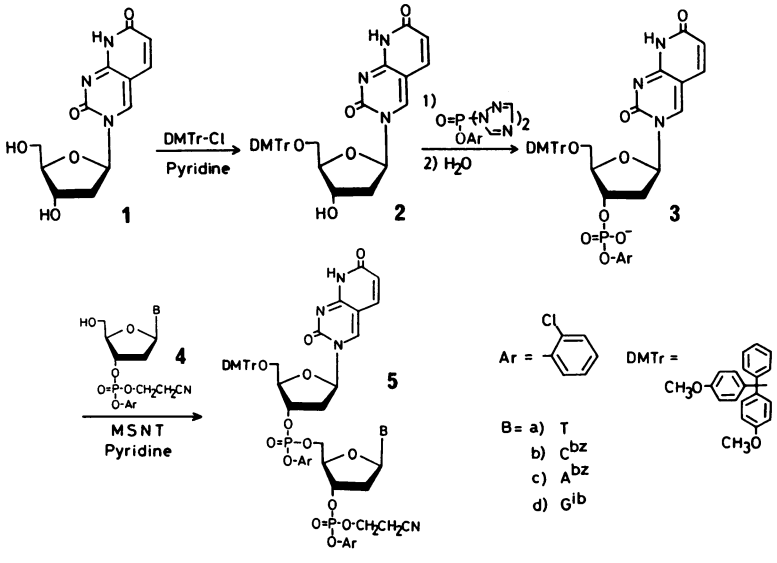

Figure 3. Synthesis of protected dinucleotides containing pyridopyrimidine

Table I. Synthesis of dinucleotides (5)

5'-OH Component 4 (mmol)	3'-Diester Component 3 (mmol)	MSNT (mmol)	Reaction time (min)	Yield (%)
a 0.41 (B=T)	0.53	0.84	25	84
b 0.40 (B=C^{bz})	0.54	0.85	25	88
c 0.39 (B=A^{bz})	0.47	0.79	30	81
d 0.40 (B=G^{ib})	0.49	0.79	40	78

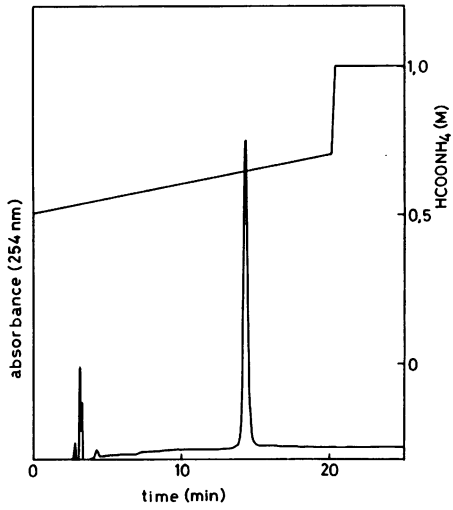

Figure 4. Ion-exchange HPLC of purified d(GGGAAFTTTCCC) (III) on a column (4 x 250 mm) of TSK gel DEAE-2SW using a gradient of ammonium formate (0.5-0.7 M) in 25 % acetonitrile in 24 min. The flow rate : 2 ml/min.

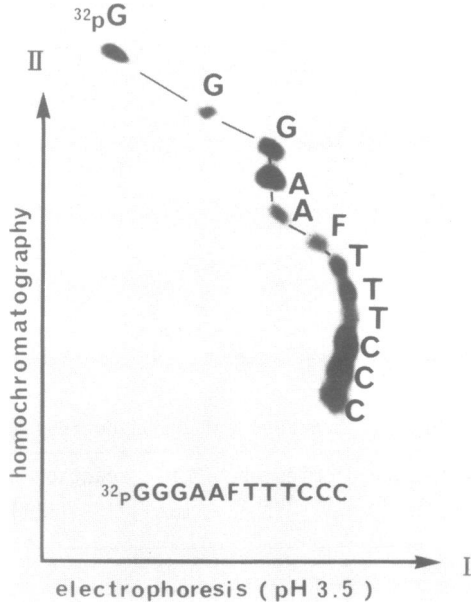

Figure 5. Mobility shift analysis [7] of dodecanucleotide (III) was performed after 5'-phosphorylation. Venom phosphodiesterase was used for the partial digestion.[8]

Figure 6. UV spectrum of d(GGGAAFTTTCCC) (III) in 0.01 M sodium cacodylate

Table II. Optical properties of dodecamers

Dodecamers	λ_{max}(nm)	Tm(°C)	fluorescence int.(%)
dF	330, 250		100
dGGGAACGTTCCC(I)	257	50.5	0
dGGGAATATTCCC(II)	258	47.9	0
dGGGAAFTTTCCC(III)	333, 258		25.3
dGGGAAFCTTCCC(IV)	333, 258		21.0
dGGGAAFATTCCC(V)	341, 257	39.4	1.7
dGGGAAFGTTCCC(VI)	338, 255	58.4	<1.0

(III) showed a characteristic absorption at 340 nm as seen in Fig. 6. The pyridopyrimidine residue in the dodecamers showed a similar contribution at the same region of wavelength as shown in Table II.

The UV-absorbance-temperature profiles for the dodecamers were measured at pH 7.0 in the presence of 0.1 M sodium chloride.

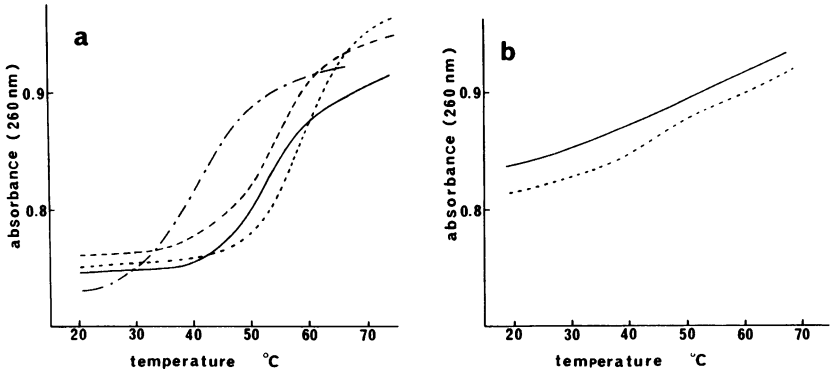

Figure 7. UV absorption-temperature profile of the dodecamers in 0.01 M sodium cacodylate (pH 7.0) and 0.1 M sodium chloride. a: ——, I; — —, II; — - —, V; ····, VI. b: ——, III; ····, IV.

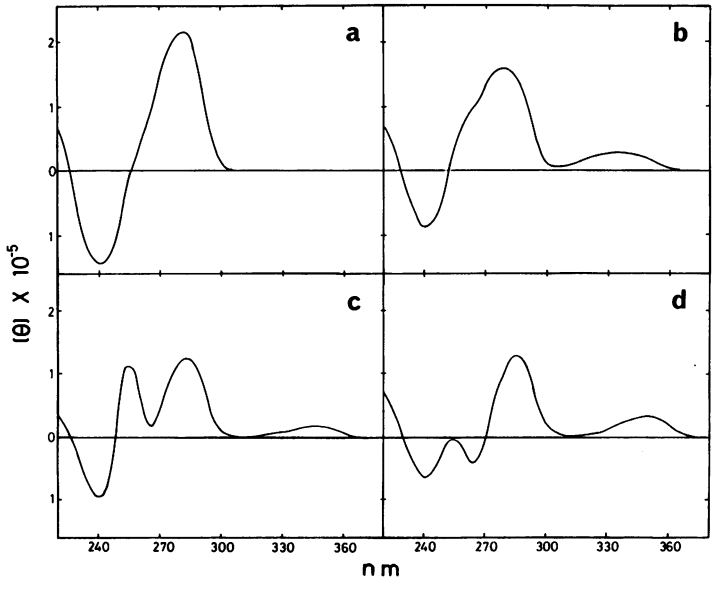

Figure 8. CD spectra of dodecamers. a, I; b, III; c, V; d, VI in the same media as shown in Fig. 7.

Fig. 7 shows the melting profiles of the dodecamers. The melting temperatures of the four duplexes shown in Fig. 7a are listed in Table II. To our surprise, the melting temperature of the FG

containing dodecamer (VI)(58.4°C) was unexpectedly higher than that of the CG containing dodecamer (I)(50.5°C). The melting temperature of the FA dodecamer (V) was lower than that of the TA dodecamer (II). This may mean that the hydrogen bonds between pyridopyrimidine and guanine are much stronger than those with adenine. Oligomers containig FT or FC did not show any distinct transitions as shown in Fig. 7b.

Fluorescence intensities were measured in the same medium as above. Relative fluorescence intensities of the dodecamers with respect to each of the analog nucleoside alone are listed in Table II. Quenching seemed to occur more distinctly in stable duplexes [e.g. less than 1 % for the FG containing dodecamer (VI)]. Duplexes in which pyrimidines and pyridopyrimidine are paired, retain 25 % of their fluorescence intensity.

The circular dichroism spectra of duplexes (I, III, V and VI) are shown in Fig. 8. The F analog duplexes show complex spectra compared to the natural duplex (I)(Fig. 8a). Presumably this could be due to more complex interaction of the pyrido-pyrimidine moiety.

DISCUSSION

Oligonucleotides containing pyridopyrimidine were succesfully synthesized by the phosphotriester method using usual procedures except that the deblocking procedure was modified in order to preserve the pyridopyrimidine ring.

We conclude that a pyridopyrimidine ring can stabilize a DNA duplex (when it pairs with guanine) more intensively than a natural GC hydrogen bond. However, the positioning of other bases pairing with a pyridopyrimidine may decrease the stability of a DNA duplex. Quenching of the fluorescence occurred most intensively in the most stable duplex (VI). Putative hydrogen bonds shown in Fig. 1 may explain these phenomena. These oligo-nucleotides could serve as a unique DNA probe, in addition to oligonucleotides containing phenyl deoxyriboside[9] or deoxy-inosine.[10] In the case of guanine and pyridopyrimidine, the third hydrogen bond involving the heterocyclic imino hydrogen may stabilize the interaction and strengthen the stacking between bases. On the other hand adenine may have to be skewed to form

hydrogen bonds with pyridopyrimidine. NMR studies on oligonucleotide duplexes containing pyridopyrimidine may elucidate these interactions. Studies along these lines are in progress.

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