Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues.

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Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues

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ABSTRACT

The synthesis of the deoxynucleoside derived from the base P, 6H,8H-3,4-dihydro-pyrimido[4,5-c][1,2]oxazin-7-one, 2, and its introduction by established phosphoramidite and H-phosphonate chemistry into oligonucleotides is described. The melting transition temperatures (T_m) of a range of heptadecamer duplexes containing P/A and P/G base-pairs are compared with corresponding ones having N^4-methoxycytosine (M) 1 and mismatched normal bases. P/A and P/G pairs allow closely similar duplex stabilities and have the potential to reduce the multiplicity of probes and primers based on amino acid sequences by removing the T/C degeneracy.

INTRODUCTION

In designing probes for genomic and complementary DNA or for messenger RNA, based on the amino acid sequence, or part thereof, of the derived structural protein a number of strategies have been employed. The fundamental problem resides in the degeneracy of the genetic code and therefore the potential multiplicity of derived synthetic complementary oligonucleotides. A number of approaches to this problem have been used. Long unique probes based on codon usage are effective, the length being dependent on genome complexity and countering the effect of inevitable base-pair mismatches (1,2). More frequently oligomer mixtures corresponding to all possible codons are used, sometimes in subsets. The problems associated with this approach have been discussed (1,3). A third method of reducing probe complexity is to use nucleotides, generally at the codon third position, the base of which has reduced hydrogen bonding specificity. Of these only hypoxanthine, the tRNA wobble-base, incorporated as deoxyinosine, dI, has found favour and has been successfully used to replace A,G,C and T (inter al. refs. 4,5). A variety of T_m studies indicate that duplexes containing I have widely varying stabilities depending on the base to which it is paired and the sequence context (3). In view of these expected findings it seemed to us that a more rational solution to probe multiplicity could be achieved if, initially, a

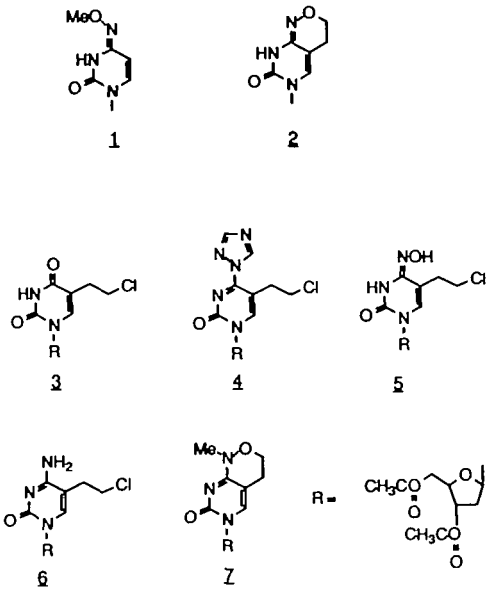
pyrimidine base resembling both C and T in its hydrogen-bonding potential could be elaborated and we took as starting-point N^4-methoxycytosine (M), 1, on the grounds that its tautomeric constant was much nearer to unity (~10-30 in favour of the imino form) than that of C or T (~10^4-10^5) (7,8,9,10). We showed that oligomers containing a methoxycytosine residue (M) vindicated this view, i.e. that the base-pairs M/A and M/G were of comparable stability. However in attempts to use probes for colony hybridisation (11) duplex stability decreased when more than one methoxycytosine was incorporated and we adduced evidence that this was due to the preferred syn conformer, 1, being inimical to Watson-Crick base-pair hydrogen-bonding (12). It followed that a base such as 2 (P), might provide a better solution since the ring structure maintains the required anti-conformation of the N-O bond and in addition the 5-methylene group might provide the same helix-stabilising effect as does the 5-methyl group of thymine.

Here we describe the synthesis of dP, 12, its conversion to the monomer 14 and the use of the latter (and the corresponding H-phosphonate salt, 15) to synthesise a number of heptadecamers containing the ring-system P. The T_m values of a number of duplexes containing P/A and P/G (and M/A and M/G) base-pairs are compared with those containing mismatches. Model studies towards the ring-system P have already been published (13).

RESULTS AND DISCUSSION

For the synthesis of the 2'-deoxyribosyl derivative, dP, 12, two routes were envisaged. In the first 5-(2-chloroethyl)deoxyuridine, kindly provided by Drs. H. Griengl and B. Rosenwirth (14), as its diacetate, 3, was converted to the 4-triazolo-derivative, 4, and thence to the N^4-hydroxycytosine, 5, by hydroxylamine. This compound could not be induced to cyclise under a variety of basic conditions. Neither did the cytosine derivative, 6, formed from 4 by ammonia in dioxan, undergo ring-closure. However 4 with N-methyl hydroxylamine hydrochloride in pyridine gave the ring-closed product, 7, directly. Similar observations were made in model experiments using corresponding N-1-methylpyrimidine derivatives and are discussed elsewhere (13).

When the tris-trimethylsilyl derivative of 5-(2-hydroxyethyl)uracil (15) is condensed with α-3,5-di-O-toluoyl-2-deoxyribosyl chloride (16) in chloroform without catalyst (17), essentially complete conversion to the β-nucleoside, 8, (14) was effected. A Mitsunobu reaction with N-hydroxyphthalimide then gave cleanly the intermediate 9. Triazolylation



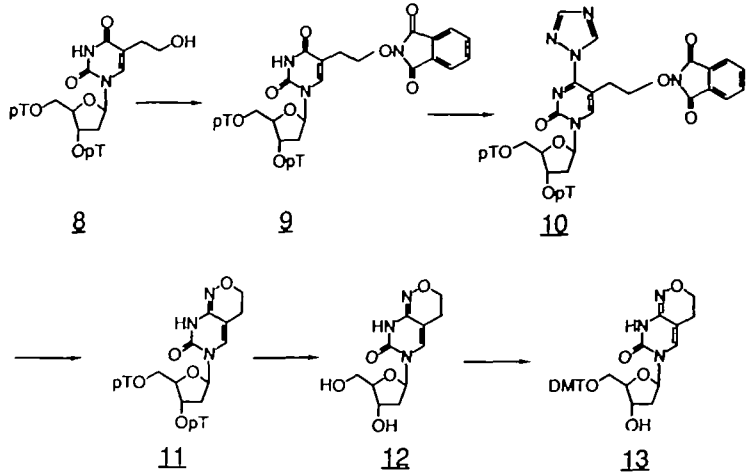
Scheme 1

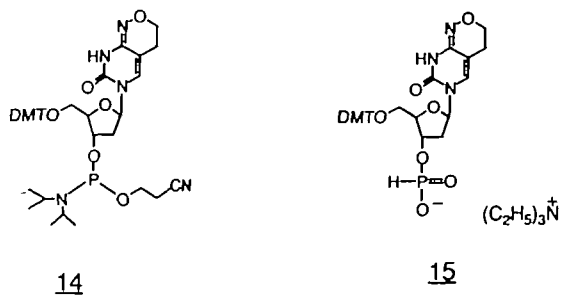
of the latter gave 10 in high yield and this with ammonia in dioxan afforded the pyrimido-oxazine, 11. Methanolic ammonia treatment then gave the nucleoside dP, 12. The 5'-O-dimethoxytrityl derivative, 13, was converted to the 3'-O-cyanoethyl-N,N-diisopropylphosphoramidite, 14, and to the corresponding triethylammonium hydrogenphosphonate by conventional methods (18,19).

Both the phosphoramidite, 14, and the hydrogenphosphonate, 15, were incorporated into oligodeoxynucleotides, behaving like the normal monomers during machine synthesis. The heptadecamers synthesised, are to be found in the Table. For comparison some corresponding oligomers containing dM, were also synthesised by phosphoramidite chemistry (Table).

Two complementary 17-mers, having A and G at position 9, but otherwise identical, are paired with those oligomers containing the bases M and P. The T_m values of these duplexes measured in a high salt buffer are given in the Table where they are compared with true complementary and with mismatched duplexes.

Some conclusions can be drawn from the results shown in the Table. Firstly, N^4-methoxycytosine pairs essentially equivalently with A and G. With one M residue in the oligomer the T_m is 6-7° lower than those of the





Scheme 2

true complements. It is evident, too, that corresponding duplexes containing two and three M residues demonstrate the essential equivalence of M/G and M/A pairs. Nevertheless duplex stability becomes lower with increasing numbers of M although not so precipitously as does that due to mismatches.

Secondly, we consider the effect of the P base. The Table shows that duplexes containing one and two such residues in P/G and P/A base-pairs appear to be as stable as the corresponding parent duplexes, the T_m values lying between the extremes of 72-75° for T/A and C/G respectively. A single P residue leads to a more stable duplex than one containing a corresponding G/T and much more stable than one with an A/C mismatch. Three P residues

Table. Observed melting temperatures (T_m) of heptadecamer duplexes.

Duplexes	T_m(°C)
ACTTGGCCACCATTTTG ————T————	72
ACTTGGCCGCCATTTTG ————C————	75
ACTTGGCCGCCATTTTG ————T————	70
ACTTGGCCACCATTTTG ————C————	64
ACTTGGCCACCATTTTG ——T—C—C———	43

	T_m(°C)		T_m(°C)
ACTTGGCCACCATTTTG ————M————	66	ACTTGGCCACCATTTTG ————P————	72
ACTTGGCCGCCATTTTG ————M————	66	ACTTGGCCGCCATTTTG ————P————	73
ACTTGGCCGCCATTTTG ————M—M———	60	ACTTGGCCGCCATTTTG ————P—P———	70
ACTTGGCCACCATTTTG ————M—M———	60	ACTTGGCCACCATTTTG ————P—P———	71
ACTTGGCCACCATTTTG ——M———M———	57.5	ACTTGGCCACCATTTTG ——P———P———	67
ACTTGGCCACCATTTTG ——M—M—M———	52.5	ACTTGGCCACCATTTTG ——P—P—P———	66
ACTTGGCCGCCATTTTG ——M—M—M———	51	ACTTGGCCGCCATTTTG ——P—P—P———	64

only reduce the T_m to 65°, that is by an average of only 8°. This, again, is to be contrasted with the much larger drop in T_m generated by mismatches. Thus for the duplex with three mismatches, giving a T_m depression of about 30°, the corresponding duplex with three P residues shows only a 6° reduction from that of the parent duplex. All P-containing duplexes show sharp melting transitions.

Although we have not yet measured the tautomeric constant for the bicyclic ring system in P, the UV spectra of model compounds (13) support

the view that the equilibrium favours the imino-form, as it does in the N^4-methoxycytosine series (9). We presume that as with the latter, the equilibrium is in the range $10-10^2$ and the present T_m measurements support this view strongly. We also think that, as mentioned above, the 5-methylene group (or even, perhaps, both methylene groups) must contribute to the helix stability as does that of the 5-methyl group of thymine (7,20).X-ray crystallographic work, with Dr. M. Moore, is in progress to determine the nature of the G/M, G/P, A/M and A/P base pairs in self-complimentary duplexes containing the M and P bases.

In view of the satisfactory base-pairing properties shown by the P residue we are investigating oligonucleotides containing it as hybridisation probes and primers.

EXPERIMENTAL

^{1}H-nmr spectra were obtained with Varian HA100 and CFT-20 instruments with tetramethylsilane as internal standard. Unless otherwise stated values given on a δ scale refer to singlet absorption, integration values and signal assignment are in parentheses; d, t, q and m refer to doublet, triplet, quartet and complex multiplet respectively. Mass spectra were recorded with a Kratos M530 instrument. For tlc, CH_2Cl_2-MeOH, 90:10(A) and 80:20(B) were used on precoated silica plates; Merck kieselgel 60H was used for column chromatography. Melting points measured on a Köfler hot stage apparatus are uncorrected.

3',5'-Di-O-p-toluoyl-5-(2-hydroxyethyl)-2'-deoxyuridine 8 - A suspension of 5-(2-hydroxyethyl)uracil (4.0g; 25.6 mmol) in hexamethyldisilazane (36 ml) and trimethylchlorosilane (5ml) was heated under reflux for 4 hr. Excess reagent was removed from the clear solution in vacuo and by co-evaporation with xylene. To the resulting oil, in pure dry chloroform (100 ml), α-3,5-di-O-p-toluoyl-2-deoxy-D-ribofuranosyl chloride (11.46 g; 29 mmol, dried over P_2O_5 and NaOH) was added and the solution stirred overnight at room temperature. After washing the chloroform solution with $NaHCO_3$ solution and then water and drying, the product (10 g; 79%) was isolated as a foam following silica gel chromatography. The β-nucleoside was contaminated with up to 15% of the α-anomer as measured by nmr spectroscopy but was used in the next step without further purification (see ref. 14).

3',5'-Di-O-acetyl-5-(2-chloroethyl)-2'-deoxyuridine 3 - 5-(2-Chloroethyl)deoxyuridine (1.5 g; 5.2 mmol) was treated with acetic anhydride in pyridine and worked up in the usual way. After column

chromatography the di-O-acetate product was obtained as a colourless foam (1.9g; 100%). ^{1}H-nmr ($CDCl_3$) 2.10 (6H, s, 2 x CH_3CO), 2.08-2.20 (1H, m, H-2'), 2.44 - 2.53 (1H, m, H-2"), 2.60-2.91 (2H, m, $-CH_2Cl$), 3.17 (2H, t, J= 5.23 Hz, 5-CH_2-), 4.22-4.42 (3H, m, H-4', H-5', H-5"), 5.18-5.22 (1H, m, H-3'), 6.29-6.34 (1H, m, H-1'), 7.40 (1H, s, H-6), 9.5 (1H, s, N-H).

5-(2-Chloroethyl)-4-triazolo-1-β-(3,5-di-O-acetyl deoxyribofuranosyl) -1-H-pyrimidin-2-one 4 To an ice-cold suspension of triazole (7.2 g) in dry acetonitrile (170 ml) was added phosphorylchloride (2.2 ml) then triethylamine (16.8 ml). After stirring for 0.5 hr the above nucleoside (3; 2.0g; 5.3 mmol) in acetonitrile (25 ml) was added dropwise. Tlc showed that reaction was complete after 2 hr. After evaporation to dryness a chloroform solution of the product was washed with $NaHCO_3$ solution and chromatographed, affording the pure product as a pale yellow gum quantitatively. ^{1}H-nmr ($CDCl_3$) 2.06 (6H, s, 2 x CH_3CO), 2.03-2.14 (1H, m, H-2'), 2.83-2.92 (1H, m, H-2), 3.12-3.50(2H, m, $-CH_2$-), 4.29-4.43(3H, m, H-4, H-5', H-5"), 5.18-5.21 (1H, m, H-3), 6.21-6.27(1H, m, H-1'), 8.08 (1H, s, H-6), 8.13 and 9.27 (each 1H, s, triazole H).

3',5'-Di-O-acetyl-5-(2-chloroethyl)-2'-deoxycytidine 6 - The above triazolo-compound (0.4 g) was dissolved in saturated ammonia-dry dioxan (15 ml) and stirred in a sealed vessel at 80°C for 1 hr. The product, after a $NaHCO_3$ wash and chromatography gave the pure product as a foam (0.26 g; 74%) (Found: C, 48.0; H, 5.5; N, 11.3; $C_{15}H_{20}O_6N_3$ Cl requires C, 48.2; H, 5.4; N, 11.2). ^{1}H-nmr ($CDCl_3$) 1.99-2.1 (1H, m, H-2'), 2.08 (6H, s, CH_3CO), 2.57-2.63 (1H, m, H-2"), 2.63-2.68 (2H, m, $-CH_2Cl$), 3.62-3.68 (2H, m, $-CH_2$-), 4.24-4.28 (1H, m, H-4'), 4.32-4.35 (2H, m, H-5', H-5"), 5.16-5.19 (1H, m, H-3'), 6.22-6.29(1H, m, H-1'), 7.45 (1H, s, H-6).

3',5'-Di-O-acetyl-N^4-hydroxy-5-(2-chloroethyl)-2'-deoxycytidine 5 - To a solution of the triazolo-compound 4 (1.5g; 3.5 mmol) in dry pyridine (20 ml) was added hydroxylamine hydrochloride (0.97 g; 14 mmol). After stirring for 3 hr at room temperature solvent was removed in vacuo. The product isolated after chromatography formed colourless crystals (1.0 g; 73%). ^{1}H-nmr ($CDCl_3$) 2.08 (3H, s, CH_3CO), 2.16 (3H, s, CH_3CO), 2.14-2.37(2H, m, H-2', H-2"), 2.52-2.78 (2H, m, CH_2Cl), 3.66 (2H, t, J= 6.68 Hz, 5-CH_2-), 4.16-4.38 (3H, m, H-4', H-5', H-5"), 5.16-5.19 (1H, m, H-3'), 6.26-6.32 (1H, m, H-1'), 6.72 (1H, s, H-6), 8.47 (1H, s, N-H).

The compound was treated with triethylamine, DBU, TMG and Hunig's base, under a variety of anhydrous conditions. Only starting material was recovered.

1-Methyl-6-(3,5-di-O-acetyl-2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-pyrimido [4,5-c][1,2]oxazin-7-one 7 - To a solution of the triazolo-compound, (0.8 g; 2 mmol) in pyridine (10 ml) was added N-methylhydroxylamine hydrochloride (0.84 g; 10 mmol). After stirring at room temperature for 4 hr solvent was removed and the product isolated in the usual way. Silica gel chromatography gave the pure product (0.6 g; 80%) as a foam. (Found: M^+ 367.1380. Calc. for $C_{16}H_{21}N_3O_7$, 367.1379). ^{1}H-nmr ($CDCl_3$) 1.97-2.02 (1H, m, H-2'), 2.03 and 2.04 (3H each, s, CH_3CO-), 2.56-2.65 (1H, m, H-2"), 2.67-2.71 (2H, m, $-CH_2-$), 3.36 (3H, s, N-CH_3), 4.11-4.16 (2H, m, $-CH_2-O$), 4.18-4.22 (1H, m, H-4'), 4.28-4.29 (2H, m, H-5', H-5"), 5.12-5.14 (1H, m, H-3'), 6.28-6.34 (1H, m, H-1'), 7.32 (1H, t, J= 1.1 Hz, H-5).

3',5'-Di-O-p-toluoyl-5-(2-phthalimido-oxyethyl)-2'-deoxyuridine 9 - The protected hydroxyethyldeoxyuridine 8 was used without removal of α-anomer contaminant. It (3.13 g; 6.36 mmol) was dissolved in anhydrous THF (200 ml) followed by addition of N-hydroxyphthalimide (1.96 g; 12 mmol), triphenylphosphine (3.15 g; 12 mmol) and then diethylazodicarboxylate (2.2 ml; 14 mmol). The exothermic reaction gave a transient deep red solution and after 1 hr the yellow solution was evaporated under reduced pressure. Addition of ether precipitated the colourless crystalline product (2.8 g; 70%), m.p. 141°C. Only traces of the α-anomer were apparent by tlc, removed by one further crystallisation from acetonitrile. (Found: C, 64.0; H, 4.7; N, 6.1. $C_{35}H_{31}N_3O_{10}$ requires C, 64.3; H, 4.8; N, 6.4%). ^{1}H-nmr ($CDCl_3$) 2.33 (3H, s, CH_3-Ar), 2.43 (3H, s, CH_3-Ar), 2.47-2.77 (4H, m, H-2', H-2", 5-CH_2-), 4.17-4.36(2H, m, $-CH_2O-$), 4.56 (1H, m, H-4'), 4.64-4.79(2H, m, H-5', H-5"), 5.67 (1H, m, H-3'), 6.52 (1H, m, H-1'), 7.12-7.28 (4H, m, Ar-H), 7.70-7.83 (5H, m, phthaloyl-H, H-6), 7.90-8.00 (4H, m, Ar-H), 8.53 (1H, s, N-H).

1-(3,5-di-O-p-toluoyl-2-deoxyribofuranosyl)-4-triazolo-5'-(2-phthalimido-oxyethyl)-1H-pyrimidin-2-one 10 - The solution of phosphoryl tristriazolide was prepared as above from triazole (1.22 g), phosphorylchloride (0.36 ml) and dry triethylamine (2.88 ml) in acetonitrile (30 ml). A solution of the above deoxyuridine derivative 9 (0.6 g; 0.096 mmol) in acetonitrile (5 ml) was added. After 2 hr solvent was removed and the product in chloroform was washed in the usual way and poured into hexane. The precipitate (0.58 g; 90%) showed one spot on tlc. ^{1}H-nmr ($CDCl_3$) 2.26 (3H, s, CH_3), 2.43 (3H, s, CH_3), 2.47-2.58 (1H, m, H-2'), 3.08-3.28 (3H, m, 5-CH_2, H-2"), 4.19-4.42 (2H, m, $-CH_2O-$), 4.63-4.85 (3H, m, H-4', H-5', H-5"), 5.65 (1H, m, H-3'), 6.46 (1H, m, H-1'), 7.05-7.29 (4H, m, Ar-H), 7.70-7.83 (5H, m, H-6

phthaloyl-H), 7.94-8.00 (4H, m, Ar-H), 8.74 and 9.28 (1H each, s, triazole-H).

6-(3,5-di-O-p-toluoyl-β-D-2-deoxyribofuranosyl)-3,4-dihydro-8H-pyrimido [4,5-c][1,2]oxazin-7-one 11 - The above triazolo-compound 10 (1.0 g; 1.5 mmol) was dissolved in saturated ammonia-dry dioxan (100 ml) and the solution stirred overnight at room temperature. Reaction was complete and after evaporation in vacuo, the crude product was chromatographed. It gave colourless crystals (0.64 g; 85%), m.p. 181°C from acetonitrile. (Found: C, 64.2; H, 5.5; N, 8.2. $C_{27}H_{27}N_3O_7$ requires C, 64.2; H, 5.4; N, 8.3%: M^+ 505.18590. Calc. for $C_{27}H_{27}N_3O_7$, 505.1849).

6-(5-O-dimethoxytrityl-β-D-2-deoxyribofuranosyl)-3, 4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazine-7-one - The above di-toluoyl derivative was stirred overnight in NH_3-MeOH solution (20 ml). Removal of solvent and thorough trituration with ether gave the free nucleoside 12 as a thick gum (82%). ^{1}H-nmr(DMSO-d_6) 1.89-2.09 (2H,m,H-2',H-2"), 2.48-2.54 (2H,m,-CH_2), 3.46-3.56 (2H,m,H-5"), 3.64-3.72 (1H,m,H-4'), 3.82 (2H,t,J=5.6HZ,-OCH_2-), 4.16-4.21 (1H,m,H-3'), 4.95 (1H,t,J=5.3Hz, -OH-5'), 5.19 (1H,d,J=4.1Hz, -OH-3'), 6.12-6.18 (1H,m,H-1'), 7.00 (1H,s,H-6), 10.51 (1H,s,N-H). This (0.5 g; 1.84 mmol) was dissolved in pyridine (10 ml) and treated with dimethoxytritylchloride (0.75 g; 2.23 mmol). Working up and chromatography in the usual way gave the pure product as a pale yellow powder (0.44 g; 70%). ^{1}H-nmr (DMSO-d_6) 2.01-2.22 (3H, m, H-2', H-2", -CH_2-), 3.11-3.32(2H, m, -CH_2O-), 3.68-3.77(2H, m, H-5', H-5"), 3.74 (6H, s, 2 x OCH_3), 3.84-3.86(1H, m, H-4'), 4.29-4.31 (1H, m, H-3'), 5.30 (1H, d, J=4.33 Hz, 3'-OH), 6.18(1H, t, J=6.51 Hz, H-1'), 6.89-7.40 (14H, m, H-5, Ar-H), 10.57 (1H, d, N-H).

5'-O-Dimethoxytrityl-N^4-methoxy-2'-deoxycytidine - 3',5'-Diacetyldeoxyuridine was converted to its 4-triazolo-derivative with the tristriazolide reagent as described above (90%). The latter was treated with methoxyamine hydrochloride in anhydrous pyridine to afford 3',5'-diacetyl-N^4-methoxydeoxycytidine (80%). ^{1}H-nmr ($CDCl_3$) 2.05 (3H, s, $COCH_3$), 2.06 (3H, s, $COCH_3$), 2.09-2.60 (1H, m, H-2), 3.78 (3H, s, N-OCH_3), 4.12-4.16 (1H, m, H-4'), 4.24-4.31 (2H, m, H-5"), 5.12-5.16 (1H, m, H-3'), 5.56-5.60 (1H, m, H-5), 6.21-6.26 (1H, m, H-1'), 6.74 (1H, d, J = 8.3 Hz, H-6), 8.11 (1H, s, N-H).

The free nucleoside was obtained by NH_3/MeOH deprotection of the above product and treatment with dimethoxytrityl chloride in anhydrous pyridine gave the corresponding 5'-O-dimethoxytrityl derivative (80%).

Monomers derived from 12. The above DMT-derivative 13 (1.27 g) was converted by a described method to its 3'-O-cyanoethyl-N,N-diisopropylphosphoramidite 14. Column chromatography afforded the pure product as a straw coloured foam (1.10 g; 64%). ^{31}P-nmr ($CDCl_3$) 148.80, 149.19 ppm.

The DMT-derivative (100 mg) was also converted to the triethylammonium salt of the 3'-O-hydrogenphosphonate 15 purified by two precipitations from $CHCl_3$ by pentane (with J.-Y. Tang). The pale yellow powder (90 mg) had ^{31}P-nmr (C_5D_5N-CD_3CN) 2.45 ppm. The 3'-O-cyanoethyl-N,N-di-isopropylphosphoramidite of 5'-O-dimethoxytrityl-N^4methoxydeoxycytidine was synthesised as above. ^{31}P-nmr (DMSO-D_6) 146.88, 147.26 ppm.

Synthesis of Oligodeoxynucleotides. The phosphoramidite monomer 14 was incorporated into oligomers using the normal programmes, on both an Applied Biosystems and on a Pharmacia gene assembler instrument. The H-phosphonate 15 was incorporated using a Biosearch 3700 instrument. Among the oligomers synthesised are those listed in the Table. Purification was by hplc on a Partisphere SAX column and by gel electrophoresis.

Melting Transitions of Oligonucleotide Duplexes. Melting transitions were measured at 260 nm in 6 x SSC buffer at an oligomer strand concentration of about 3μM. Absorbance V/S temperature profiles for each duplex were determined with a temperature controlled, six cuvette cell holder installed in a programmable Kontron Uvikon 810 spectrophotometer interfaced to a BBC microcomputer. The T_m was taken as the maximum of the first derivative of the transition curve, with an error limit of better than ± 1°C. In earlier work[7], T_m values of some duplexes were lower, but consistent in order, with those recorded here. Several of the present values were confirmed on independently synthesised oligomers with a Unicam SP500 spectrometer fitted with a Gilford 222 photometer and a Gilford 2527 thermoprogrammer.

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