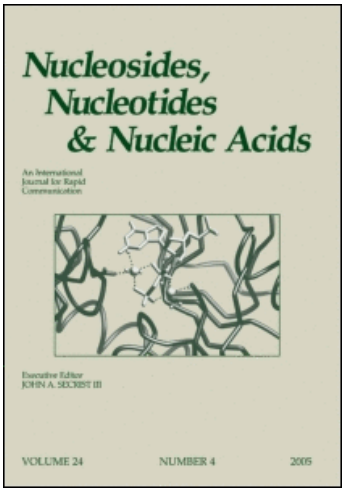

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Synthesis and Stability of Oligonucleotide Duplexes Containing N4 Aralkyl-Substituted Cytosine Bases

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SYNTHESIS AND STABILITY OF OLIGONUCLEOTIDE DUPLEXES CONTAINING N4 ARALKYL-SUBSTITUTED CYTOSINE BASES

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ABSTRACT: N4 aralkyl-substituted cytosine nucleosides, available directly by displacement of the PfpO group at C4 of 5'-*O*-DMT-protected nucleoside **4**, were efficiently incorporated into short oligonucleotides. Aralkyl substitution at the N4 of cytosine was entropically stabilising but offset by loss in enthalpy resulting overall in duplex destabilisation.

Alkylation of the heterocyclic bases[1] usually alters the stabilities[2] and subsequently the molecular recognition properties of duplexes formed from base-modified 2'-deoxy-oligoribonucleotides. The duplex destabilisation[3] resulting from methylation at the exocyclic amino group of adenine (m^6A)[4,5] is attributable to the preference of the methyl group of m^6A to adopt the *syn* orientation with respect to N1 disrupting Watson-Crick base pairing between m^6A and its thymine partner on the neighbouring strand[2]. Methylation at the exocyclic amino group of cytosine (m^4C) monomer also imposes a *syn* orientation[1] with respect to the cytosine N3, and when incorporated into oligonucleotides causes destabilisation of duplexes in solution[6,7]. However, m^4C is accommodated in RNA oligomers without loss to duplex stability[8]. Further, in the crystal structure of the self-complementary DNA duplex formed from CGCGm^4CG, which adopts the Z-conformation, the methyl groups of m^4C are oriented *anti* with respect to N3 maintaining Watson-Crick base pairs with their G partners[9]. *Quasi*-linear[10,11] hexose oligonucleotides containing 2',3'-dideoxyglucose[12-14] in place of 2'-deoxyribose, form Watson-Crick purine-purine base pairs (FIGURE 1(a); R_1 = 2',3'-dideoxyglucose). Duplex stability is significantly enhanced by aralkyl substitution[15] in the hexose series (FIGURE 1(b)) and this observation prompted us to investigate how aralkyl substitution might influence the hybridisation of 2'-deoxyoligoribonucleotides (FIGURE 1(c),(d); R_2 = 2'-deoxyribose).

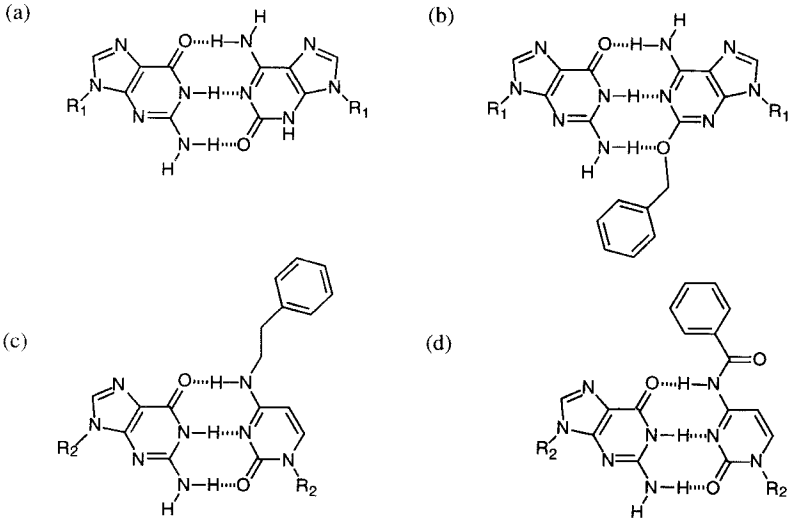
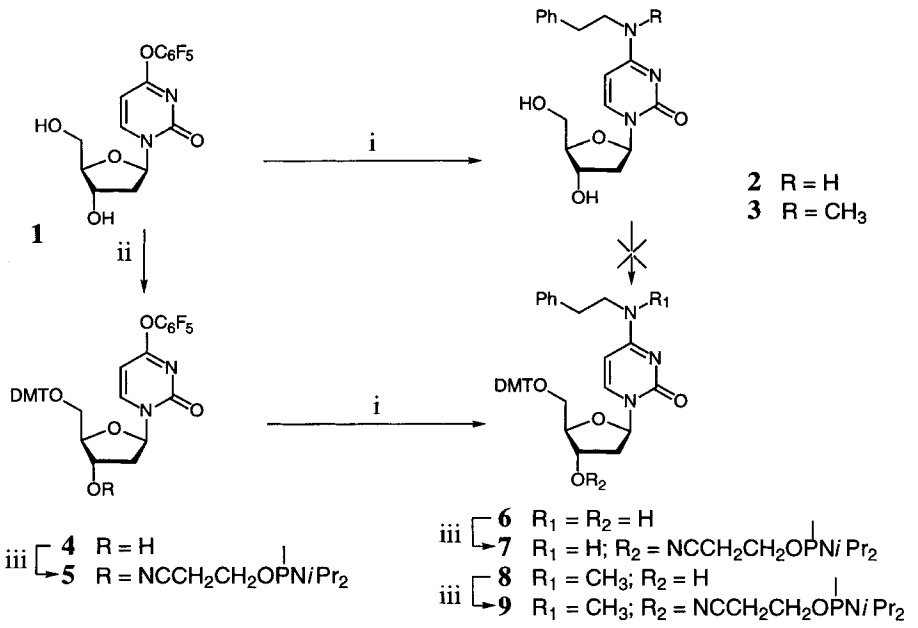


FIGURE 1

We chose to attach aralkyl substituents at the N4 of cytosine to allow comparison with the methylated analogues described previously in the literature and to avoid inadvertent steric clashes in the minor groove. A variety of procedures for the preparation of N4-substituted pyrimidine nucleosides exist with some requiring formal protection of the reactive 5'- and 3'-OH groups and isolation of intermediates[16-24], and others relying on transient protection of these hydroxyl groups[25-26]. We recently reported synthesis[27] of cytosine nucleoside precursor **1**, directly and efficiently from 2'-deoxyuridine using transient *in situ* trifluoroacetate protection.[25] We demonstrate here the use of **1**, with its displaceable pentafluorophenoxyl (PfpO) at C4, for the synthesis of N4-substituted cytosine nucleoside phosphoramidites, and assess the duplex stability of oligonucleotides made from them.

RESULTS AND DISCUSSION

Pentafluorophenoxy-substituted nucleoside **1** underwent substitution at elevated temperature with *N*-phenethylamine to give 4-*N*-phenethyl-2'-deoxycytidine **2** in 85% yield (SCHEME). Similarly, N4-disubstituted analogue **3** was isolated in near quantitative yield after reaction of **1** with *N*-methyl-*N*-phenethylamine. Substituted cytosine nucleosides **2** and **3** resisted attempts to etherify at O5' using either 4,4'-dimethoxytrityl



Reagents and conditions: i. $PhCH_2CH_2NHR$, 1,4-dioxane, 70°C; ii. DMTCl, C_5H_5N, r.t.; iii. $NCCH_2CH_2OP(Cl)NiPr_2$, Et_3N, THF, r.t.

SCHEME

chloride (DMTCl) in pyridine or the tetrafluoroborate salt $DMT^+BF_4^-$ in non-donor solvents in the presence of hindered bases[28,29]. Alternatively, **1** underwent etherification smoothly using DMTCl in pyridine at room temperature giving 5'-*O*-DMT-protected cytosine nucleoside precursor **4** in 80% yield. The PfpO group attached at C4 was readily displaced by *N*-phenethylamine to give protected 2'-deoxycytidine nucleoside **6** in 87% yield from **4**. A similar displacement reaction using *N*-methyl-*N*-phenethylamine gave the methylated analogue **8** from **4** but in substantially poorer isolated yield (37%). Phosphitylation of nucleosides **6** and **8** proceeded smoothly giving protected cyanoethyl phosphoramidites **7** and **9** in high yield for use in oligonucleotide synthesis. Acceptable coupling yields (> 98%) were obtained for modified phosphoramidites **7** and **9** when used in conventional solid phase synthesis of the phosphodiester-linked oligonucleotides **11** (**P** = *N*-phenethylcytosine) and **12** (**M** = *N*-methyl-*N*-phenethylcytosine) shown in TABLE 1. These, together with the unmodified parent sequence dGCGCGC **10**, were purified to homogeneity by reversed phase HPLC.

TABLE 1

	Oligonucleotide	T_m/°C
10	dGCGCGC	49
11	dGCG**P**GC	25
12	dGCG**M**GC	< 5
13	dTTTCCTTT + **14**	32
14	dAAAGGAAA	-
15	dTTTC^{Bz}C^{Bz}TTT + **14**	22
16	dGCG**F**GC	-

T_m values were determined at 260 nm for each oligomer (9 μM) at pH 7.0 in aqueous solution containing 0.10 M NaCl and 0.05 M Tris.HCl.

Thermal denaturation of duplexes formed from **10** to **12** was studied by variable temperature UV spectrophotometry (TABLE 1) at low salt concentration to promote formation of the B-DNA conformation and to discourage formation of the A- or Z-DNA conformations[30] in solution[31,32]. Self-complementary pentose oligonucleotide **10** was chosen as the parent sequence since previous hybridisation studies had shown that, although short, **10** forms stable duplexes[33,34]. Replacement of one cytosine in **10** by **P** gives **11** which places a phenethyl group at the N4 of two neighbouring cytosine bases in duplexes formed from **11**. Here, the possibility to form hydrogen bonds between the remaining cytosine N-H group as donor and the O6 of the partner G bases on the neighbouring strand (FIGURE 1(c)) is maintained. However, the duplex formed by **11** was less stable than the duplex formed by **10** with T_m lowered by 24 °C (TABLE 1). Replacement of the remaining N4-H of **P** by N4-Me in the methylated analogue **M** gives **12**. This proved particularly destabilising (T_m < 5 °C) since there was no longer the possibility of hydrogen bond formation between the disubstituted amino group of **M** and the O6 of the G partner base in duplexes formed from **12**.

The hybrid stabilities of **10** and **11** were investigated at various oligomer concentrations to construct ($1/T_m$ vs ln C_t) plots from which thermodynamic parameters for duplex formation were estimated[35] (TABLE 2). Aralkyl substitution in **11** promoted greater entropic stabilisation of duplexes compared to the unmodified parent sequence **10**, although this entropic gain was outweighed by the loss in enthalpic stabilisation[36]. The phenethyl substituents in duplexes formed from **11** contribute favourably to the entropy of duplexation presumably as a result of their hydrophobic nature by displacing

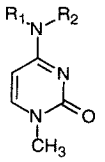
TABLE 2

	Oligonucleotide	$\Delta G^{25\ °C}$ [kcal.mol^{-1}]	ΔH [kcal.mol^{-1}]	$T\Delta S$ [kcal.mol^{-1}.K^{-1}]
10	dGCGCGC	-4.4	-34.5	-30.1
11	dGCGPGC	-1.3	-22.7	-21.4

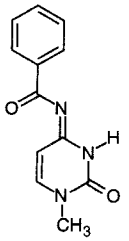
water from the duplex major groove. The loss to the enthalpy of duplexation could result from the following: displacement of stabilising, hydrogen bonded water molecules by the phenethyl substituents, steric clashes between these substituents on adjacent strands, the *+I* effect of the phenethyl substituent rendering the N4-H to which it is attached a marginally poorer hydrogen bond donor compared to the N4-H of unsubstituted cytosine, or a preferred *cis* orientation of the N4-phenethyl substituent with respect to N3 disrupting Watson-Crick base pairing with G. Yet, if oriented correctly, the N4-H groups of both **P** bases in the duplexes formed from **11** should still form reasonably strong hydrogen bonds with the O6 of partner G bases (FIGURE 1(c)). To probe the generality of duplex destabilisation due to substitution at the N4 of cytosine, we prepared two further duplexes by annealing unsubstituted **13** with **14**, and benzoyl-substituted **15**[37] with **14** (TABLE 1). Benzoylation at the N4 (FIGURE 1(d)) of the two contiguous cytosine bases decreased the affinity of **15** for its complementary sequence **14** (ΔT_m = 10 °C) compared to the unmodified parent sequence **13**. If oriented correctly, the N-H of benzoyl cytosine retains the ability to form a reasonably strong hydrogen bond with its partner G base (FIGURE 1(d)) assisted by the *-R* effect of the appendant benzoyl substitutent.

To assess whether substitution at the N4 of cytosine had a significant effect on the preferred substituent orientation (*syn* or *anti* with respect to N3) of the cytosine derivatives at the monomer level, we performed theoretical calculations on model bases where methyl replaces the pentose sugar at N1 (TABLE 3). Attachment of a methyl substituent at the N4 of **17** raises the calculated heat of formation with the *cis* isomer **19** 1.1 kcal.mol^{-1} lower in ΔH_f than the *trans* isomer **18**. Replacement of methyl by phenethyl further raises the calculated heat of formation but with the *cis* isomer **21** only marginally lower in ΔH_f by 0.3 kcal.mol^{-1} compared with the *trans* isomer **20**. The order is reversed for benzoyl substituents. The *trans* isomer **22** is the lower in ΔH_f by 3.8 kcal.mol^{-1} compared with its *cis* isomer **23** suggesting that C^{Bz} should have good prospects for Watson-Crick base

TABLE 3



17 to **23**



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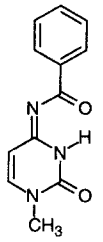


25

Compound	R_1	R_2	ΔH_f [kcal.mol^{-1}]
17	H	H	8.2
18	Me	H	12.5
19	H	Me	11.4
20	$PhCH_2CH_2$	H	32.5
21	H	$PhCH_2CH_2$	32.2
22	PhCO	H	8.9
23	H	PhCO	12.7
24	-	-	11.0
25	-	-	5.8

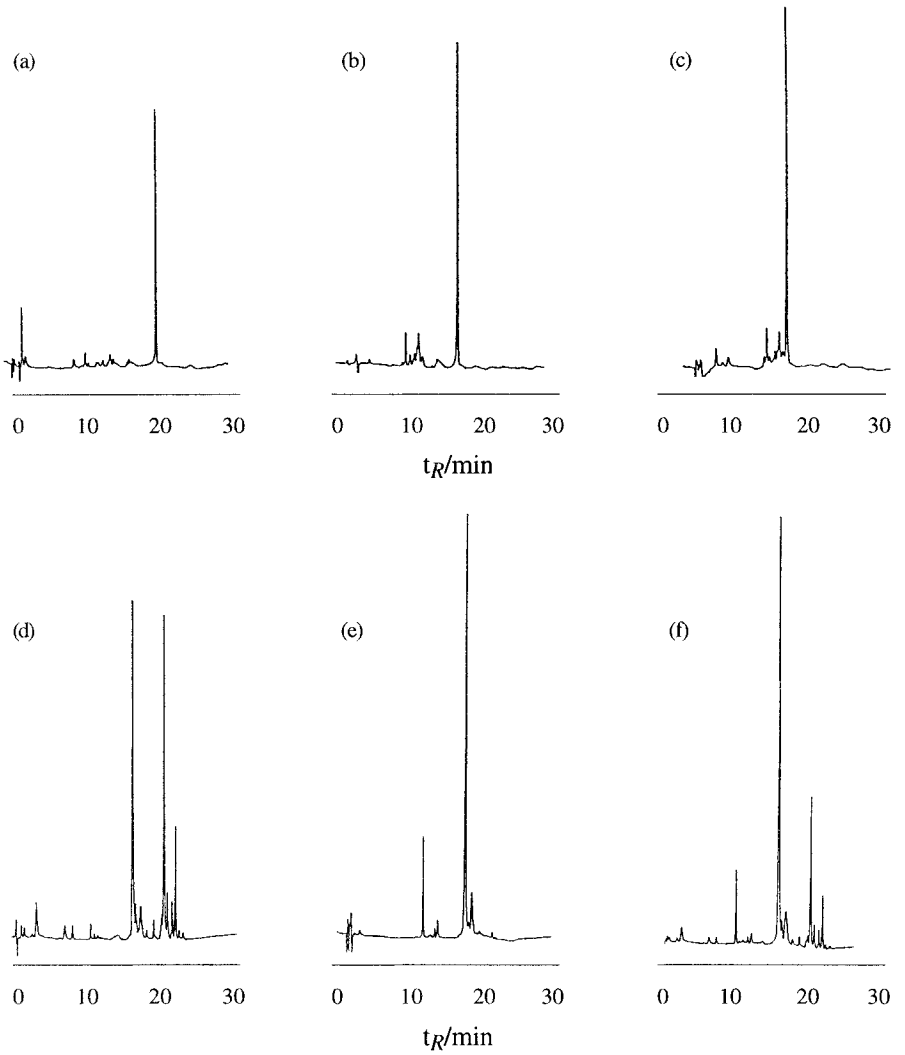
Structure optimisation and ΔH_f calculation was provided by MOPAC[38] with AM1 parameters and concluded with GAMESS[39] where convergence criteria (gradient norm < 0.1) were not met in MOPAC.

pairing with G. This assumes that the N4-H tautomeric form predominates, unaffected by benzoylation. Yet, when incorporated into an oligonucleotide, C^{Bz} clearly causes significant duplex destabilisation (Table 1). To assess the affect of benzoylation on the tautomerisation of cytosine, and consequently its hydrogen bonding pattern and ability to pair with G, we calculated the heats of formation of the *trans* and *cis* imino tautomers **24** and **25** (TABLE 3). The heat of formation *in vacuo* is lower for **25** than for any alternative tautomer **22** to **24**. This favourable value is attributable to its extended conjugated system and its intramolecular N3-H...O hydrogen bond. Involvement of **25** in normal Watson-Crick base pairing would thus be prevented by alternative presentation of donor and acceptor groups as well as the bulk of the substituent.

Post-synthetic modification of oligonucleotides circumvents the need for modified phosphoramidites[40]. Post-synthetic displacement of PfpO-substituted purines in short oligonucleotides has been achieved on prolonged treatment with amine nucleophiles at elevated temperature[25]. We were interested in determining whether the PfpO group of **1** could be displaced by amines to install N4-modified cytosine bases in pre-formed oligonucleotides. Phosphitylation of **4** gave phosphoramidite **5** in quantitative yield (SCHEME). Solid phase synthesis using **5** provided solid support bound dGCG**F**GC **16** where **F** = 4-PfpO-pyrimidin-2-one. Treatment of **16** with NH_3 (aq) at 60 °C during 20 h resulted in complete conversion of **16** to the parent, unmodified sequence dGCGCGC **10** as anticipated. Reversed phase HPLC analysis of the crude product **10** (FIGURE 2(a)) from this post-synthetic substitution procedure showed one major peak which, on co-injection with the same crude sequence **10** made by conventional methods (FIGURE 2(b)), revealed that the sequences made by both methods were identical (FIGURE 2(c)). Similarly, **16** was treated with *N*-methyl-*N*-phenethylamine at 60 °C for 20 h followed by NH_3 (aq) at 55°C during 18 h to facilitate deprotection and cleavage from the solid support. HPLC analysis (FIGURE 2(d)) showed the presence of two main products. Co-injection of this crude sample with an authentic sample of crude **12** (FIGURE 2(e)), made by conventional methods using modified phosphoramidite **9**, showed that **12** had indeed formed during post-synthetic substitution although inefficiently, and that this corresponded to the shorter retention time (t_R) component (FIGURE 2(f)). The other major component at longer retention time (FIGURE 2(d)) corresponded to **10** which had formed presumably during NH_3 (aq) treatment from the remainder of **16** which failed to react with *N*-methyl-*N*-phenethylamine. Similar inefficient formation of **11** resulted from reaction of **16** with *N*-phenethylamine.

EXPERIMENTAL

General: NMR spectra were recorded on a Bruker AC-250 spectrometer at ^1H (250.1 MHz), ^{13}C (62.9 MHz), ^{31}P (101.3 MHz) and ^{19}F (235.3 MHz) with positive chemical shifts downfield of TMS (^1H, ^{13}C), 85% phosphoric acid (^{31}P), and negative chemical shifts upfield of CF_3CCl_3 (^{19}F). Mass spectra were recorded using VG Quatro II or VG AutoSpec instruments. IR and UV spectra were recorded on Mattson Galaxy 2020 FT-IR and Unicam PU8730 instruments. Flash column chromatography[41] was performed using Sorbsil C60 silica and TLC was performed using plastic-backed Kieselgel 60 silica plates containing a fluorescent indicator and visualised under UV (254 nm) and with acidic anisaldehyde solution. Elemental Analyses were performed by Butterworth Laboratories (Middlesex). Oligonucleotides **10** to **16** were prepared on an Applied Biosystems 392



HPLC elution profiles of crude sequences: (a) **10** from reaction of **16** with NH_3 (aq); (b) authentic **10**; (c) co-injection of (a) and (b); (d) **12** from reaction of **16** with *N*-methyl-*N*-phenethylamine; (e) authentic **12**; and (f) co-injection of (d) and (e).

FIGURE 2

DNA Synthesiser following the standard protocol recommended by the manufacturer[42] except for **15** which was prepared as described previously[37]. Cyanoethylphosphoramidites (dT, dA^{Bz}, dG^{iBu}, dC^{Bz}) and CPG supports were purchased from Cruachem. HPLC analyses and purifications were carried out on a Waters Multi-solvent Delivery System using a HPLC Technology C18 reversed phase column (250 x 4.6 mm) with UV detection (260 nm). Gradient elution was achieved by mixing A with B (0-35%) during 20 min then with B (50%) during a further 10 min where A (pH 7.0) contained 1 M aqueous triethylammonium acetate (TEAA, 10%) and MeCN (2%), and B (pH 7.0) contained 1 M aqueous TEAA (20%) and MeCN (80%). T_ms were recorded on a Varian-Cary E1 UV-Visible Spectrophotometer with a multi-compartment heating block in a N_2 atmosphere. Oligonucleotide samples were cooled from 80 to -2 °C at a rate of 0.1 °C.min^{-1} to aid annealing before heating at a rate of 0.5 °C.min^{-1} from 0 to 80 °C and absorbance (260 nm) vs temperature recorded automatically at 0.5 °C intervals using Cary Thermal Analysis Software running on a Victor 433D Personal Computer. Thermodynamic data for duplex formation were derived as described previously[35].

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4-pentafluorophenoxypyrimidine-2(1*H*)-one 4

To PfpO-substituted pyrimidine nucleoside[27] **1** (1.00 g, 2.54 mmol) in dry pyridine (40 mL) was added DMTCl (1.03 g, 3.05 mmol) and the mixture stirred under Ar at room temperature for 5 h. The reaction was quenched with MeOH and concentrated under vacuum. Flash column chromatography, eluting with EtOAc-hexane 1:1 containing Et_3N (2%), gave a semi-solid which was dissolved in EtOAc (5 mL) and precipitated into hexane (100 mL) to yield the title compound **4** (1.41 g, 80%) as a colourless solid; m.p. 104°C; TLC (EtOAc-Hexane 1:1): R_f 0.58; IR (KBr disc): υ_{max} 3463, 3417, 3097, 3060, 2931, 1670, 1552, 1525, 1289, 1249, 1180 cm^{-1}; ^{1}H NMR [$(CD_3)_2SO$]: δ 2.18 (m, 1 H, 2'-CH), 2.36 (m, 1 H, 2'-CH), 3.28 (m, 2 H, 5'-CH_2), 3.73 (s, 6 H, CH_3O), 3.99 (d, 1 H, *J* 3.9 Hz, 4'-CH), 4.29 (t, 1 H, *J* 4.0 Hz, 3'-CH), 5.40 (d, 1 H, *J* 4.5 Hz, 3'-OH), 6.20 (t, 1 H, *J* 6.1 Hz, 1'-CH), 6.24 (d, 1 H, *J* 7.3 Hz, 5-CH), 6.91 (d, 4 H, *J* 8.9 Hz, CH (DMT)), 7.25 (m, 9 H, CH (DMT)), 7.29 p.p.m. (d, 1 H, *J* 7.3 Hz, 6-CH); ^{13}C NMR [$(CD_3)_2SO$]: δ 40.5 (2'-CH_2), 55.0 (CH_3O), 62.9 (5'-CH_2), 69.6 (3'-CH), 85.9 (C DMT), 86.0 (1'-CH), 86.7 (4'-CH), 92.4 (5-CH), 113.2 (CH), 126.4 (CH), 126.8 (CH), 127.4 (CH), 127.7 (CH), 128.9 (CH), 129.8 (CH), 135.1 (C (DMT)), 135.4 (C (DMT)), 140.2 (4-C), 144.6 (C (DMT)), 147.2 (6-CH), 153.3 (C (Pfp)), 158.1 (CO (DMT)), 168.0 p.p.m. (2-CO); ^{19}F NMR [$(CD_3)_2SO$]: δ -14.78 (t, 2 F, *J* 21.1 Hz, 2 x *meta* CF), -19.63 (t, 1 F, *J* 21.2 Hz, *para* CF), -21.52 p.p.m. (d, 2 F, *J* 24.2 Hz, 2 x *ortho* CF); MS (FAB$^+$): *m/z* (I_r)

719 (M + Na, 86%), 696 (M, 60%), 619 (8%), 570 (25%), 447 (35%), 418 (13%), 377 (90%), 303 (100%); Anal. calcd. for $C_{36}H_{29}F_5N_2O_7$: C, 62.0; H, 4.2; F, 13.7; N, 4.0. Found: C, 62.1; H, 4.3; F, 13.7; N, 4.1.

1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-4-*N*-phenethylcytosine 2

To PfpO-substituted pyrimidine nucleoside[27] **1** (191 mg, 0.48 mmol) in dioxane (10 mL) was added *N*-phenethylamine (0.06 mL, 0.53 mmol) and the solution stirred at 80°C overnight. Concentration of the product solution followed by flash column chromatography, eluting firstly with EtOAc then with EtOAc-MeOH 4:1, gave the title compound **2** (135 mg, 85%) as a hygroscopic semi-solid. TLC (EtOAc-MeOH 4:1): R_f 0.60; UV (95% EtOH): λ_{max} 274 nm (12700); IR (KBr disc): υ_{max} 3341, 3130, 3025, 2933, 1647, 1565, 1514, 1452, 1325, 1089, 1050 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 1.97 (m, 1 H, 2'-CH), 2.09 (m, 1 H, 2'-CH), 2.79 (t, 2 H, *J* 7.0 Hz, CH_2Ph), 3.45 (d, 2 H, *J* 3.0 Hz, 5'-CH_2), 3.54 (m, 2 H, CH_2N), 3.76 (d, 1 H, *J* 3.1 Hz, 4'-CH), 4.12 (s, 1 H, 3'-CH), 4.96 (t, 1 H, *J* 5.1 Hz, 5'-OH), 5.19 (s, 1 H, *J* 4.0 Hz, 3'-OH), 5.73 (d, 1 H, *J* 7.5 Hz, 5-CH), 6.16 (t, 1 H, *J* 6.6 Hz, 1'-CH), 7.33 (m, 5 H, Ph), 7.74 p.p.m. (m, 2 H, 6-CH, NH); ^{13}C NMR [$(CD_3)_2SO$]: δ 34.5 (2'-CH_2), 40.2 (CH_2Ph), 41.3 (CH_2N), 61.4 (5'-CH_2), 70.5 (3'-CH), 84.9 (1'-CH), 87.2 (4'-CH), 94.2 (5-CH), 126.1 (CH (Ph)), 128.4 (CH (Ph)), 128.7 (CH (Ph)), 139.5 (6-CH), 139.9 (C (Ph)), 156.4 (4-C), 163.3 p.p.m. (2-CO); MS (FAB^+): *m/z* (I_r) 354 (M + Na, 8%), 332 (M + H, 26%), 216 (100%); HRMS calcd. for $C_{17}H_{22}N_3O_4$ (M + H): 332.161. Found: 332.161; Anal. calcd. for $C_{17}H_{21}N_3O_4 \cdot 0.5H_2O$: C, 60.0; H, 6.5; N, 12.3. Found: C, 60.2; H, 6.2; N, 12.3.

Similarly prepared were:

1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-4-*N*-methyl-4-*N*-phenethylcytosine 3

From **1** (900 mg, 2.28 mmol), dioxane (30 mL) and *N*-phenethyl-*N*-methylamine (0.4 mL, 2.74 mmol). Flash column chromatography, eluting with EtOAc-MeOH 4:1, gave the title compound **3** (779 mg, 98%) as a hygroscopic semi-solid; TLC (EtOAc-MeOH 4:1): R_f 0.57; UV (95% EtOH): λ_{max} 281 nm (13100); IR (KBr disc): υ_{max} 3447, 3317, 3091, 2914, 1631, 1521, 1448, 1417, 1326, 1265, 1189, 1116, 1049 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 1.95 (m, 1 H, 2'-CH), 2.15 (m, 1 H, 2'-CH), 2.81 (d, 2 H, CH_2Ph), 2.89 (s, 1.5 H, CH_3N), 2.94 (s, 1.5 H, CH_3N), 3.35 (m, 4 H, CH_2N, 5'-CH_2), 3.80 (m, 1 H, 4'-CH), 4.21 (m, 1 H, 3'-CH), 4.98 (s, 1 H, 5'-OH), 5.16 (s, 1 H, 3'-OH), 6.02 (m, 1 H, 5-CH), 6.16 (t, 1 H, 1'-CH), 7.25 (m, 5 H, Ph), 7.88 p.p.m. (m, 1 H, 6-CH); ^{13}C NMR [$(CD_3)_2SO$]: δ 32.6 (CH_2Ph), 33.4 (CH_2Ph), 35.3

(CH_3N), 36.3 (CH_3N), 40.2 (2'-CH_2), 50.1 (CH_2N), 51.4 (CH_2N), 61.3 (5'-CH_2), 70.4 (3'-CH), 85.0 (1'-CH), 87.3 (4'-CH), 91.5 (5-CH), 126.4 (CH (Ph)), 128.4 (CH (Ph)), 128.7 (CH (Ph)), 129.0 (CH (Ph)), 138.5 (C (Ph)), 139.2 (C (Ph)), 140.9 (6-CH), 141.4 (6-CH), 154.3 (4-C), 162.7 p.p.m. (2-CO); MS (FAB^+): *m/z* (I_r) 368 (M + Na, 40%), 346 (M + H, 91%), 230 (100%), 105 (32%); HRMS calcd. for $C_{18}H_{24}N_3O_4$ (M + H): 346.177. Found: 346.176; Anal. calcd. for $C_{18}H_{23}N_3O_4 \cdot 0.25H_2O$: C, 61.8; H, 6.8; N, 12.0. Found: C, 61.9; H, 6.8; N, 12.2.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4-*N*-phenethylcytosine 6

From **4** (350 mg, 0.503 mmol), dioxane (10 mL) and *N*-phenethylamine (63 μL, 0.503 mmol). Flash column chromatography, eluting with in EtOAc containing Et_3N (1%), gave the title compound **6** (302 mg, 95%) as a semi-solid; TLC (EtOAc): R_f 0.57; IR (KBr disc): υ_{max} 3463, 3417, 3097, 2930, 1670, 1525, 1449,1388, 1290, 1249, 1180 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 2.06 (m, 1 H, 2'-CH), 2.16 (m, 1 H, 2'-CH), 2.80 (t, 2 H, *J* 7.3 Hz, CH_2Ph), 3.19 (s, 2 H, 5'-CH_2), 3.44 (t, 2 H, *J* 7.3 Hz, CH_2N), 3.73 (s, 6 H, CH_3O), 3.86 (m, 1 H, 4'-CH), 4.24 (s, 1 H, 3'-CH), 5.31 (s, 1 H, 3'-OH), 5.59 (d, 1 H, *J* 7.4 Hz, 5-CH), 6.19 (t, 1 H, *J* 6.8 Hz, 1'-CH), 6.89 (d, 4 H, *J* 8.3 Hz, CH (DMT)), 7.30 (m, 14 H, CH (DMT), CH (Ph)), 7.61 (d, 1 H, *J* 7.4 Hz, 6-CH), 7.79 p.p.m. (t, 1 H, *J* 4.5 Hz, NH); ^{13}C NMR [$(CD_3)_2SO$]: δ 34.5 (2'-CH_2), 39.8 (CH_2Ph), 40.4 (CH_2N), 55.1 (CH_3O (DMT)), 62.4 (5'-CH_2), 70.0 (3'-CH), 84.6 (1'-CH), 85.2 (4'-CH), 85.8 (C (DMT)), 94.6 (5-CH), 113.2 (CH) 126.2 (CH), 126.8 (CH), 127.7 (CH), 127.9 (CH), 128.4 (CH), 128.6 (CH), 129.7 (CH), 130.2 (CH), 135.3 (C (DMT)), 135.6 (C (DMT)), 139.4 (4-C, C (Ph)), 139.5 (6-CH), 144.7 (C (DMT)), 158.4 (CO (DMT)), 163.5 p.p.m. (2-CO); MS (FAB^+): *m/z* (I_r) 656 (M + Na, 10%), 634 (M + H, 38%), 303 (100%), 216 (25%), 105 (12%); Anal. calcd. for $C_{38}H_{39}N_3O_6$: C, 72.0; H, 6.2; N, 6.6. Found: C, 72.1; H, 6.1; N, 6.5.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4*N*-methyl-*N*-phenethylcytosine 8

From **4** (1.0 g, 2.54 mmol), dioxane (50 mL) and *N*-phenethyl-*N*-methylamine (0.41 mL, 2.79 mmol). Flash chromatography, eluting with EtOAc-MeOH 9:1 containing Et_3N (1%), gave the title compound **8** (600 mg, 37%) as a semi-solid; TLC (EtOAc-MeOH 9:1): R_f 0.53; IR (KBr disc): υ_{max} 3433, 3080, 2935, 1638, 1523, 1505, 1448, 1407, 1301, 1253, 1174, 1099 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 2.07 (m, 1 H, 2'-CH), 2.17 (m, 1 H, 2'-CH), 2.77 (m, 2 H, CH_2Ph), 2.90 (s, 1.5 H, CH_3N), 2.96 (s, 1.5 H, CH_3N), 3.21 (m, 2 H, CH_2N), 3.56 (m, 1 H, 5'-CH), 3.72 (m, 7 H, CH_3O,

5'-CH), 3.87 (s, 1 H, 4'-CH), 4.25 (m, 1 H, 3'-CH), 5.31 (d, *J* 7.6 Hz, 5-CH), 6.12 (m, 1 H, 1'-CH), 6.90 (d, 4 H, *J* 8.8 Hz, CH (DMT)), 7.34 (m, 14 H, CH (DMT), CH (Ph)), 7.66 p.p.m. (m, 1 H, 6-CH); ^{13}C NMR [$(CD_3)_2SO$]: δ 32.9 (CH_2Ph), 35.8 (CH_3N), 40.5 (2'-CH_2), 55.0 (CH_3O (DMT)), 63.4 (5'-CH_2), 70.4 (3'-CH), 85.0 (1'-CH), 85.4 (C (DMT)), 85.7 (4'-CH), 91.8 (5-CH), 113.2 (CH), 126.2 (CH), 127.7 (CH), 128.4 (CH), 129.8 (CH), 135.1 (C (DMT)), 135.4 (C (DMT)), 138.8 (C (Ph)), 141.0 (6-CH), 144.8 (C (DMT)), 158.1 (CO (DMT)), 162.4 p.p.m. (2-CO); MS (FAB$^+$): *m/z* (I_r) 670 (M + Na, 15%), 648 (M + H, 14%), 344 (12%), 303 (100%), 230 (65%), 165 (10%); HRMS calcd. for $C_{39}H_{42}N_3O_6$ (M + 1): 648.307. Found: 648.309; Anal. calcd. for $C_{39}H_{41}N_3O_6$: C, 72.3; H, 6.3; N, 6.5. Found: C, 72.0; H, 6.4; N, 6.6.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4-*O*-pentafluorophenoxypyrimidine-2(1*H*)one-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropyl]-phosphoramidite 5

To 5'-*O*-DMT-protected nucleoside **4** (500 mg, 0.718 mmol) in dry THF (10 mL) was added Et_3N (0.29 mL, 2.16 mmol) and 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite (0.25 mL, 1.44 mmol) and the mixture stirred under Ar at room temperature for 3 h. Concentration of the product solution followed by flash column chromatography, eluting with EtOAc-hexane (1:1) containing Et_3N (2%), gave the title compound **5** (645 mg, 100%) as a semi-solid; TLC (2% Et_3N in EtOAc-hexane, 1:1): R_f 0.60; IR (KBr disc): υ_{max} 3086, 2970, 1679, 1629, 1517, 1453, 1284, 1249, 1176, 1026 cm^{-1}; ^{1}H NMR [$(CD_3)_2SO$]: δ 1.15 (m, 12 H, CH_3 (*i*Pr)), 2.31 (m, 1 H, 2'-CH), 2.45 (t, 1 H, *J* 6.4 Hz, CH_2CN), 2.63 (t, 1 H, *J* 6.4 Hz, CH_2CN), 2.75 (m, 1 H, 2'-CH), 3.49-3.73 (m, 6 H, 5'-CH_2, CH_2O, CH (*i*Pr)), 4.14 (s, 1 H, 4'-CH), 4.66 (s, 1 H, 3'-CH), 5.83 (m, 1 H, 1'-CH), 6.23 (m, 1 H, 5-CH), 6.85 (m, 4 H, CH (DMT)), 7.18-7.38 (m, 9 H, CH (DMT)), 8.41 (d, 0.5 H, 7.2 Hz, 6-CH), 8.46 p.p.m. (d, 0.5 H, 7.2 Hz 6-CH); ^{13}C NMR [$(CD_3)_2SO$]: δ 16.2 (*C*H_2CN), 24.1 (CH_3), 24.2 (CH_3), 24.3 (CH_3), 24.4 (CH_3), 42.5 (CH (*i*Pr)), 42.7 (CH (*i*Pr)), 55.0 (CH_3O (DMT)), 55.2 (CH_3O (DMT)), 58.2 (CH_2O), 67.0 (5'-CH_2), 72.0 (3'-CH), 85.9 (C (DMT)), 86.0 (1'-CH), 87.1 (4'-CH), 92.5 (5-CH), 113.2 (CH), 118.9 (CN), 125.0 (CH), 126.8 (CH), 127.6 (CH), 127.8 (CH), 129.8 (CH), 135.0 (C (DMT)), 144.5 (C (DMT)), 147.5 (6-CH), 153.2 (4-C), 162.0 (CO (DMT)), 168.4 p.p.m. (2-CO); ^{19}F NMR [$(CD_3)_2SO$]: δ -14.87 (t, 2 F, *J* 21.3 Hz, *meta* CF), -19.75 (t, 1 F, *J* 23.1 Hz, *para* CF), -24.15 p.p.m. (d, 2 F, *J* 20.7 Hz, *ortho* CF); ^{31}P NMR [$(CD_3)_2SO$]: δ 143.2, 143.7 p.p.m.; MS (FAB$^+$): *m/z* (I_r) 919 (M + Na, 62%), 897 (M + H, 7%), 679 (93%), 593 (8%), 577 (22%), 303 (100%); HRMS calcd. for $C_{45}H_{46}F_5N_4NaO_8P$ (M + Na): 919.287. Found: 919.283; Anal. calcd.

for $C_{45}H_{46}F_5N_4O_8P$: C, 60.3; H, 5.3; N, 6.3; P, 3.5; Found: C, 60.7; H, 5.3; N, 6.2; P, 3.5.

Similarly prepared were:

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4-*N*-phenethylcytosine-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropyl]-phosphoramidite 7

From 5'-*O*-DMT-protected nucleoside **6** (460 mg, 0.79 mmol), anhydrous THF (10 mL), Et_3N (0.28 mL, 1.98 mmol) and 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (0.15 mL, 0.87 mmol). Flash column chromatography, eluting with EtOAc-MeOH (9:1) containing Et_3N (2%), gave the title compound **7** (535 mg, 88%) as a hygroscopic semi-solid; TLC (2% Et_3N in EtOAc-MeOH, 9:1): R_f 0.56; IR (KBr disc): υ_{max} 3452, 3096, 2966, 2931, 1648, 1503, 1466, 1410, 1365, 1305, 1250, 1178, 1031 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 1.20 (m, 12 H, CH_3 (*i*Pr)), 2.32 (m, 2 H, 2'-CH_2), 2.73 (t, 1 H, *J* 5.9 Hz, CHCN), 2.83 (m, 3 H, CHCN, $PhCH_2$), 3.27 (m, 2 H, 5'-CH_2), 3.44-3.68 (m, 6 H, CH_2N, CH_2O, 2 x CH (*i*Pr)), 3.73 (s, 6 H, CH_3O), 4.01 (m, 1 H, 4'-CH), 4.5 (m, 1 H, 3'-CH), 5.59 (d, 1 H, *J* 7.5 Hz, 5-CH), 6.17 (m, 1 H, 1'-CH), 6.69 (m, 4 H, CH (DMT)), 7.30 (m, 14 H, CH (DMT), CH (Ph)), 7.61 (m, 1 H, 6-CH), 7.81 p.p.m. (t, 1 H, *J* 4.5 Hz, NH); ^{13}C NMR [$(CD_3)_2SO$]: δ 19.8 (CH_2CN), 22.6 (CH_3), 24.1 (CH_3), 24.2 (CH_3), 24.3 (CH_3), 41.2 (2'-CH_2), 42.5 (CH (*i*Pr)), 42.7 (CH (*i*Pr)), 45.6 (CH_2N), 53.6 (CH_3O (DMTr)), 58.2 (CH_2O), 62.9 (5'-CH_2), 72.8 (3'-CH), 83.5 (1'-CH), 84.8 (4'-CH), 85.8 (C (DMT)), 94.8 (5-CH), 113.2 (CH (DMT)), 118.9 (CN), 126.1 (CH), 126.8 (CH), 127.7 (CH), 128.3 (CH), 128.6 (CH), 129.7 (CH), 135.2 (C (DMT)), 135.3 (C (DMT)), 139.4 (C (Ph)), 139.7 (6-CH), 144.6 (C (DMT)), 154.9 (4-C), 158.1 (CO (DMT)), 163.3 p.p.m. (2-CO); ^{31}P NMR [$(CD_3)_2SO$]: δ 147.6, 148.1 p.p.m.; MS (FAB$^+$): *m/z* (I_r) 856 (M + Na, 25%), 834 (M + H) (11%), 616 (18%), 303 (100%), 216 (37%); HRMS calcd. for $C_{47}H_{57}N_5O_7P$ (M + H): 834.980. Found: 834.981.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-4-*N*-phenethyl-*N*-methylcytosine-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropyl]-phosphoramidite 9

From 5'-*O*-DMT-protected nucleoside **8** (916 mg, 1.42 mmol), THF (10 mL), *i*Pr_2NEt (0.74 mL, 4.25 mmol) and 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (0.47 mL, 2.12 mmol). Flash column chromatography, eluting with EtOAc containing Et_3N (2%), gave the title compound **9** (1.04 g, 87%) as a semi-solid; TLC (2% Et_3N in EtOAc): R_f 0.32; IR (KBr disc): υ_{max} 3452, 3030, 2966, 2931, 1648, 1504, 1465, 1410,

1364, 1305, 1250, 1178, 1031 cm^{-1}; ^1H NMR [$(CD_3)_2SO$]: δ 1.11 (m, 12 H, CH_3 (*i*Pr)), 2.06 (m, 1 H, 2'-CH), 2.49 (m, 1 H, 2'-CH), 2.67 (t, 1 H, *J* 5.9 Hz, CHCN), 2.84 (m, 3 H, CHCN, CH_2Ph), 2.90 (s, 1.5 H, CH_3N), 2.96 (s, 1.5 H, CH_3N), 3.30 (m, 2 H, 5'-CH), 3.55 (m, 6 H, CH_2N, CH_2O, CH (*i*Pr)), 3.79 (s, 6 H, CH_3O), 4.01 (m, 1 H, 4'-CH), 4.49 (m, 1 H, 3'-CH), 5.78 (d, 1 H, *J* 7.1 Hz, 5-CH), 6.15 (t, 1 H, *J* 6.5 Hz, 1'-CH), 6.90 (m, 4 H, CH (DMT)), 7.30 (m, 14 H, CH (DMT), CH (Ph)), 7.61 p.p.m. (m, 1 H, 6-CH); ^{13}C NMR [$(CD_3)_2SO$]: δ 19.8 (CH_2CN), 20.9 (CH_3), 24.3 (CH_3), 32.1, 33.4 (CH_2Ph), 35.2 (CH_3N), 36.1 (CH_3N), 39.5 (2'-CH_2), 42.5 (CH), 42.7 (CH), 50.0 (CH_2N), 51.2 (CH_2N), 55.1 (CH_3O (DMT)), 58.1 (CH_2O), 82.8 (5'-CH), 72.4 (3'-CH), 83.8 (1'-CH), 85.0 (4'-CH), 91.7 (5'-CH), 113.2 (CH (DMT)), 118.4 (CN), 127.9 (CH), 128.4 (CH), 128.9 (CH), 129.5 (CH), 129.8 (CH), 135.3 (C (DMT)), 138.2 (C (Ph)), 139.0 (6-CH), 144.2 (C (DMT)), 154.0 (4-C), 158.2 (CO (DMT)), 162.1 p.p.m. (2-CO); ^{31}P NMR [$(CD_3)_2SO$]: δ 147.7, 148.1 p.p.m.; MS (FAB^+): *m/z* (I_r) 870 (M + Na, 15%), 848 (M + H, 9%), 630 (24%), 303 (100%), 230 (52%); HRMS calcd. for $C_{48}H_{59}N_5O_7P$ (M + H): 848.415. Found: 848.416.

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