Supplementary Material (ESI) for Chemical Communications

Recognition of CG inversions in DNA triple helices by methylated 3*H*-pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one nucleoside analogues

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Synthesis of Oligonucleotides

All oligonucleotides were synthesised on an Applied Biosystems ABI 394 automated DNA/RNA synthesiser on the 0.2 or 1 μM scale using the standard cycles of acid-catalysed detritylation, coupling, capping and iodine oxidation procedures, using phosphoramidite monomers and other reagents purchased from Applied Biosystems, Cruachem or Link Technologies. All 2-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. Stepwise coupling efficiencies were determined by the trityl cation conductivity monitoring facility and in all cases were >95 %. Oligonucleotides were deprotected in concentrated aqueous ammonia (temp, hrs), apart from triplex forming oligonucleotides (TFOs) containing MP, ^{A}EP and ^{A}PP for which 10 % aqueous methylamine containing 2.5 mg.mL^{-1} phenol was used (temp, hrs). This requires the use of 5'-(4,4'-dimethoxytrityl)-*N*4-acetyl-2'-deoxycytidine-3'-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (Ac-dC CE phosphoramidite). Deprotected oligonucleotides were purified by RP-HPLC on a Gilson system using an ABI Aquapore column (C8), 8 mm x 250 mm, pore size 300 Å. The system was controlled by Gilson 7.12 software and the following protocol was used: Run time 30 minutes, flow rate 4 mL min^{-1}, binary system, gradient: Time in minutes (% buffer B);0 (0); 3 (0); 5 (20); 21 (100); 25 (100); 27 (0); 30 (0). Elution buffer A, 0.1 M ammonium acetate, pH 7.0, buffer B, 0.1 M ammonium acetate (35 % acetonitrile) pH 7.0.

Elution of oligonucleotides was monitored by UV absorption at 295 nm. Purified oligonucleotides were desalted using Sephadex® containing NAP-10 columns (Pharmacia Biotech), and their optical densities recorded on a Lambda 15 UV/Vis Spectrophotometer (Perkin Elmer). The optical densities recorded were used to calculate their concentrations. Purity after HPLC was >95 %. The molecular masses of oligonucleotides were determined by MALDI-TOF MS on a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer (Table 1). Internally and externally calibrated data were obtained in positive ion mode using delayed extraction and an initial accelerating voltage of 20 kV. Spectra were recorded from a matrix containing 4:1 3-hydroxypicolinic acid: picolinic acid in 1:1 acetonitrile:water in the presence of Dowex 50WX8-200 ion exchange beads according to the method of Langley *et al.* [1]

Table 1. Sequences and MALDI-TOF data for oligonucleotides containing the modified nucleotides.

Code	Sequence	*m/z* expected $[M+H]^+$	*m/z* found $[M+H]^+$
FM1	MR-TCTCTCTT**MP**TCCTCCTCC	5720.9	5720.5
FM2	MR-TCTCTCTT^{A}**EP**TCCTCCTCC	5749.9	5749.6
FM3	MR-TCTCTCTT^{A}**PP**TCCTCCTCC	5764.0	5764.1
FP1	TCTCTT**MP**TTTCT	3581.4	3581.8
FP2	TCTCTT^{A}**EP**TTTCT	3610.5	3610.3
FP3	TCTCTT^{A}**PP**TTTCT	3624.5	3624.5
UV1	TTTTT^{m}CT**MP**T^{m}CT^{m}CT^{m}CT	4535.1	4535.7
UV2	TTTTT^{m}CT^{A}**EP**T^{m}CT^{m}CT^{m}CT	4564.1	4564.1
UV3	TTTTT^{m}CT^{A}**PP**T^{m}CT^{m}CT^{m}CT	4578.2	4577.8

Fluorescence Melting

Fluorescent melting profiles were recorded using a Roche LightCycler® as previously described.[2] All oligonucleotides were prepared in 50 mM Sodium acetate, pH 5.5 or 6.0, containing 200 mM NaCl. Assays consisted of each duplex-forming oligonucleotide (0.25 μM) and TFO (3 μM) in a buffer solution (50 mM Sodium acetate, 200 mM NaCl at pH 5.5 or 6.0). At pH 5.5 the complexes were denatured by heating to 95 ºC at a rate of 0.1 ºC.sec^{-1} and maintained at this temperature for 5 minutes before cooling to 30 ºC at 0.1 ºC.sec^{-1} whilst monitoring fluorescence in Channel 1 of the LightCycler® ($\lambda_{obs.} \sim 520$ nm). Samples were then held at 30 ºC for 5 minutes before heating to 95 ºC at 0.1 ºC.sec^{-1}, again monitoring fluorescence in

Channel 1 of the LightCycler®. At pH 6.0 the same temperature profile was used except that samples were cooled to 20 ºC due to the low melting temperatures obtained at this pH. T_m values were determined from the first derivatives of the melting profile using Roche LightCycler® software. Each experiment was performed in triplicate, and the T_ms of replicates differed by less than 0.5 ºC.

DNase I footprinting studies

DNA fragments. The *tyr*T (43-59) fragment contains a 17-base oligopurine tract.[3] This was modified to produce four fragments, each containing a different base pair at position ZY. Radiolabelled fragments were produced by digesting each plasmid with *Eco*RI and *Ava*I and labelling at the 3'-end of the *Eco*RI site using reverse transcriptase and [α-^{32}P]dATP. Each fragment was separated from the remainder of the plasmid DNA on an 8 % (w/v) non-denaturing polyacrylamide gel. After elution the fragment was dissolved in 10 mM tris-HCl, pH 7.5 containing 0.1 mM EDTA to give approximately 10 cps.μL^{-1} as determined on a hand held Geiger counter (<10 nM).

DNase I footprinting. Radiolabelled DNA (1.5 µL) was mixed with TFOs (3 µL) dissolved in 50 mM sodium acetate pH 5.0, containing 10 mM $MgCl_2$ to produce final oligonucleotide concentrations between 0.3 µM and 20 µM. The complexes were left to equilibrate at 20 °C overnight. DNase I digestion was carried out by adding 2 µL of DNase I (typically 0.01 units/ml) dissolved in 20 mM NaCl containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$. The reaction was stopped after 1 min by the addition of 4 µL of 80% formamide containing 10 mM EDTA, 10 mM NaOH, and 0.1 % (w/v) bromophenol blue. The products of digestion were separated on 9 % polyacrylamide gels containing 8 M urea. Samples were heated to 100 ºC for 3 minutes, before rapidly cooling on ice and loading onto the gel. Polyacrylamide gels (40 cm long, 0.3 mm thick) were run at 1500 V for approximately 2 hr. and then fixed in 10 % (v/v) acetic acid. These were transferred to Whatman 3MM paper and dried under vacuum at 86 ºC for 1 hr. The dried gels were subjected to phosphorimaging using a Molecular Dynamics Storm phosphorimager.

The intensity of bands within each footprint was estimated using ImageQuant software. These intensities were then normalised relative to a band in the digest which is not part of the triplex target site, and which was not affected by addition of the TFO. Footprinting plots[4] were constructed from these data and fitted using simple binding curves using Sigmaplot for Windows to calculate C_{50} values, indicating the TFO concentration that reduces the band intensity by 50 %.

UV-melting

UV-melting experiments were performed on a Varian Cary 400 Scan UV-Visible spectrophotometer, in Hellma® SUPRASIL synthetic quartz, 10 mm pathlength cuvettes, monitoring at 260 nm. All three oligonucleotide strands were used at a concentration of 0.53 μM, in a 10 mM sodium cacodylate, 100 mM NaCl, 0.25 mM spermine buffer, at pH 7.0. The sample chamber was flushed with nitrogen to prevent condensation on the cells. Samples were filtered through Kinesis regenerated cellulose 13 mm, 0.45 μM syringe filters prior to analysis. Following an initial heat and cool cycle (5 to 80 to 5 °C at 0.5 °C.min^{-1}) to ensure uniform annealing of the strands, the UV melting curves were recorded for three consecutive heat and cool cycles (5 to 45 to 5°C at 0.5 °C.min^{-1}). Samples were made up and run in triplicate and the average T_m reported.

Fluorescence melting curves

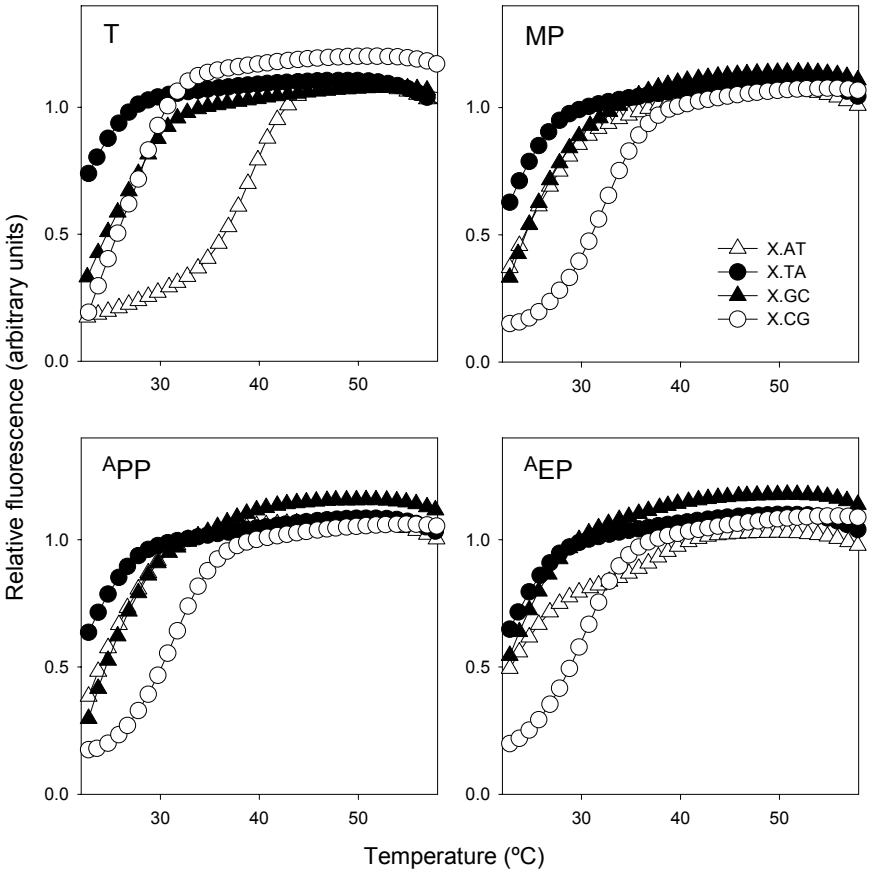

Fig. 1. Representative fluorescence melting curves showing the interaction of triplex forming oligonucleotides containing T, MP, ^{A}EP and ^{A}PP with target duplexes containing a variable central base pair (AT, TA, GC or CG). The experiments were performed in 50 mM sodium acetate, pH 6.0 containing 200 mM NaCl.

References

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